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NOVEL STRATEGIES IN THE MANAGEMENT OF EXPOSED NECROTIC BONE AND BONE DEFECTS IN ORAL AND MAXILLOFACIAL SURGERY

[Management of Osteomyelitis and Medication-related Osteonecrosis of the Jaw "MRONJ"]

Dissertation

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LIST OF ABBREVIATIONS

µCT: Micro computed tomography
911 helper: Human embryonic retinoblasts

AAV: Adeno-associated virus
ALP: Alkaline phosphatase
b-FGF: Basic fibroblast growth factor
BMD: Bone mineral density
BMP-2: Bone morphogenetic protein 2
BMP-7: Bone morphogenetic protein 7
CHA: Coral hydroxyapatite

CMPC: Calcium magnesium phosphate cement
EGFP: Enhanced green fluorescence protein
FACS: Fluorescence-activated cell sorting
FEA: Finite element analysis
HA/TCP: Hydroxyapatite/beta-tricalcium phosphate
HGF: Hepatocyte growth factor,
HVJ: Hemagglutinating virus of Japan
IGF 1: Insulin growth factor
LMP-3: LIM mineralization protein 3,
MKP-1: Mitogen-activated protein kinase phosphatase 1
MBG: Mesoporous bioglass
N/R: Not reported
NGF-β: Nerve growth factor beta
NNB: Natural non-organic bone
OF: Orthodontic force
OSX: Osterix
OSTEOBONE: Calcium silicon phosphorus
pOBs: Periosteal derived osteoblasts

PBS: Phosphate buffered saline
PDGF-A: Platelet derived growth factor A
PDLSCs: Periodontal stem cells
PFU: plaque forming unit
Pg-LPS: Lipopolysaccharide mediated bone loss
RANKL: Receptor activator of nuclear factor kappa-B ligand
RUNX2: Runt-related transcription factor 2
SEM: Scanning electron microscope
TM: Tooth movement
TRAP: Tartarate resistance acid phosphatase
TU: Transduction units
VEGF: Vascular endothelial growth factor

WEHI 164: Mouse skin fibroblast
JM 109: Escherichia Coli
MSCs: Mesenchymal stem cells
DMEM: Dulbecco's modified eagle medium
SA β-Gal: Senescence-associated β-galactosidase
cDNA: Complementary DNA
ARS: Alizarin red staining
OPN: Osteopontin
MV: Matrix vesicles

AAOMS: American association of oral and maxillofacial surgery
MSP: Main Spectral Projection

β-TCP: Beta-tricalcium phosphate
293FT: Human embryonic kidney cells with the SV40 large T antigen
ADSCs: Adipose derived stem cells
AV: Adenovirus
BGC: Bioactive glass ceramic
BMMSCs: Bone marrow mesenchymal stem cells
BMP-4: Bone morphogenetic protein 4
BMP-9: Bone morphogenetic protein 9
CFSE: Carboxyfluorescein diacetate succinimidyl ester
CRE8: Cre-expressing 293 cells
DPSCs: Dental pulp stem cells
ERR: External root resorption
ELISA: Enzyme linked immunosorbent assay
GAM: Gene activated matrix
HA/COL: Hydroxyapatite/ Collagen
HA/PA: Hydroxyapatite/polyamide
HEK293: Human embryonic kidney 293 cell line
HIF-1α: Hypoxia-inducible factor-1 alpha
iPSCs: Induced pluripotent stem cells
IFU: Infectious units per ml
LacZ: β-galactosidase
Luc: Firefly luciferase
MOI: Multiplicity of infection
mSS: Premineralized silk fibroin protein scaffolds
NB: Nano-bubbles
NIH3T3: Mouse embryo fibroblast
NOD/SCID mice: Non-obese/severe combined immunodeficient
OPG: Osteoprotegrin
PCR: Polymerase chain reaction
PDGF-B: Platelet derived growth factor B
PDLA: Poly D, L-lactide
PF127: Pluronic F127
PG13: Mouse embryonic fibroblast

PLGA: Poly lactic co glycolic acid
RSV: Respiratory syncytial virus
SDF: Syngeneic dermal fibroblasts
TGF-b: Transforming growth factor beta
TNFR: Tumour necrosis factor alpha receptor
TSG-6: Tumour necrosis factor alpha-stimulated gene-6
US: Ultra-sound
WB: Western Blot
OM: Osteomyelitis
FBS: Fetal bovine serum
ALP: Alkaline Phosphatase
OD: Osteogenic media
Col1α1: Collagen Type I
OCN: Osteocalcin
MRONJ: Medication-related osteonecrosis of the jaw
EDT: Extended Direct Transfer method

PUBLICATION LIST

BOOK CHAPTERS

- ***Riham Fliefel*** and Sven Otto. Pathogenesis of antiresorptive drug-related osteonecrosis of the jaw. In: Kenneth E Fleisher, Risto Kontio, Sven Otto. Antiresorptive Drug-related Osteonecrosis of the Jaw (ARONJ)—a Guide to Research. Switzerland: AOCMF; 2016. p 64. ISBN: 978-3-905363-10-4.
- ***Riham Fliefel*** and Pit Voss. New and Innovative Treatment Strategies for Medication-Related Osteonecrosis of the Jaw. In: Sven Otto. Medication-Related Osteonecrosis of the Jaws: Bisphosphonates, Denosumab, and New Agents. Heidelberg: Springer; 2015. p 220. ISBN: 978-3-662-43732-2.

JOURNAL PUBLICATIONS

- ***Riham Fliefel***, Cvetan Popov, Matthias Tröltzsch, Jan Kühnisch, Michael Ehrenfeld, Sven Otto. Mesenchymal stem cell proliferation and mineralization but not osteogenic differentiation are strongly affected by extracellular pH. J Craniomaxillofacial Surgery 2016; 44(6): 715–24.
- Sven Otto, Oliver Ristow, Christoph Pache, Matthias Tröltzsch, ***Riham Fliefel***, Michael Ehrenfeld, Christoph Pautke. Fluorescence-guided surgery for the treatment of medication-related osteonecrosis of the jaw: a prospective cohort study. J Craniomaxillofac Surg. 2016 Aug; 44(8):1073-80.
- ***Riham Fliefel***, Matthias Tröltzsch, Jan Kühnisch, Michael Ehrenfeld, Sven Otto. Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. Int J Oral Maxillofac Surg 2015; 44(5): 568–85.
- Sappasith Panya, ***Riham Fliefel***, Florian Probst, Matthias Tröltzsch, Michael Ehrenfeld, Sören Schubert, Sven Otto. Role of microbiological culture and PCR in Medication- related osteonecrosis of the jaw (MRONJ). Under review in J Craniomaxillofacial Surgery 2016.
- ***Riham Fliefel***, Jan Kühnisch, Michael Ehrenfeld, Sven Otto. Gene Therapy for Bone Defects in Oral and Maxillofacial Surgery: A Systematic Review and Meta-Analysis of Animal Studies. Stem Cells Dev. 2016.
- Benjamin Palla, Egon Burian, John Richard Klecker, ***Riham Fliefel***, Sven Otto. Systematic review of oral ulceration with bone sequestration. J of Craniomaxillofacial Surgery 2015; 44(3): 257–64.
- Florian Probst, Sven Otto, Matthias Cornelsen, ***Riham Fliefel***, Egon Burian, M. Seitz, M. Berger, Michael Ehrenfeld. Custom-made vitalized scaffolds for bone tissue reconstruction in craniomaxillofacial surgery. Int J Oral Maxillofac Surg 2013; 42(10): 1375.
- Florian Probst, Egon Burian, ***Riham Fliefel***, Michael Ehrenfeld, Sven Otto. Zukünftige Optionen zur Rekonstruktion bei ausgedehnten knöchernen Defekten im Kiefer-, Gesichts- und Schädelbereich mittels CAD/CAM-gefertigter bioaktiver Leitschienen. OP-Journal 2014; 29(02):200-4.

- Sven Otto, Robert E Marx, Matthias Tröltzsch, Oliver Ristow, Thomas Ziebart, Bilal Al-Nawas, Knut A Groetz, Michael Ehrenfeld, Valeria Mercadante, Stephen Porter, Alberto Bedogni, Giuseppina Campisi, Vittorio Fusco, Ezher Dayisoylu, ***Riham Fliefel***, Bente Brokstad Herlofson, Christoph Pautke, Tae-Geon Kwon, Stefano Fedele: Comments on "Diagnosis and Management of Osteonecrosis of the Jaw: A Systematic Review and International Consensus": LETTER TO THE EDITOR. J Bone Miner Res. 2015; 30(6):1113–5.

GENERAL INTRODUCTION

INTRODUCTION

Oral and Maxillofacial Surgery and its branches

Oral and Maxillofacial surgery is a multidisciplinary field involving plastic, orthopaedic, general, ENT and neurosurgery. It deals with minor and major surgeries including simple and complicated extractions of teeth, treatment of cysts and tumours, management of maxillary sinuses disorders or traumatic injuries of orofacial soft and hard tissues, temporomandibular joint disorders, salivary gland diseases, dentofacial deformities and infections, pre-prosthetic surgical procedures, reconstruction of soft and hard tissues defects and management of facial neuropathy [1].

Type of defects in Oral and Maxillofacial Surgery

Severe maxillofacial bone defects secondary to trauma, congenital anomalies, ischemic diseases as osteoradionecrosis, infectious diseases as osteomyelitis, tumours, surgical resection or cranioplasty, odontogenic cysts lead to aesthetic deformities and functional damage greatly influencing the quality of life of patients with psychological consequences. These defects may vary from few millimetres to critical-sized large segmental defects. The vast majority of the small defects heal spontaneously under suitable physiological environmental conditions due to the regeneration ability of bone. However, the healing process of bone defect is slow and time consuming. Large defects are difficult to heal due to the size of defects or unstable biomechanical properties, unfavourable wound environment, suboptimal surgical technique, metabolic factors, hormones, nutrition and applied stress resulting in complex three-dimensional structure difficult to restore complicated with the absence of the overlying periosteum and soft tissues [2-5].

Reconstruction of maxillofacial bone defects

Reconstruction of maxillofacial bone defects is challenging for the oral and maxillofacial surgeons due to the potential exposure of grafted tissue to infection complicated by the direct contact with the mouth, sinuses, nasal passages and external environment characterized by high moisture content, significant bacterial populations and physiological functional loads as chewing. In addition to contaminated wound sites, tissue constructs may be exposed to complicated mechanical loads [6, 7].

The standard approaches widely used for reconstructive surgery including distraction osteogenesis or bone grafts [8] have significant limitations as shortage of availability, donor site morbidity, post-operative pain, hypersensitivity, infection, inflammation and resorption of the implanted bone. Although alternatives as the use of allografts or synthetic grafting materials overcome these limitations, both alternatives are also limited by immunorejection or lack of osteoinduction [9].

Exposed bone as a problem faced by Oral and Maxillofacial (OMFS) Clinicians

One of the most common problems OMFS clinicians face is the presence of exposed necrotic bone in the oral cavity. Various pathological conditions have been attributed to this condition as osteomyelitis (OM), osteoradionecrosis (ORN), medication-related osteonecrosis of the jaws (MRONJ) which are presented with similar signs, symptoms and radiographic findings. However, each condition is a separate entity with different treatment approaches [10].

Osteomyelitis as a common infection of the maxillofacial region

Osteomyelitis of the jaw is one of the most important oral and maxillofacial severe bone infections. It is a debilitating disease [11] with severe bone infection leading to dysfunction, progressive inflammatory destruction, [12] marked bone resorption at sites of infection and proximal abnormal bone formation [13]. It occurs more frequently in the mandible than in the maxilla originating from dental infection of root canal, periodontal ligament or extraction of a

tooth, fracture site, soft tissue wound or surgical site [14]. Many mechanisms of bone loss in osteomyelitis have been proposed in the literature [15-17]. During infection, localized pH reduction is manifested [18] with accumulation of an inflammatory exudate causing compression of the blood supply to the bone. Necrotic tissue promotes the proliferation of bacteria resulting in incomplete healing [19]. Furthermore, increased formation and activity of osteoclasts is noticed and elimination of osteoblasts responsible for new bone matrix deposition [20].

Some studies have proved that bacteria, such as, staphylococcus aureus create an acidic environment during proliferation under static culture conditions attributed to the metabolic production of acidic substances like lactic acid [21]. Secondly, the human immune system is notable for its ability to combat infectious microorganism by eliciting inflammatory responses [22]. During this process, local acidosis occurs due to massive infiltration of neutrophils and macrophages [23] to the site of infection. These pathological conditions can decrease the pH to 5.5–7.0 [24]. Infection interferes with the process of bone healing and regeneration by excessive bone resorption as well as impaired bone formation [25, 26].

Medication-related osteonecrosis of jaw (MRONJ) as an emerging disease

Recently, another common disease associated with exposed necrotic bone is medication-related osteonecrosis of the jaw (MRONJ). It is a devastating complication of anti-resorptive (ARD) drugs used globally to treat bone disorders as osteoporosis, skeletal complications associated with osseous metastasis and multiple myeloma [27, 28]. The American Association of Oral and Maxillofacial Surgeons (AAOMS) had changed the nomenclature of bisphosphonate-related osteonecrosis of the jaw (BRONJ) to medication-related osteonecrosis of the jaw (MRONJ) due to the rise in the number of osteonecrosis cases involving the maxilla and mandible associated with other anti-resorptive (Denosumab) and antiangiogenic therapies [29]. Multiple factors had played role in MRONJ pathogenesis. However, none of

them had been proved to be the exclusive reason. Some main theories had been proposed for MRONJ as over-suppression of bone turnover rate, anti-angiogenic properties of ARD leading to necrosis, constant micro-trauma, soft tissue toxicity and inflammation or infection. However, the presence of infection was almost always an initiating role rather than bone turnover [30, 31].

The unique structure of the maxillofacial region and certain bacterial infection has been suggested as key factors for the pathogenesis and progression of MRONJ. The oral cavity comprises of more than 750 bacterial species existing as mixed biofilm communities [32]. The mandible and maxilla are covered by thin layer of mucosa in close proximity to the external environment. After invasive dental procedures, oral trauma or soft tissue infection, microbial biofilms in the mouth and saliva gain access to the exposed jaw bone and play a significant role in the necrosis of the bone, inhibition of oral wound healing and facilitating bacterial colonization on bone surface [33, 34].

Management of exposed necrotic bone

Management of exposed necrotic bone is controversial and difficult to perform due to the increased potential of bacterial adhesion to the exposed surface with high risk of resistance to the antibiotics. For decades, classical methods for treatment of necrotic bone have been used ranging from simple treatment as administration of antibiotics, oral antibacterial mouth rinses, pain control, surgical debridement and removal of sequestrum to aggressive surgical interventions as debridement of large area of bone to include a segmental mandibulectomy and partial maxillectomy, mandibular reconstruction and covering the exposed areas with tissue flaps. However, new treatments have been studied recently as therapeutic tools as hyperbaric oxygen (HBO), fluorescence-guided bone surgery, low-intensity laser therapy and the use of ozone in combination with antibiotics and surgery [35, 36].

Tissue engineering paradigm in Maxillofacial Surgery

A new paradigm is emerging in the field of oral and maxillofacial surgery in the recent years due to the advances in technology in both materials and methods used had led to refining the surgical procedures and achieving precision with minimally invasive techniques. In the maxillofacial region, nearly all disorders have become research subjects of regenerative medicine which is considered to be advantageous because the weight load is smaller than in the long bones and the amount of tissue needed for reconstruction is generally small with its increasing ability to replace, repair or regenerate damaged and injured tissues and restore their physiological function by means of stem cell-based technologies [37, 38].

Bone tissue engineering raised as an alternative to the conventional surgical techniques to regenerate oral and maxillofacial defects by combining the principles of orthopaedic surgery with knowledge from biology, physics, materials science and engineering [8, 39]. As tissue engineering becomes more of a clinical reality through the ongoing bench to bedside transition, nowadays, research in this field focus on addressing relevant clinical situations. While most in-vivo work in the area of bone tissue engineering focuses on bone regeneration within sterile, surgically created defects, there is a growing need for investigation of bone tissue engineering approaches within contaminated or scarred wound beds, such as those that may be encountered following traumatic injury or during delayed reconstruction/regeneration [40].

Gene therapy as a recent therapeutic technique in Maxillofacial Surgery

Recently, progress of genome sciences and molecular biology has enabled to analyse biological phenomena genetically and promote basic research of gene biology and medicine in which genes can be used as a medicine by curing a wide range of serious diseases or healing of defects [41, 42]. Gene therapy has emerged as a new and promising tool for delivery of additional gene or removal of defective gene for the purposes of treating a disease

process or regenerating tissues and hence the improvement of the clinical status of the patient [43-45].

In contrast to traditional replacement gene therapy, craniofacial regeneration via gene therapy has been somewhat different in seeking to transfer the gene encoding desired growth factor or recombinant protein into cells for osteoinduction, tissue growth and repair [46]. This application of gene therapy does not replace a defective gene but rather delivers specific genetic information to cells to start synthesis and secretion of a gene product resulting in higher and more constant levels of protein production for gene therapy-directed osteogenesis. Since the effect is within a local environment for craniofacial bone regeneration, systemic administration is not necessary [2].

OBJECTIVES OF THE THESIS

The main objectives of this thesis were to:

1) Determine the effect of pH on viability and proliferation of human mesenchymal stem cells (hMSCs) and investigate the role of the pH on hMSCs mediated osteogenesis, expression of osteoblast markers and matrix mineralization that may contribute for understanding how changing pH modulates biological and biochemical processes during bone healing in osteomyelitis.
2) Examine the success rate of fluorescence-guided surgery in MRONJ patients in terms of postoperative mucosal integrity and absence of bone exposure with monitoring pain, infection rates as well as disturbances of sensitivity.
3) Identify the bacterial profiles that colonize MRONJ bone samples determined by polymerase chain reactions (PCR) and culture approach with clinical features of patients. This line of investigation could provide rationale in the future for MRONJ therapeutics and targeted antimicrobial therapy.
4) Determine the treatment strategies available for BRONJ by performing a systematic review describing the outcome variables measured for each treatment modality and the success of the treatment expressed by the outcome.
5) Outline the efforts done in gene therapy worldwide in the field of oral and maxillofacial surgery by conducting a systematic review and meta-analysis.
6) Address the pathogenesis of anti-resorptive drug-related osteonecrosis of the jaw.
7) Determine the new and innovative treatment strategies for medication-related osteonecrosis of the jaw.

1. PUBLICATION I

MESENCHYMAL STEM CELLS (MSCS) PROLIFERATION AND MINERALIZATION BUT NOT OSTEOGENIC DIFFERENTIATION ARE STRONGLY AFFECTED BY EXTRACELLULAR PH.

Riham Fliefel, Cvetan Popov, Matthias Tröltzsch, Jan Kühnisch, Michael Ehrenfeld, Sven Otto. J of Craniomaxillofacial Surgery 2016;44(6):715–24.

ABSTRACT

Background: Osteomyelitis is a serious complication in oral and maxillofacial surgery affecting bone healing. Bone remodelling is not only controlled by cellular components but also by ionic and molecular composition of the extracellular fluids in which calcium phosphate salts are precipitated in a pH dependent manner. Objective: To determine the effect of pH on self-renewal, osteogenic differentiation and matrix mineralization of mesenchymal stem cells (MSCs). ***Methods:*** We selected three different pH values; acidic (6.3, 6.7), physiological (7.0-8.0) and severe alkaline (8.5). MSCs were cultured at different pH ranges, cell viability measured by WST-1, apoptosis detected by JC-1, senescence was analysed by β-galactosidase whereas mineralization was detected by Alizarin Red and osteogenic differentiation analysed by Real-time PCR. ***Results:*** Self-renewal was affected by pH as well as matrix mineralization in which pH other than physiologic inhibited the deposition of extracellular matrix but did not affect MSCs differentiation as osteoblast markers were upregulated. The expression of osteocalcin and alkaline phosphatase activity was upregulated whereas osteopontin was downregulated under acidic pH. ***Conclusion:*** pH affected MSCs self-renewal and mineralization without influencing osteogenic differentiation. Thus, future therapies, based on shifting acid-base balance toward the alkaline direction might be beneficial for prevention or treatment of osteomyelitis

INTRODUCTION

Osteomyelitis (OM) of the jaw is a debilitating disease [11] in which severe bone infection leads to dysfunction, progressive inflammatory destruction, marked bone resorption at sites of infection and abnormal bone formation [12, 13]. It occurs more frequently in the mandible than in the maxilla [14] with staphylococcus aureus creating an acidic environment decreasing the pH to 5.5–7.0 [24] caused by massive infiltration of neutrophils and macrophages [18, 19, 21, 23, 47, 48]. It is well known that infection and inflammation interfere with the process of bone healing and regeneration by excessive bone resorption as well as impaired bone formation by activation of several cell populations producing inflammatory cytokines having an impact on bone remodelling [25, 26, 49, 50].

Bone remodelling is not only controlled by osteoblasts and osteoclasts [51] but also by the ionic and molecular composition of the extracellular fluids in which calcium phosphate salts are precipitated in a pH dependent manner [52-54]. Osteoblasts are the most affected cells by pH and acidity of the extracellular microenvironment [52, 55-57]. On a cellular level, even modest reduction in extracellular pH have an effect on osteoblast mineralization and energy metabolism as it was suggested that changes in acid-base balance in the extracellular microenvironment can direct bone formation and resorption [52, 55, 58, 59]. It was shown that alkaline pH enhance mineralization of osteoblasts and decrease the activity of osteoclasts while acidic surroundings can activate osteoclasts as well as impair osteoblast differentiation and in severe cases can cause osteoblast death [60-62].

MSCs are adult stem cells originating from the mesoderm possessing self-renewal ability and multi-lineage differentiation into mesoderm lineages, as chondrocytes, osteocytes and adipocytes, also ectodermic cells and endodermic cells [63] and existing in almost all tissues including bone marrow, adipose tissue, synovium, periosteum, perichondrium as well as cartilage [64]. They have the ability to migrate into sites of injury releasing trophic and

growth factors and differentiated towards terminally-committed cells making them prime candidates for use in regenerative medicine [65-69]. Recently, MSCs showed great potential in clinical practice upon activation by biological or pharmacological means leading to improvement in bone healing by modulating their differentiation into osteoblasts [70, 71]. The chemical and physical environment of MSCs has a strong influence on their behaviour in which matrix acidity is a crucial factor [72, 73]. The effect of the pH of the tissue microenvironment on bone mineralization and repair has been previously reported [74-76]. However, the insight into the mechanism underlying pH-related destruction of bone in osteomyelitis and osteogenic differentiation of human mesenchymal stem cells under various pH conditions have not been discussed. As tissue engineering becomes more of a clinical reality through the ongoing bench to bedside transition, research in this field must focus on addressing relevant clinical situations. While most in vivo work in the area of bone tissue engineering focuses on bone regeneration within sterile, surgically created defects, there is a growing need for investigation of bone tissue engineering approaches within contaminated or scarred wound beds, such as those that may be encountered following traumatic injury or during delayed reconstruction/regeneration [40]. Our study is novel and of importance when considering bone infections as it might be used in future clinical applications for prevention and treatment of some bone infections or diseases. It explains what happens in bone microenvironment during pH changes which could be a key study not only for bone infection/disease but also adds an important facet to the linkage between pH and other hard tissues mineralization. Thus, in the present study, we aimed to 1) determine the effect of pH on viability and proliferation of hMSCs, 2) investigate the role of the pH on hMSCs mediated osteogenesis, expression of osteoblast markers and matrix mineralization that may contribute for understanding how changing pH modulates biological and biochemical processes during bone healing in osteomyelitis.

MATERIALS AND METHODS

Cell culture

All experiments were performed with commercially available human MSCs (hMSCs; Lonza, Basel, Switzerland). Cells were cultured in high glucose Dulbecco's modified eagle medium (DMEM; Life Technologies, California, USA), supplemented with 10 % fetal bovine serum (FBS; Life Technologies, California, USA), 1% penicillin/streptomycin (GE Healthcare, Little Chalfont, UK) at 37°C in a humidified atmosphere of 5% CO_2. Cells between passages 5 and 10 were used from three donors for the experiments.

Preparation of pH culture media

The pH of the culture medium was adjusted to one of six values: 6.3, 6.7, 7.0, 7.4, 8.0 and 8.5 by adding an appropriate amount of 6M HCl or 10M NaOH to the supplemented DMEM. Before resuspending the cells, the culture media were kept in the incubator for 24 hours under culture conditions to allow the desired pH value to equilibrate (CO_2-dependent). After incubation, a small adjustment in pH was occasionally required to create the desired final pH. The pH was monitored with a pH meter (Mettler Toledo GmbH, Giessen, Germany). The pH media were filtered using a syringe driven through a 0.22 µm sterile filter and stored at 4°C to be used later. For pH experiments, normal medium was replaced with various pH media 24 hours upon cell plating and was kept through the experiment.

Self-renewal analysis and WST-1 assay

Long-term cell growth was evaluated by calculation of increased cell number as described previously [77]. The effect of pH on hMSCs proliferation in monolayer culture was evaluated over a five day time course. Cells were plated into 35 mm dishes at a density of 3.0×10^4 and incubated in different pH media. At each time point, cell yield was divided by the number of cells plated at the start of the experiment to obtain a fold-change in cell number. The experiment was repeated twice.

Cell viability was assessed with WST-1 assay (Roche Diagnostics, Risch-Rotkreuz, Switzerland) as previously described [78]. Cells were seeded at a density of 1.7×10^3 cells/well in 96-well plates and incubated with different pH media for 3 days. The WST-1 was mixed with the fresh complete medium, added to the wells and incubated for 4 hours at 37°C in 5% CO_2. WST-1 was quantified by measuring the absorbance at 450 nm using Multiskan FC microplate plate reader (Thermo Scientific, Massachusetts, USA). Each experiment was repeated at least twice with two different donors to obtain the mean values.

JC-1 staining for apoptosis detection

One of the hallmarks of apoptosis is mitochondrial disruption, which is characterized by changes in the mitochondrial membrane potential. These changes were detected by using the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylimidacarbocyanine iodide (JC-1; Life Technologies, California, USA), a membrane-permeable dye which accumulates in mitochondria in a membrane potential-dependent manner. To ascertain whether pH induced apoptosis, slides were coated with collagen, hMSCs (7.0×10^3 cell) were cultured in different pH media for 24 hours. They were stained with JC-1 at 37 °C for 60 min and Hoechst 33342 (Thermo Scientific, Massachusetts, USA) was used as the counterstain [79]. Cells were mounted on slides and pictured with Axio-Observer.Z1 fluorescence microscope (Zeiss, Oberkochen, Germany). Positive control was cells treated with hydrogen peroxide for 5 minutes and negative control was cells cultured in normal media.

Detection and Quantification of Senescent Cells

Senescence-associated β-galactosidase (SA β-Gal; Sigma Aldrich, Missouri, USA) staining was used to detect senescent cells as previously described [78]. Cells were seeded at a density of 3.0×10^4 in 35 mm dishes and cultured at different pH media for 72 hours. Fresh staining mixture was added and incubated at 37 °C overnight. The cells were observed under Axiovert 40 CFL microscope (Zeiss, Oberkochen, Germany). The percentage of blue cells expressing

β-galactosidase (senescent cells) was calculated. The proportion of cells positive for SA-βgal activity was determined by counting the number of blue cells in the total population.

Osteogenic differentiation of hMSCs

Osteogenic differentiation was performed [77]. Shortly, cells were counted and plated at density of 3.2×10^4 on 35 mm dishes. After 24 hrs, normal media were replaced with pH adjusted osteogenic media with cells being cultured for 21 days. The osteogenic media consisted of DMEM supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate and 150 μM ascorbic-2-phosphates (Sigma Aldrich, Missouri, USA). Media were changed twice per week. As a control, hMSCs were cultured at different pH media without osteogenic reagents.

Alizarin red staining (ARS) was performed on day 21. Mineralized nodules were visualized and photographed with Axiovert 40 CFL microscope (Zeiss, Oberkochen, Germany). Osteogenic quantification kit was used for quantification of the staining (Merck Millipore, Darmstadt, Germany). The osteogenic differentiation was calculated versus standard curve and the absorbance was measured at 405 nm using Multiskan FC microplate reader plate reader (ThermoScientific, Massachusetts, USA).

Alkaline Phosphatase (ALP) activity and mineralization

The differentiation of cells to osteoblasts was evaluated as a function of ALP activity. The ALP assay was performed on day 0, 2, 5, 7, 10 and 14 of culture. For this, cells were seeded in 35 mm dishes and cultured at different pH media. The media were changed twice per week. ALP released from the cells was measured with a commercially available ALP assay kit (StemTAG; Cell Biolabs, California, USA). The amount of enzyme released by the cells was quantified by comparison with a standard curve. The experiment was repeated twice with two different donors. The enzyme activities expressed as nmol protein.

RT-PCR analysis of osteogenic genes

RT-PCR was used to evaluate the osteogenic differentiation at different pH after 21 days. RNA was isolated as previously described [77] by QIAzol reagent (Qiagen, Hilden, Germany). RNA concentration and quality was analysed by NanoDrop (ThermoScientific, Massachusetts, USA). Reverse transcription of RNA into complementary DNA (cDNA) was done using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). RT-PCR was used to analyse the expression of the osteogenic genes. The primers for the target genes used and PCR conditions are shown in Table 1.1. The gel electrophoresis was visualized and photographed using gel imager (Vilber Lourmat, Eberhardzell, Germany). Bands were quantitatively analysed by ImageJ (http://imagej.nih.gov/ij/). Gene expression was calculated as the ratio to the housekeeping gene (GAPDH).

Table 1.1: Sequences of the PCR primers with the annealing temperatures and the expected sizes of the amplified products.

Gene	Name	Primer Sequence (F, R, 5'-3')	$T_{annealing}$ (°C)	Product size (bp)
GAPDH	Glyceraldehyde 3 phosphate dehydrogenase	F: CAA CTA CAT GGT TTA CAT GTT C R: GCC AGT GGA CTC CAC GAC	50°C	181
RUNX2	Runt-related transcription factor 2	F: TCT TCA CAAATC CTC CCC R: TGG ATT AAA AGG ACT TGG TG	55°C	230
OCN	Osteocalcin	F: GGC ACA AAG AAG CCG TAC TC R: CAC TGG GCA GAC AGT CAG AA	56°C	242
OPN	Osteopontin	F: CTG ATG AAC TGG TCA CTG ATT TTC R: CCG CTT ATA TAA TCT GGA CTG CTT	60°C	347
Col1α1	Collagen 1α 1	F: AGG GCT CCA ACG AGA TCG AGA TCC G R: TAC AGG AAG CAG ACA GGG CCA ACG TCG	54°C	223

Statistical Analysis

All the experiments were repeated at least two times with 3 different donors each and the results were expressed as means ± standard deviations. Statistical analysis was performed by using GraphPad Prism (GraphPad, California, USA) using one way ANOVA, followed by

Tukey test to determine the statistical significance among the different groups. Levels of significance were indicated at *P < 0.05, **P < 0.01, ***P < 0.0001.

RESULTS

hMSCs self-renewal under different pH conditions

First, we analysed self-renewal by examining the effect of pH on the cell proliferation and viability. For this, we cultured hMSCs in the six different pH conditions for 5 days. We found that the exposure of hMSCs to pH (6.3, 6.7 and 8.5) had a negative effect on proliferation capability in comparison to physiologic pH (7.0, 7.4 and 8.0) indicating that the latter pHs are optimal for cell growth (Figure 1.1A). Then we analysed cell activity by measuring the enzymatic catabolism of formazan to WST-1. Our results showed that similarly to proliferation, the viability of hMSCs was influenced by pH and more viable cells were observed at physiologic pH (7.0, 7.4 and 8.0) while cell viability at pH (6.3, 6.7 and 8.5) decreased (Figure 1.1B).

These findings suggested that the physiological pH (7.0, 7.4 and 8.0) was suitable for hMSCs growth. Since the cell viability at pH 8.5 was severely decreased, this result indicated that alkaline environment up to a certain limit was advantageous for cell growth.

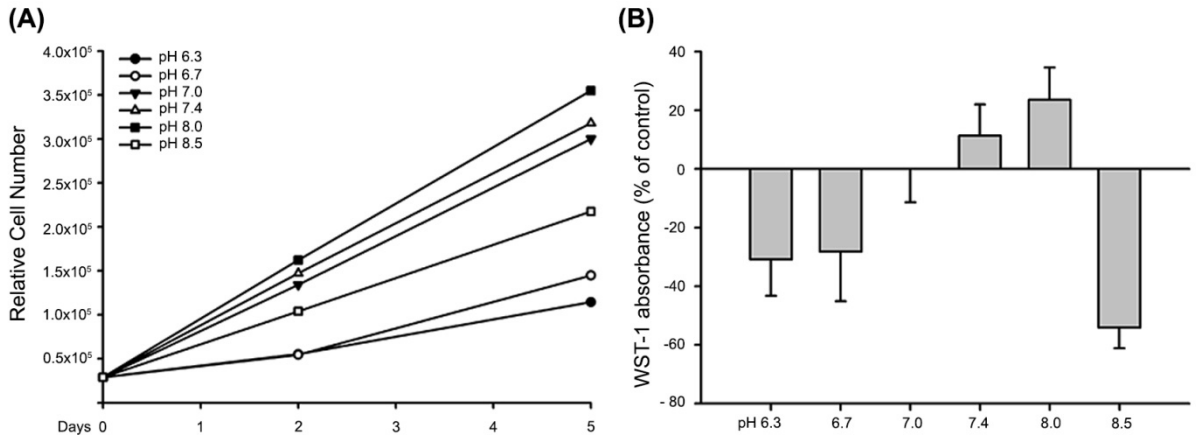


Figure 1.1: Effect of pH on proliferation and viability of hMSCs.
A) Proliferation of human bone marrow stem cells (hMSCs) in different pH media from day 0 to day 5. hMSCs grown in pH (7.0, 7.4 and 8.0) showed the highest proliferation rate compared with those grown in pH (6.3, 6.7 and 8.5); B) Effect of pH on viability of hMSCs cultured at different pH for 3 days was measured at the indicated time points using WST-1 assay and expressed as optical density at 450 nm (A_{450}) as described in Materials and Methods. Error bars represent standard deviations (n=2).

pH effect on hMSCs apoptosis and senescence

Observing the fact that pH (6.3, 6.7 and 8.5) resulted in less self-renewal of hMSCs, we next investigated the reasons behind. We checked whether the cells had undergone apoptosis or senescence. Apoptosis was inspected using JC-1 staining that shows the loss of the mitochondrial membrane potential. In healthy cells, the dye stains the mitochondria bright red while in apoptotic cells, the mitochondrial membrane potential collapses and JC-1 stained the cells green. The results showed that cells cultured in different pH media appeared orange-red and the only green cells were the positive control suggesting that pH did not induce apoptosis in cells (Figure 1.2A).

Besides, we tested if different pH triggered senescence. We found that treatment of hMSCs with different pH media for 3 days resulted in senescent cells in cultures. Cells incubated at pH (6.3, 6.7 and 8.5) appeared flattened and were more positive for β-gal staining while at physiologic pH (7.0, 7.4 and 8.0), cells maintained their spindle shape and only few stained

blue (Fig.2B). Quantification of β-gal staining demonstrated that the staining frequency of hMSCs was approximately 58% blue-positive at pH 6.3, 56% at pH 6.7 and 25% for pH 8.5. In contrast, the frequency for pH 7.0 was 30% whereas at pH 7.4, it was 18% and at pH 8.0, it was about 15% which nearly lacked detectable β-gal activity (Figure 1.2B).

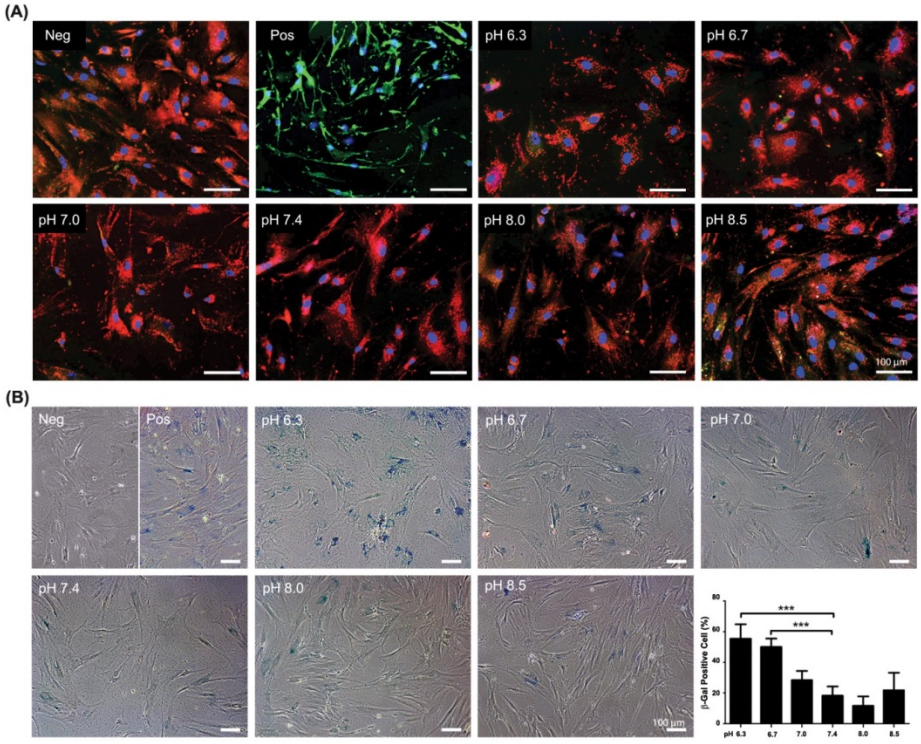


Figure 1.2: Apoptosis and Senescence of hMSCs at different pH.
A) Morphological observation of JC-1 and Hoechst 33342 staining of cells treated at different pH examined with fluorescence microscope at 10× magnification, scale bar represents 100 µm. The experiments were performed on two different donors. Cells at different pH appeared orange red while for positive control (hydrogen peroxide treated cells), showed strong green fluorescence and indicated typical apoptotic morphology; B) hMSCS senescence at different pH condition measured by SA ß-Gal activity assay. The nuclei of senescent cells are surrounded by cyan dye and a significant increase in cell size was detected at pH (6.3, 6.7 and 8.5). Staining was quantified by positive cell count. Error bars represent the means ± SD, n = 2; ($P < 0.0001$).

Osteogenic differentiation of hMSCs and Mineralization Assay

We performed osteogenic differentiation of hMSCs in different pH osteogenic media (OD) or control media. At day 21, alizarin red staining (ARS) confirmed osteogenic differentiation and matrix mineralization of hMSCs. Cells grown in OD exhibited red staining at pH (7.0, 7.4

and 8.0), among them pH 8.0 showed the strongest staining. At pH other than physiologic, the cells showed weaker or no mineralization (Figure 1.3A&B).

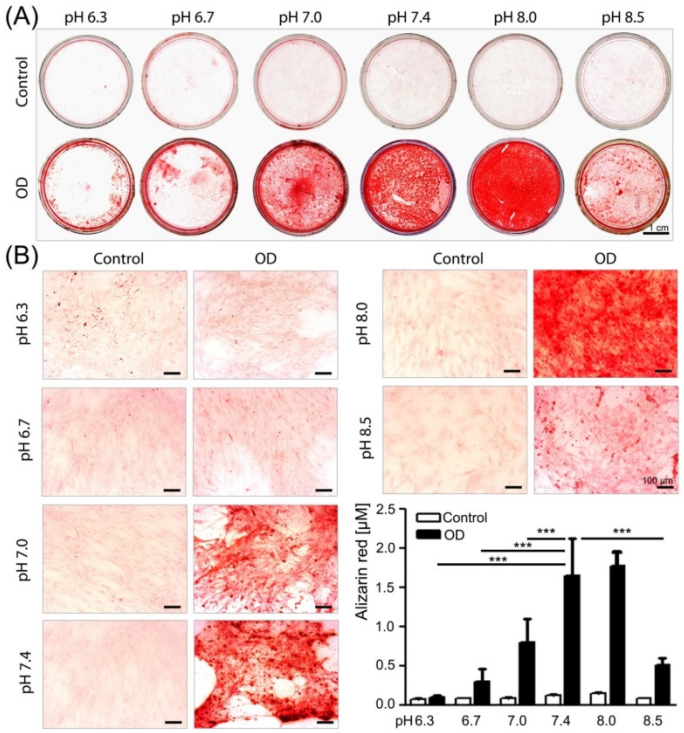


Figure 1.3: Osteogenic differentiation of hMSCs and quantification of ARS.
A) Osteogenic differentiation of hMSCs stained with ARS, scale bars represent 1 cm; B) Morphology of hMSCs grown in control or osteogenic medium (OD) at different pH (magnification 10×, scale bars = 100 µm). Cells were incubated for 21 days in DMEM containing 10% FBS and observed under phase contrast microscope with ARS quantification. Osteogenic differentiation showed significant difference of the amount of soluble alizarin red. The average absorbance value at 405 nm. Error bars represent standard deviations (n=2) ($P < 0.0001$).

Quantitative estimation of alkaline phosphatase (ALP) activity and RT-PCR of osteogenic genes

In order to validate the defected mineralization under various pH conditions, we first investigated the changes in ALP activity. Our results showed no significant differences at different pH conditions at day 0, 2, 5 and 7. However, from day 10-14, ALP activity showed significant difference as its activity increased proportionally at lower pH (6.3 and 6.7)

(Figure 1.4A). Additionally, our RT-PCR analysis showed that all the important osteogenic markers were expressed by the cells in comparison to control media. From the assessed genes, pH media had an effect on OPN and OCN while Col1α1 and Runx2 was pH independent. OPN increased gradually with increasing the pH of the media till pH 8.0 and then down regulated at pH 8.5. The expression of OPN in osteogenic differentiated cells was always higher compared to control media. In contrast to OPN, OCN had an opposite correlation where pH (6.3 And 6.7) showed higher expression followed by pH 8.5 and then the physiologic pHs (Figure 1.4B).

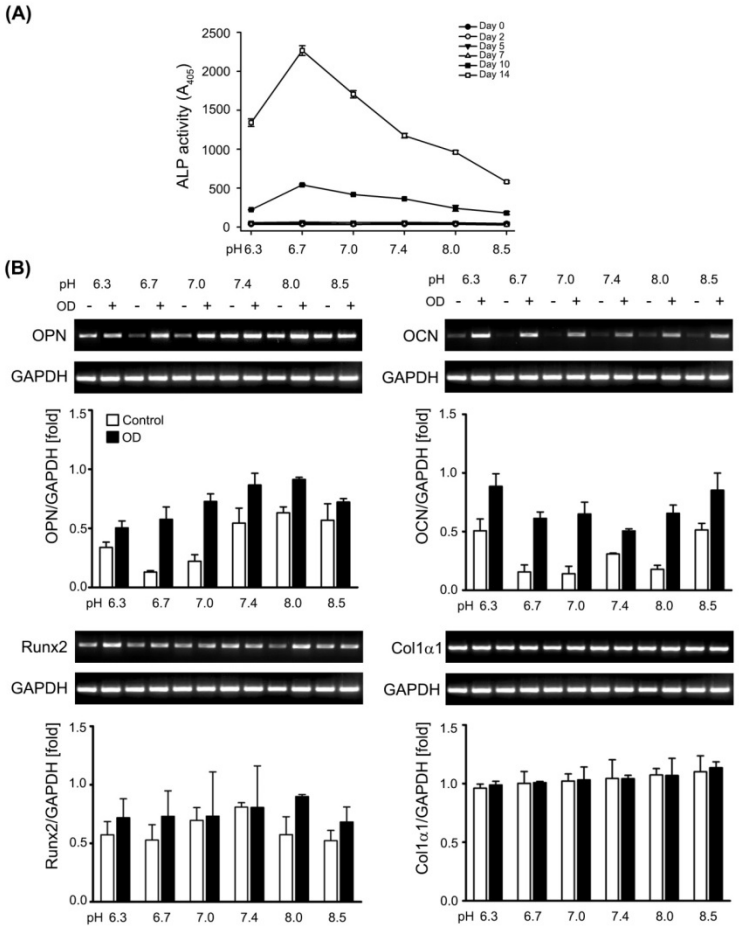


Figure 1.4: ALP activity of osteogenic differentiated MSCs at different pH with expression level of bone-related markers (OPN, OCN, Runx2 and Col1A1) in control and osteogenic media.

A) ALP activity was measured during the course of osteogenic differentiation from day 0 to day 14 showing that it was inversely proportional to the pH: when the pH increased, the ALP activity increased and vice versa; B) RT-PCR data of OPN, OCN, Runx2 and Col1α1 representative of 3 independent experiments from three different donors were combined together and analysed. Runx2 code for major osteogenic transcription factors; Col1α1 is an early marker of osteogenic differentiation; OCN and OPN are markers of late stages of osteogenesis. GAPDH was used as the control Housekeeping gene for this study. Graphs representing mean values of relative optical densities of PCR results are shown in the mRNA expression patterns of osteogenic marker genes in cells at day 21 and the results are expressed as the fold change relative to the respective control.

DISCUSSION

In this study, we confirmed that hMSCs are sensitive to pH as their self-renewal and mineralization were significantly affected. Our study provides new insight into the mechanism underlying pH-related bone destruction and adds an important facet to the linkage between pH and bone infections which might be used clinically in the future to treat osteomyelitis of the jaw. We have selected the used pH values in accordance to their relevance in vivo as follows: pH 6.3-6.7 is common in infection [18] and in cultures with high cell numbers but limited nutrients [80]; pH 7.0-7.4 are commonly used conditions in cell culture [81] and typical value in blood stream [82]; pH 8.0-8.5 are recommended pH for stronger ability for production of osteocytes [83]. An in-vitro approach was used to disclose several clinical important questions: What is the effect of pH on self-renewal and differentiation; how can we make use of this knowledge to be directed for preventing or treating osteomyelitis of the jaw?

Osteomyelitis is prevalent in the facial skeleton associated with abnormal bone remodelling and massive bone resorption. It also presents a major complication ensuing orthopaedic and maxillofacial surgeries as well as routine dental extractions [84]. There is increased formation and activity of osteoclasts in osteomyelitis together with the elimination of the osteoblasts responsible for new bone matrix deposition following infection [20]. Infection causes some essential changes in the extracellular milieu. On these occasions, the pH of the bone tissue environment often falls below pH 7.0, whereas in healthy tissues this pH value varies in the range 7.35 to 7.45 [85].

During early embryonic development, pH regulation is critical for cell metabolism, intracellular ionic signalling, differentiation, quiescence and proliferation [86, 87]. pH controlled self-renewal (proliferation and viability) as well as expression of extracellular matrix proteins not only in fibroblasts but also several cell types by affecting the cytoskeleton

and cell adhesion molecules in addition to arrested cell cycle at the G1 phase [88-90]. Our results demonstrated that changes of pH other than the physiologic can negatively influence cell proliferation and viability of MSCs which might be caused by several factors as apoptosis or senescence.

It is not clear what determines whether cells undergo senescence or apoptosis. One determinant is cell type; for example, damaged fibroblasts and epithelial cells tend to senesce, whereas damaged lymphocytes tend to undergo apoptosis [91]. While it is well known that pH regulates many vital cell functions [92], the effect of pH on apoptotic signalling is poorly defined. Loss of the mitochondrial membrane potential is a hallmark of intrinsic apoptosis, because it is associated with the release of pro-apoptotic proteins into the cytosol [93]. Some studies demonstrated that severe extracellular acidification or alkalization induced pro-apoptotic effect [94, 95] in addition others revealed a link between acidosis and apoptosis [96, 97] while different study showed that pH had no effect on mitochondria-mediated apoptosis in hMSCs [98]. Even though viability test revealed a pH dependency, it was difficult to make conclusions about apoptotic processes. Comparing the apoptotic events in our experiment, we did not find increased apoptosis throughout the different pH conditions. It is possible that this may represent a time-dependent phenomenon and that 7 days or more may be required to observe an enhancement in hMSCs apoptosis. Cellular senescence occurs in response to various cellular stresses with the loss of proliferative capacity, despite continued viability and metabolic activity [99]. From our results, we saw that the strongest senescence occurred under the acidic pH (6.3 and 6.7). Taken together, we found that the effect of pH on proliferation or viability is modulated through increased senescence.

MSCs are characterized not only by the capacity for self-renewal but also by the ability to differentiate into osteoblasts and deposition of matrix minerals in which pH plays a regulatory role in the process of mineralization and bone repair [52, 100]. Poor mineralization at alkaline

conditions beyond pH 8.0 affected the solubility of calcium and magnesium pyrophosphate with no longer beneficial effect on bone mineralization [101]. It was also suggested that acidic pH reduce bone mineralization via increased hydroxyapatite solubility and systemic alkali therapy can be used to treat osteomalacia and the bone pain associated with it [98, 102, 103]. The physicochemical mechanism play also role in matrix mineralization based on the fact that low pH decreases calcium and phosphate tissue deposition because it increases their solubility [53, 64]. The most effective ways to destroy the ability of the nucleation core to induce mineral formation is exposure to acidic citrate buffer [104]. Also the nucleation activity and core is operative only within a very narrow pH range between 7.4–7.8 [105]. Either below or above this range, its ability to nucleate mineral formation was very reduced but in the studies by Wu et al [106], the pH range in which rapid mineral formation occurred was broader (pH 7.4– 8.0) indicating that at pH 8.0, the nucleation core is highly stable and insoluble. In accordance to this data, our results showed that a slight elevation in pH from 7.4 to 8.0 significantly increases the mineralization and the rise of pH to 8.5 does not further drive differentiation. This implies that small pH fluctuations will facilitate bone formation by elevating the phosphate ratio at least in the very narrow pH zone where the nucleation core is operative, up to a maximum of pH 8.0.

Since we have found defective mineralization at certain pH conditions, a question regarding the reason for the defective mineralization remained. It occurred due to impairment of osteogenic differentiation or due to the change in the extracellular environment. So we performed PCR to analyse the key osteogenic markers for differentiation and mineralization. From our results, PCR was different from the alizarin red staining where late markers of osteogenesis were expressed on the PCR with the lack of mineralization in the staining.

Osteoblasts arise from mesenchymal stem cells and determine the formation and structural organization of bone extracellular matrix and its mineralization [107]. Alkaline phosphatase is

synthesized by the osteoblasts and is presumed to be involved in the calcification of bone matrix [108]. Some researchers showed that pH 8.5 was optimum for ALP activity toward inorganic pyrophosphate during bone formation, while the activity was retained at the pH 7.3-7.4 [109, 110]. It was reported that decreasing the extracellular pH reduced the amount of collagen and alkaline phosphatase activity in mesenchymal stem cells, while others reported that alkaline pH decreased the alkaline phosphatase activity and could delay the differentiation of MSCs [54, 111]. It was shown in the literature that a higher calcium concentration inhibits the ALP activity but stimulates the expression of OPN associated with the osteogenic differentiation [112]. ALP activity appeared to decrease during mineralization [113]. In another study, it was also reported that a consistent marked loss of ALP activity occurs during mineralization. The time of onset and the extent of decline in ALP activity were found to mirror almost exactly the time of onset and the extent of calcium accumulation by the matrix vesicles (MV) [114]. Our results showed that ALP was decreased at higher pH indicating that mineralization down regulated the ALP activity.

In parallel, we also investigated the changes of the expression levels of several key osteogenic genes like Runx2, collagen I (Col1α1), OPN and OCN. We reported that among the analysed genes only OPN and OCN have been slightly influenced by the different pH values. In body, OPN is normally linked to mineralization of the tissues [115] and similarly to our data was found to be sensitive to pH [98, 116]. The highest expression we observed under pH 8.0, while the least was detected at acidic pH (6.3 and 6.7). The other osteogenic marker, OCN is linked to terminally differentiated osteoblast; however, its role in bone mineralization remains unclear since in OCN-deficient mice, it was discovered that osteocalcin does not necessarily ensure normal osteoblast function [117]. The trend in OCN expression in our hMSCs showed increased levels under lower and higher pH values (different to physiologic). Analysis of the other two osteogenic markers, collagen I and Runx2, showed no significant changes upon pH

treatment. Throughout all different pH conditions during differentiation, we found strong upregulation of both genes. Collagen I is the main building protein of bone, while Runx2 is the master regulator of osteoblast lineage [118-120] that control expression of several osteogenic genes among which is collagen I [121]. Expression of Runx2 and collagen I can be affected by the pH was depending on the MSC donor [98, 116, 122, 123].

The difference between the osteogenic markers expression and the matrix mineralization can be explained by initiation of matrix vesicle-mediated mineralization followed by collagen-mediated mineralization. The matrix vesicle mineralization is characterized by an initial formation of apatite or primary nucleation intracellularly within matrix vesicles (MV) that transport hydroxyapatite (HA) crystals outside of the cells [124-127]. During collagen-mediated mineralization (secondary nucleation), MV membranes break down and exposure of preformed HA to the extracellular fluid, allowing for propagation of HA deposition onto the collagenous ECM [125, 127] leading to mineralization by physicochemical and biochemical processes [128]. At low pH, calcium and phosphate tissue deposition decreases by increasing HA solubility with 10-fold for each unit decrease in pH [53, 64, 129]. According to our data, pH had an effect on hMSCs mineralization potential where induction of mineralization was more efficient at physiologic pH (7.0, 7.4 and 8.0) and much less at pH (6.3, 6.7 and 8.5).

Taken together, our study demonstrated that different pH conditions can strongly affect both cell self-renewal and mineralization. However, the same pH did not affect cell osteogenic potential since the main lineage–specific markers were expressed.

A number of limitations of this study needed to be considered. For instance, one question still not answered is whether comparison to diseased tissue would have been advantageous to determine cell responses to alterations in the physicochemical environment. Direct comparison can often be complicated due to inherent heterogeneity of both normal and diseased tissue and the difficulty in obtaining bone samples. Another thing is that cells from

different lots or donors were used resulting in the variability of the results represented by big means and standard deviations. Despite these limitations the effect of pH on the gene expression is preserved.

CONCLUSION

Within this study, it was proven that MSCs were highly sensitive to small shifts in external pH as their viability, proliferation and mineralization were affected. However, the osteogenic differentiation was not affected by pH. Thus, we think that in the injured sites, MSCs behaviour could be altered by the extracellular pH. The results of our study indicate that changing the pH of culture medium from normal to alkaline medium could improve the differentiation of MSCs to osteoblasts. There are currently various treatments clinically available and used for treating osteomyelitis of the jaw due to the complex nature of the infection, including the presence of microorganisms and change in pH. Future therapies for treating osteomyelitis could be based on shifting the pH of the local environment in the alkaline direction in order to overcome the acidic inflammatory exudates released during infection.

2. PUBLICATION II

FLUORESCENCE-GUIDED SURGERY FOR THE TREATMENT OF MEDICATION-RELATED OSTEONECROSIS OF THE JAW: A PROSPECTIVE COHORT STUDY

Sven Otto, Oliver Ristow, Christoph Pache, Matthias Troeltzsch, Riham Fliefel, Michael Ehrenfeld, Christoph Pautke. J of Craniomaxillofacial Surgery 2016. In Press Accepted Manuscript.

ABSTRACT

Introduction: The delineation of the necrotic bone is a crucial step in the surgical treatment of Medication-related osteonecrosis of the jaw (MRONJ). Several different approaches have been described including the innovative technique of fluorescence-guided surgery. However, until now there is a lack of data regarding the outcome. Therefore, the aim of the present study is to investigate the long-term success rates of fluorescence-guided surgery in the treatment of MRONJ. ***Patients and Methods:*** 54 Patients were prospectively assigned for surgical treatment of medication-related osteonecrosis of the jaw using fluorescence-guided surgery. Patients received doxycycline 100 mg twice a day for at least seven days preoperatively. Surgical treatment of MRONJ included complete removal of necrotic bone, which was monitored using the visual enhanced lesion scope (Velscope), followed by smoothening sharp bony edges and meticulous wound closure. Procedure success was assessed as postoperative maintenance of full mucosal coverage without pain, infection or bone exposure during regular follow-up. ***Results:*** The study included a total of 54 patients (32 female and 22 male, mean age of 71.4 ±9.2 years). In the last follow-up an intact mucosa and absence of exposed bone, pain or signs of infection was identified in 47 of 54 patients (87%) and 56 of 65 lesions (86.2%) after first surgery using fluorescence-guidance. In 4 patients with 6 lesions a second fluorescence-guided surgery was necessary to achieve complete

mucosal closure. Respectively, including the case with second surgical attempt 51 of 54 patients (94.4%) and 62 of 65 lesions (95.4%) showed complete mucosal healing. ***Conclusion:*** The study shows that fluorescence-guided surgery is a safe and successful treatment option which can be considered for all stages of MRONJ. The technique seems also promising for MRONJ cases under Denosumab.

INTRODUCTION

There is an ongoing debate on treatment strategies for medication-related osteonecrosis of the jaw (MRONJ): namely non-surgical (conservative) versus surgical treatment. The success rates for surgical strategies in MRONJ cases under bisphosphonates are significantly higher [130-133] than conservative treatment regimens [134-139] even though a direct prospective comparison between surgical and non-surgical treatment is missing till date.

MRONJ is currently diagnosed by the presence of exposed jawbone for a period that exceeds 8 weeks [140, 141]. Consequently, a successful therapy should aim for absence of bone exposure and restoration of mucosal integrity [133, 142]. Due to the fact that the infected necrotic and exposed bone will not be revitalized and resurrected, MRONJ should be removed even if only small bone areas are affected. Thus, the aim of the surgical therapy should be a complete removal of the necrotic bone. But even among those who favour surgical therapy there is an uncertainty as to which surgical technique is more effective. Indeed, the challenge as well as the limitations of the MRONJ therapy is that the margins of the osteonecrosis cannot be exactly determined, and therefore a clear demarcation of the necrotic bone is difficult if not impossible [140, 143]. The complete removal of necrotic bone is of crucial importance because otherwise there is the risk of disease recurrence or progression [133, 144]. Furthermore, it must be avoided to unintentionally and unnecessarily remove healthy bone without signs of osteonecrosis. Still, surgical experiences supported by various imaging modalities are used to remove only as much as necessary and as less as possible of necrotic bone [145-148]. Therefore, surgical therapy is dependent on the surgeon and can neither be comparable nor reproducibly objectified.

Fluorescence-guided bone surgery has shown promising results in the surgical MRONJ management [149-151]. Providing a controllable therapeutic approach, this technique may help to define the transitions between necrotic and non-necrotic bone during the surgical

procedure. Due to the fact that this surgical approach is easy to apply and reproducible, it may help to objectify surgical MRONJ therapy auguring an improvement of the treatment.

Therefore, the aim of this study is to examine the success rate of fluorescence-guided surgery in MRONJ patients in in terms of postoperative mucosal integrity and absence of bone exposure. Furthermore, pain, infection rates as well as disturbances of sensitivity are monitored.

PATIENTS AND METHODS

Patients

Over a period of 5 years (2010-2014), 54 patients were recruited and prospectively included in our monocentric cohort study (Department of Oral and Maxillofacial Surgery, Ludwig-Maximilians-University, Munich, Germany). 32 female and 22 male patients were enrolled with a mean age of 71.4 (standard deviation ±9.2 years; age range, 45-91 years). Inclusion criteria were: Exposed necrotic jawbone over a period of more than 8 weeks; with a history of antiresorptive drug treatment (bisphosphonates and / or Denosumab) in the absence of radiotherapy to the head and neck region according AAOMS [29, 141]. Exclusion criteria were a history of head and neck irradiation, metastatic bone disease of the maxillofacial region and contradictions for surgery under general anaesthesia. After obtaining the approval of the institutional ethics committee (LMU 189/10), patients were informed about all treatment options and provided written informed consent.

Surgical procedure

All surgical procedures were performed by the same board-certified and specialized Oral and Maxillofacial Surgeons (SO) under general anaesthesia using a nasal intubation. The surgeries were performed under sterile conditions following a standardized operation protocol [130].

All patients received 100 mg doxycycline twice a day for at least 7 days preoperatively. Surgical procedures were performed as the fluorescence guided surgery technique described

previously by our group using the VELscope® system (LED Dental, White Rock, British Columbia, Canada) to induce and visualize fluorescence of the jaw bone [130, 143, 151, 152]. After surgical bone exposure was performed the bone fluorescence showed viable bone in a bright greenish fluorescence and necrotic bone areas showed none or only pale fluorescence. Reddish fluorescence was considered as a bacterial colonization or infection of necrotic bone parts and the respective areas were removed. Necrotic bone was removed using a burr a homogenous greenish bone fluorescence was observed as described in previous studies [130, 143, 149, 153, 154]. It should be stressed that only necrotic and infected bone parts were removed and the surrounding vital bone was preserved which means that no resections including safety margins have been performed. Thereafter, sharp bony edges were smoothened using burrs and diamante burrs. A tension free wound closure was achieved using mucoperiosteal flaps and simple as well as back stiches (Serafit 3-0, SERAG-Wiesner GmbH Germany). In extensive cases of the maxillary molar and premolar region (stage 2 and 3) a second layer of wound closure was achieved using the buccal fat pad before mucoperiosteal closure.

All patients stayed in hospital for at least 48 hours after surgery. Patients received the routine postoperative instructions and routine postoperative analgesic drug therapy; antibiotic treatment was continued using Augmentin 2.2 g or Unacid 3g intravenously three times per day for 3-5 days. In case of a penicillin allergy clindamycin 600 mg was used. In cases of severe infection (mainly stage 2 and 3) metronidazole 500 mg (1-0-1) was administered additionally. In cases of renal function disturbances the doses were adjusted accordingly. The antibiotic treatment was continued orally after discharge from hospital for 2-4 weeks orally.

Measurements

Regular clinical examinations were performed daily during in-patient treatment, weekly during the first month and monthly during first year of out-patient treatment. The surgical

treatment was only considered a success if full mucosal coverage without signs of residual infection or exposed bone was achieved at the time of last follow-up. Furthermore, all patients were asked for pain and were examined for signs of sinusitis and checked for oro-antral fistula in cases of upper jaw lesions and checked for sensitivity in the lower lip area in cases of MRONJ of the lower jaw.

Statistical analysis

Descriptive statistics were computed using SPSS version 16. Results are expressed as percentages or as mean values including standard deviation and range. Means were compared by statistical testing (students t-test), where $p < 0.05$ was considered to be significant.

RESULTS

Base line characteristics

54 patients (32 female and 22 male) patients with a mean age of 71.4 years (standard deviation 9.2 years) were included in the study. The mean age of the female patients was 70.4 years (standard deviation 7.6 years); the mean age of all male patients was 72.9 years (standard deviation 7.0 years). Respectively, there was no significant difference (Figure 2.1).

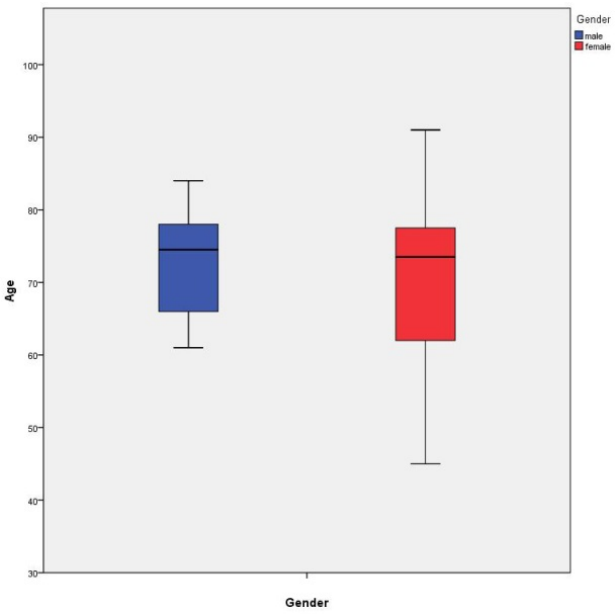


Figure 2.1: Age range of patients with MRONJ.

45 of the patients (83.3%) suffered from an underlying malignant disease, specifically breast cancer (n=20; 37%), prostate cancer (n=16; 29.6%), and multiple myeloma (n=4; 7.4%). There were also cases of metastatic thyroid cancer (n=2), squamous cell carcinoma (n=1), bronchial cancer (n=1), and endometrial cancer (n=1) in the study cohort. In the remaining 9 (16.7%) patients osteoporosis was the cause of the antiresorptive treatment. An overview is given in (Figure 2.2).

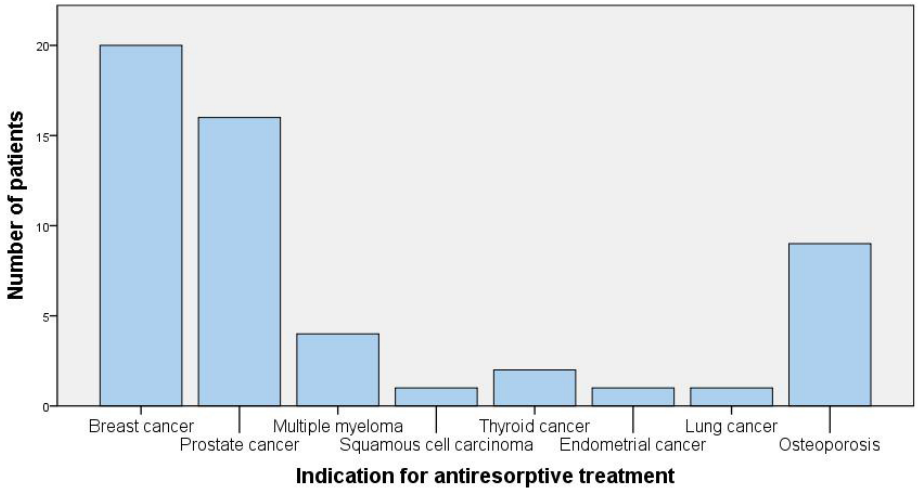


Figure 2.2: Overview of primary cause of MRONJ.
Overview of the underlying diseases leading to anti-resorptive treatment with bisphosphonates and Denosumab in patients suffering from MRONJ.

Of the 54 patients included, 47 were treated with nitrogen-containing bisphosphonates (87%), 3 had a history of Denosumab intake (5.5%) and the remaining 4 patients (7.4%) reported a sequential intake of bisphosphonates and Denosumab. The most common anti-resorptive drugs within the cohort were Zoledronate (n=40; 74.1%), Alendronate (n=5; 9.3%), Ibandronate (n=2; 3.7%) and Denosumab (n=3; 5.5%) or the combination of bisphosphonate and Denosumab (n=4; 7.4%). The mean duration of intake of the anti-resorptive drugs was 46.3 months (SD 31.8 months).

The 54 patients revealed 65 MRONJ lesions. 40 of the lesions (61.5%) were located in the mandible and 25 (38.5%) were located in the maxilla. The majority of the lesions referred to stage 2 (n=42; 64.6%) and stage 3 (n=8; 12.3%) according to AAOMS 2014 (Ruggiero et al.,

2014a). It is worth mentioning that also stage 1 lesions were included (n=14; 21.5) and even a singular case of stage 0 (n=1; 1.5%). The mean follow-up of the patients was 12.9 months (median 11 months; range 1-39 months).

Results of fluorescence-guided bone surgery

The first surgical intervention using fluorescence-guided bone surgery resulted in complete mucosal healing in 47/54 of the evaluated patients (87%) and 56/65 lesions (86.2%) without any kind of bone exposure and without complaints at the time of last follow-up. Typical cases are illustrated in Figure 2.3 and Figure 2.4.

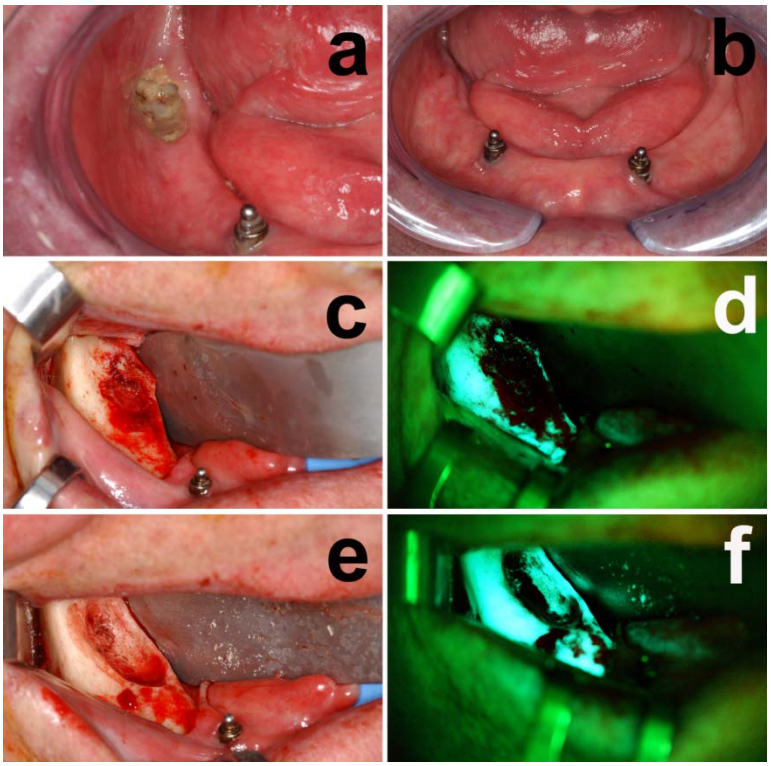


Figure 2.3: 58-years old female presented with MRONJ. Illustration of a 58-year old female patient suffering from breast cancer who received 56 months zoledronate and developed a medication-related osteonecrosis of the jaw in her left mandible (region 37/38 mainly lingual aspect). The illustration depicts the clinical intraoral situation prior to (a) and 3 months after surgery (b). The intraoperative clinical and fluorescence view prior to removal of the necrotic bone (c and d) and after the removal of necrotic bone and smoothening of sharp bony edges (e and f) are also illustrated. Note the weak green fluorescence in the lingual aspect region 37/38 corresponding to the necrotic bone area (d) as well as the reddish fluorescence in this area corresponding to the bacterial infection of this region prior to removal of the necrotic and infected bone parts as well as the homogenous greenish fluorescence after the removal and the absence of red fluorescence after the removal of necrotic bone parts.

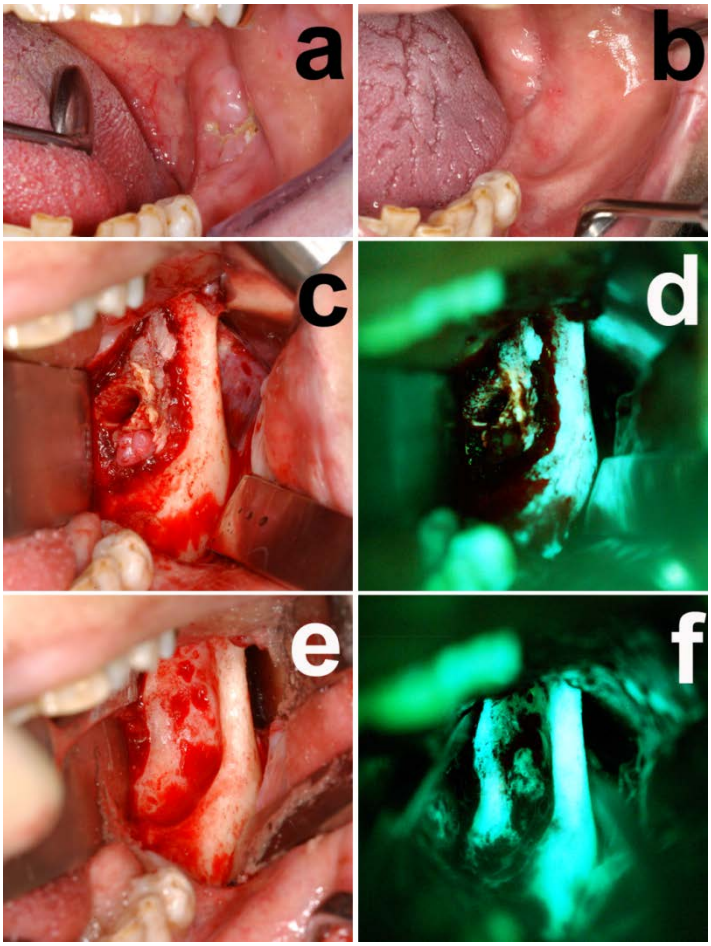


Figure 2.4: 74-year old male presented with MRONJ. Illustration of a 74-year old male patient suffering from prostate cancer who has received intravenous treatment with zoledronate over 2 years and exposed necrotic bone and putrid exudation of the right mandible (region 47/48) according to a medication-related osteonecrosis of the jaw (region 47/48) prior to (a) and one year after fluorescence-guided surgery (b). During surgery there was necrotic bone with diminished fluorescence in the lingual aspect of the mandible (c and d). After complete removal of the necrotic bone parts and smoothening of sharp bony edges the fluorescence was homogenously green (e and f).

2/54 (3.7%) patients were also free of complaints and had no bone exposure and a complete mucosal coverage of the bone. However, in these patients the lesions in the maxilla were that extensive (AAOMS stage 3) that oro-antral fistula persisted. Both patients preferred an obturator prosthesis instead of another surgical approach to close the oro-antral fistula. One of these two cases is illustrated in Figure 2.5.

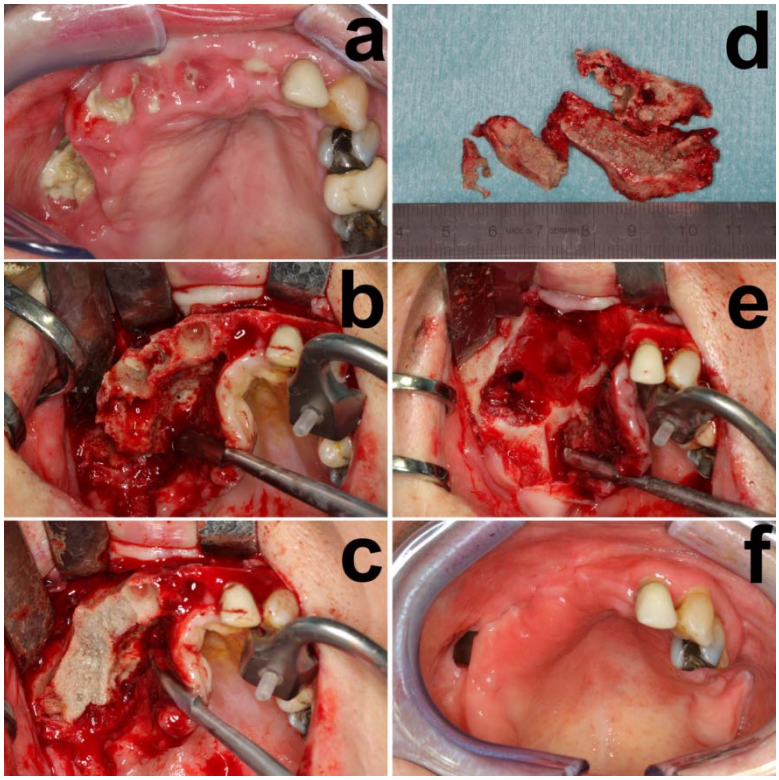


Figure 2.5: 62-year old female presented with MRONJ. The patient suffered from metastatic breast cancer who received zoledronate intravenously (4mg every 4 weeks) for more than 3 years and developed an extremely extended stage III MRONJ in her right maxilla with bone exposure suppuration which was also extremely painful on palpation (a). After antibiotic pre-treatment the patient was treated surgically. After exposure (b) the whole extent of the MRONJ lesion became visible which included parts of the hard palate and parts of the facial wall of the maxillary sinus. After removal of parts of the necrotic bone (c and d) it became obvious that the whole alveolar process of the right maxilla was necrotic and infected. The necrotic bone was completely removed using fluorescence-guided surgery (e) and a double-layered plastic wound closure was performed using the buccal fat pad and mucoperiosteum. In the postoperative course the patient was free of pain but developed a wound healing disturbance and an oro-antral fistula. After complete healing there was no bone exposure but the oro-antral fistula persisted (f). As the patient was free of complaints she did want to go for another surgery to close the oro-antral fistula. So she was treated using an obturator prosthesis as described in detail elsewhere [155].

5/54 patients (9.3%) with 7/65 lesions (10.8%) showed stage improvement and were free of pain after first surgery but still had bone exposure present. 4 of these patients (with 6 of the 7

lesions) underwent a second surgery using fluorescence-guided bone surgery, which in all 4 patients and all 6 lesions resulted in complete mucosal healing. An overview of the treatment outcome after first surgery and including the 4 cases with second surgery is provided in Table 2.1.

Table 2.1: Overview of the treatment outcome after first surgery including the 4 cases with second surgery

	Pre-operative total n=54 (65)	**After first surgery total n=54 (65)**	**After second surgery in n=4 (6); total 54 (65)**
Bone exposure	53 (63)	5 (7)	1 (1)*
Pain / complaints	43 (51)	1 (1)	1(1)**a
Impaired sensitivity N. V3	7 (7)	1 (1)	1 (1)**b
Sinusitis/ oro-antral fistula	7 (8)	2 (2)	2 (2)**b
Pathological fracture	0	0	0

*change due to complete mucosal healing in 4 patients with 6 lesions who underwent second surgery, **no change as none of the affected patients was treated surgically again, a due to worsening of underlying malignant disease, b due to patients wish and no need for second surgery

Only in one single patient (1 lesion) the bone exposure persisted and was subsequently treated conservatively as the patients systemic condition had worsened over time caused by the underlying malignant disease. The initial stage improvement (stage 2 prior to surgery and stage 1 after surgery) gradually worsened over time back to the initial stage 2.

Taken together the results of the first and second surgery 51/ 54 patients (94.4%) and 62 / 65 lesions (95.4%) showed complete mucosal healing and no bone exposure. Two further patients were free of complaints and had no bone exposure but developed oro-antral communication. Only one patient with a single lesion showed persistent bone exposure which could not be addressed by a second surgery due to the worsened general condition of the patient.

It is worth mentioning that no continuity resection had to be performed in the mandible, whereas the removal of MRONJ in the maxilla resulted in resection-like defects in 4 cases.

Two of those cases developed a persistent oro-antral fistula. None of the patients showed a recurrence of MRONJ in the respective area after complete mucosal healing in the further postoperative course. None of the patients developed a pathological fracture of the mandible.

DISCUSSION

There is an ongoing debate and certainly no consensus yet regarding the management of patients with MRONJ. Moreover, there is not even consensus regarding the main treatment aim and the optimal outcome measures.

While some authors recommend conservative treatment protocols mainly aiming in relief of pain and control of infection, a number of papers have suggested that in patients with a good performance status the primary aim of treatment should be mucosal healing as this is the physiological status, rather than bone exposure without symptoms [130, 133, 149, 156, 157]. Conservative treatment cannot achieve this aim, neither considering the frequency nor the predictability especially in oncological patients who have received long term intravenous courses of nitrogen-containing bisphosphonates. In this respect, Hoff et al.[135] reported 23% healing (3/13 patients) and similarly Nicolatou-Galitis et al.[158] reported mucosal healing in only 14.9% of BRONJ cases (7/47) managed conservatively, notably after a median time of 8 months (range 2-36 months, mean 14.7 months), while pain subsided in 80.9% (38/47). It is also worth mentioning that 4 of the 7 patients who showed complete healing referred to stage 0 according to the AAOMS definition [29, 141]. This in turn means that the outcome results for cases with bone exposure are even less convincing. Regardless of the type of definition or staging system applied, the vast majority of patients with BRONJ (especially oncological patients) cannot be cured using conservative measurements and have long lasting jaw bone exposure which can not only affect their quality of life [159], but may also limit the oncological treatment options including immuno- or chemotherapy and possibly further anti-resorptive treatment with bisphosphonates or Denosumab [160, 161]. Conservative treatment

might be adequate if the aim of treatment is to slow down or stop disease progression and to alleviate pain and superinfection of the exposed bone, while there is increasing evidence supporting surgical protocols if the aim of treatment is mucosal healing.

In this respect our study showed that fluorescence-guided bone surgery is a reliable and promising treatment option for patients suffering from MRONJ.

This is in line with the recent literature where Carlson et al. reported mucosal integrity of 92% after surgical resection in a case series of 95 patients [133]. Likewise, other authors stated a healing rate up to 89% (12 month follow up; n=50) [131] as well as 88 % (60 weeks follow up; n=24) after surgical treatment. Prospective case series further support the benefit of a surgical treatment of BRONJ: Bedogni et al. 2011 [162] (n = 30) surgical treatment: 90 % healing 6 months follow up, Schubert et al., 2012 [163] (n = 54) surgical treatment: 89 % healing (min. 3 months follow up), Jacobsen et al., 2012 [164]. (n = 64 surgical treatments: 78 % healing (7 years follow up). It is however hard to compare the different studies because the underlying study cohorts were composed of different populations regarding the proportion of oncological and osteoporotic patients, regarding the surgical protocol applied (e.g. only removal of necrotic bone versus resection) and regarding the outcome evaluation and postoperative follow-up but the bottom line of all of the above mentioned studies was that patients suffering from MRONJ can successfully be treated using surgical treatment protocols.

Comparative studies also seem to substantiate these findings. The multivariate analysis of Mücke and co-workers showed a lower recurrence rate for surgically-treated ONJ patients when compared to conservative treatment (n=108) [144], as well as the multivariate analysis of Graziani et al., 2013 [165] (n = 347) confirmed significantly more mucosal healing for surgical treatment versus conservative protocols. Finally, a 2014 systematic review by Rupel et al. [166], and another very recent systematic review meeting PRISMA guidelines [167]

which analysed data from 97 studies and 4,867 patients suggest that surgical treatment protocols are superior to conservative management [139].

The most important parts for a successful surgical treatment of MRONJ include pre- and postoperative antibiotic treatment, complete removal of the necrotic and often infected bone parts, smoothening of sharp bony edges and a complete and reliable plastic wound closure. The aim of the preoperative antibiotic treatment is to stop disease progression and to reduce infection in order to provide optimal conditions for the surgical treatment. The complete removal of necrotic bone is essential to provide the conditions for bone and soft tissue healing and in order to avoid reinfection of necrotic bone parts. Fluorescence-guidance might be a tool to optimize the completeness of removal of necrotic bone parts. Smoothening of sharp bony edges is of special importance because of the remodelling suppression caused by anti-resorptive drugs and seems therefore even more important when the anti-resorptive activity is high (e.g. after multiple years of intravenous bisphosphonate intake or shortly after the last application of anti-resorptive drugs with short half-life e.g. Denosumab). The aim of the plastic wound closure is to ensure that the delayed and endangered healing of the jaw bone treated with anti-resorptive drugs can take place in an undisturbed manner. In the experience of the authors of this article safe and reliable mucoperiostal flaps closed with multiple back stiches seems sufficient. However, it is recommended to perform double layered wound closure whenever possible. In this respect for example the use of the buccal fat pad in cases of MRONJ of the molar and premolar region of the maxilla and the use of the mylohyoid flap in the mandibular molar region might have advantages. The postoperative antibiotic treatment should protect the wound healing period and avoid reinfection of the bone. A prolonged antibiotic treatment seems to have advantages.

According to several guidelines including the AAOMS position paper and the ASBMR expert panel recommendation early stages of MRONJ should be treated conservatively and surgical

treatment should only be applied to stages 2 and 3 [29, 168, 169]. The authors of this paper disagree with these opinions especially in patients receiving intravenous administrations of bisphosphonates in the oncological setting [157]. In fact treatment of all stage 0 and 1 lesions resulted in complete mucosal healing with minimal morbidity and a predictable and reasonable time frame. Furthermore, after complete mucosal healing the respective patients had no restrictions regarding their further oncological or osteological treatment including further anti-resorptive treatment. Actually, surgical treatment of early MRONJ lesions offers a lot of advantages including the usually smaller extent of the lesions leading to less extended surgical removal of bone and minor functional impairments. Besides that lack of infection usually offers better conditions for surgical treatment. Therefore, the authors of this paper call for a re-evaluation of concepts and aim for a change of paradigms. Instead of long lasting, unpredictable conservative treatment approaches usually resulting in improvements of symptoms but rarely leading to complete mucosal healing should be replaced by early surgical interventions aiming in complete mucosal healing in a predictable timeframe and resulting in optimized functional outcomes as respective surgeries which frequently occur after unsuccessful conservative treatment approaches can be avoided. Indeed, it is worth mentioning that after changing our treatment concept to early surgical intervention we did not experience MRONJ cases, in which we had to perform continuity resections of the mandible and no microvascular reconstructions were necessary any more, which we experienced during the timeframe where we applied a more conservative treatment approach in early stages. So in fact so called conservative treatment protocols might lead to the necessity of more aggressive and large resections including all functional impairments over the long run [169]. The authors of this paper do not doubt that ablative surgery including continuity resections of the mandible and microvascular reconstructions are necessary in selected cases of MRONJ whereas a lot more cases of osteoradionecrosis require this radical treatment. We think that

the progression of MRONJ cases presenting in early stages can be avoided when treated adequately. However, conservative treatment approaches and the role of drug holidays might well be different in MRONJ cases under Denosumab especially in cases without prior bisphosphonate treatment because of the much shorter half-life of Denosumab (26 days) when compared to bisphosphonates in bone [157].

Regarding the specific technique of fluorescence-guided bone resection it needs to be mentioned that it is not yet certain what exactly causes the intraoperative fluorescence. Recent reports suggest that there is an auto-fluorescence without tetracycline bone labelling, leading to similar bone fluorescence of tetracycline-exposed tissue [170, 171]. Indeed, it is well known that not only tetracycline but also components of the extracellular matrix e.g. calcified tissues (bone or teeth) have fluorescence properties [130, 154]. A combination of these components might contribute to the fluorescence effects that can be used in the treatment of MRONJ. Therefore, further basic and clinical research is needed in order to investigate the fluorescence properties and their differences. Once the causes for fluorescence-guided surgical approaches might be suitable not only for MRONJ but also for osteoradionecrosis and osteomyelitis [154].

Limitations of the present study include the inhomogeneous recall intervals of some of the patients which were mainly due to their underlying diseases and respective oncological treatment protocols. Furthermore there were only very few cases of MRONJ due to Denosumab intake. Given the much shorter half-life of Denosumab when compared to nitrogen-containing bisphosphonates there might be a different and more important role of conservative treatment protocols especially when there is no pre-treatment with bisphosphonates and no further necessity of anti-resorptive treatment. However, up to now there is no study which directly compares the outcome of conservative and surgical treatment

and there is also no study comparing conventional surgical treatment versus fluorescence-guided surgery.

The available data might not yet be robust enough to inform guidelines on the treatment of MRONJ, especially as there is hardly any data on how to manage patients exposed to Denosumab where conservative treatment might theoretically play a different role due to its much shorter half-life. There is an urgent need of prospective randomized trials comparing surgical and non-surgical treatment of MRONJ and including patient-centred outcome measures like quality of life before, during and after treatment. Ultimately, the clinical decision making will always be based on individual risk assessment, especially as most patients with MRONJ have multiple comorbidities, which require knowledge about the predictable efficacy and limitations of the all treatment options.

CONCLUSION

We conclude that fluorescence-guided bone resection is a reliable surgical treatment option for patients suffering from medication-related osteonecrosis of the jaw.

3. PUBLICATION III

ROLE OF MICROBIOLOGICAL CULTURE AND PCR IN MEDICATION-RELATED OSTEONECROSIS OF THE JAW (MRONJ)

Sappasith Panya, Riham Fliefel, Florian Probst, Matthias Tröltzsch, Michael Ehrenfeld, Sören Schubert, Sven Otto. Under review in J of Craniomaxillofacial Surgery 2016.

ABSTRACT

We hypothesized that local infection plays a critical role in the pathogenesis of medication-related osteonecrosis of the jaw (MRONJ). Recent developments in molecular methods have revolutionized new approaches for the rapid detection of microorganisms including those difficult to culture. The aim of our study is to identify the bacterial profiles in MRONJ by microbiological culture and polymerase chain reactions (PCR). A retrospective analysis was performed on MRONJ patients from 2008 to 2014. The bacterial profile from MRONJ bone samples was determined using microbiological culture and PCR. Ninety five patients fulfilled the inclusion criteria with mean age of 69.85 ± 8.71 years. A female predilection was detected. The mandible was more commonly affected than maxilla. Tooth extraction was the frequent triggering factor. Breast cancer was the primary cause for administration and intravenous bisphosphonates were the most commonly administrated anti-resorptive drugs. The majority of patients were classified as stage 2. Posterior teeth were most commonly affected. Based on bone culture results, the most common microorganism were both actinomyces and mixed flora. PCR confirmed the presence of actinomyces in 55 patients. Our data suggest that PCR might be an innovative method for detection of microorganisms difficult to culture using traditional microbiological techniques.

INTRODUCTION

Medication-related osteonecrosis of the jaw (MRONJ) is a potentially devastating complication of anti-resorptive drugs used globally to treat bone disorders as osteoporosis, skeletal complications associated with osseous metastasis and multiple myeloma [27, 28]. Nowadays, the pathophysiology of MRONJ is not clearly understood. Numerous theories have been proposed, neither of which can provide an adequate explanation of the disease. MRONJ was perceived as a type of avascular necrosis due altered bone turnover or direct toxicity to the soft tissue, infection, inflammation, inhibition of angiogenesis or suppression of innate or acquired immunity have been identified as possible explanations of the disease process [172].

Bacterial infection to the maxillofacial region has been suggested as key factor for the pathogenesis and progression of MRONJ [18, 173]. The oral cavity comprises of more than 750 bacterial species existing as mixed biofilm communities [32]. The mandible and maxilla are covered by thin layer of mucosa in close proximity to the external environment. After invasive dental procedures, oral trauma or soft tissue infection, microbial biofilms in the mouth and saliva gain access to the exposed jaw bone and play a significant role in the necrosis of the bone, inhibition of oral wound healing and facilitating bacterial colonization on bone surface [33, 34]. Actinomyces were regularly found in MRONJ suggesting a latent role of infection in the pathogenesis [174-176]. Actinomyces are filamentous gram-positive anaerobic bacteria that usually can be found in calculus, periodontal pockets, carious lesions and oral mucosal surfaces, in addition to the upper respiratory, gastrointestinal tracts and vagina. They are common saprophyte bacteria of low virulence in nature causing no disease as long as they stay on the surface of the mucosa but in certain conditions where the integrity of the mucosal barrier is compromised, the bacteria may be pathogenic and gain access to the

oral tissues or jawbones initiating a prolonged chronic inflammatory process, creating a tumour-like mass, tissue destruction, osteolysis and multiple sinus tracts [177-179].

MRONJ lesions are usually colonized by oral bacteria and the use of systemic antibiotics failed to restrict the bacterial colonization and effective healing of the lesion. It is important to identify the bacterial species colonizing jaw bone associated with the disease to delineate the pathogenesis. Moreover, it is not well understood whether the bacteria involved in MRONJ is similar or different to other biofilm associated bone infections in the oral cavity [180]. Recently, bone abnormalities were studied by various modalities but none proved to be reliable in describing the infectious nature of the disease. Recent advances using biomolecular profiling to describe MRONJ flora have decreased this gap [181].

Here, we identify the bacterial profiles that colonize MRONJ bone samples determined by culture approaches and polymerase chain reactions (PCR) with clinical features of patients. This line of investigation could provide rationale in the future for MRONJ therapeutics and targeted antimicrobial therapy.

PATIENTS AND METHODS

Study design

This is a retrospective study of MRONJ patients treated at the Department of Oral and Maxillofacial Surgery, Ludwig-Maximillians-University Clinic, Munich from January 2008 to December 2014. Inclusion criteria were based on the American association of oral and maxillofacial surgery (AAOMS) Position paper [30]. Patients missing clinical, radiographic or follow-up data were excluded or if they had a history of head and neck radiation. Appropriate Institutional Review Board approval was obtained.

Data collection

Clinical data relevant to the study were extracted and entered into an excel datasheet with a detailed history concerning: age, gender, location and teeth involved in the lesion, primary

cause of the disease, comorbidities, clinical presentation, MRONJ clinical staging, type of anti-resorptive drug, route of administration and pathological/microbiological findings of bone samples. Bone samples were obtained from bone resection surgeries and were sent for microbiological investigations and PCR. Due to high likelihood of false positive culture from environmental exposure, we considered only at least strongly positive culture result (+2) as positive culture. One bone sample from each MRONJ patient was cut into fragments and prepared for microbiological analysis as described below.

Microbiological culture of bone samples from MRONJ

Bone samples have been introduced in classical bacterial diagnostics. For this, aerobic cultures were prepared on Columbia blood-agar, MacConckey-agar and Columbia-CAN-agar, anaerobic cultures on Schaedler-agar and Schaedler-KV-agar (all agar plates from BD, Heidelberg, Germany). Besides, the swabs were cultivated in thioglycolate broth. All aerobe cultures have been read after 24h, 48h and 72h, the anaerobic cultures after 2d, 5d and 7d. The bacterial counts have been enumerated semi-quantitative and bacterial colonies were objected to MALDI-TOF MS for further species identification.

Samples were evaluated by the use of Microflex LT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in linear positive-ion mode across the m/z range of 2,000 to 20,000 Da. Each spot was measured by using 240 laser shots at 60 Hz in groups of 40 shots per sampling area of the spot. Spectra were analysed by using MALDI Biotyper software (v 3.1 – Build 65). Sample preparation included either the “direct transfer method”, the “Extended Direct Transfer method (EDT)” or the “ethanol/formic acid extract method” as previously described [40]. Resulting spectra were compared against reference spectra using Bruker MALDI-TOF Biotyper software to obtain identification with a confidence score. For most isolates, the MSP (Main Spectral Projection) reference spectra were those contained in the Bruker database of 2013 (database version V 3.3.1.2) containing 364 genera, 2185 species

and 4613 individual MSP. Results with score values >2 were considered as correct species identification, results displaying values of 1.5≤ and ≤2 were accepted as correct genus identification.

PCR of Actinomyces

Identification of bacteria by sequencing of 16S rDNA has been performed as described previously with some modifications [182]. In brief, crude bacterial lysates were prepared directly from culture plates by suspending bacteria from a clonal culture in 100 µl of RT-PCR grade water (approximately McFarland Standard 2.0) and placed in a hot block at 100 °C for 10 min. A ~800 bp-fragment of 16S rDNA was amplified using the universal primer pair FD1 5′-AGAGTTTGATCCTGGCTCAG-3′ and 800r 5′-GAGTACCAGGGTATCTAATCC-3′. Resulting PCR amplicons were sequenced using the same primers and standard sequencing methods. Data from both strands was aligned in SeqMan (DNASTAR Lasergene 8 Suite) to generate a contig of around 800 bp. The consensus sequences were then used to compare with online databases (NCBI BLAST—http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (http://rdp.cme.msu.edu/). Identification criteria of 99% sequence identity for identification to species level were applied [183] where matches had to be to the species type strain. The identities of type strains, as well as accession numbers in NCBI for equivalent 16S rDNA sequences, are available at http://www.bacterio.cict.fr/ for all validly published bacterial species.

Statistical analysis

Descriptive statistics were computed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as mean values including standard error of the mean and range. Means were compared by statistical testing (Student's t-test), where $P < 0.05$ was considered to be significant.

RESULTS

A total of 150 patients were diagnosed with MRONJ from 2008 to 2014. However, 95 patients satisfied the inclusion criteria and form the basis of this study. Flow chart of the number of patients included in the study is illustrated in (Figure 3.1).

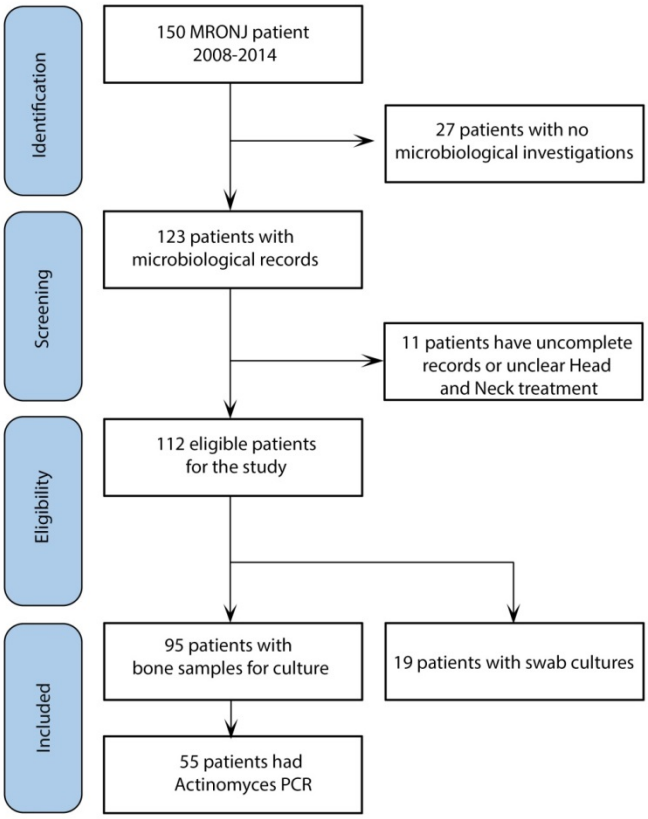


Figure 3.1: Flow chart.
Flow chart of the number of patients included in the study.

The mean age of the patients was 69.9 ± 8.7 years; with a male to female ratio of 1:1.4 (39 males and 56 females). Breast cancer was the primary cause for the administration of antiresorptive drugs (n=35; 36.8%), followed by prostate cancer (n=24; 25.3%) and osteoporosis (n=13; 13.7%) in addition to multiple myeloma (n=10; 10.5%), lung cancer (n=4; 4.2%) and finally other cancers (n=9; 9.5%). The relevant comorbidities identified included: diabetes mellitus (n=17; 17.9%), cardiovascular diseases (n=29; 30.5%), chemotherapy (n=57; 60%), irradiation other than head and neck (n=51; 53.7%), steroid

intake (n=28; 29.5%), anti-angiogenic drugs (n=2; 2.1%) and smoking (n=28; 29.5%). The most commonly administrated anti-resorptive drugs (ARD) were bisphosphonates (BPs) in 85 patients (89.5%) of which, zoledronate in 58 (61.1%), pamidronate in 3 (3.2%), ibandronate in 2 (2.1%), combination of BPs in 22 (23.1%). Only ten patients received Denosumab (10.5%). Among the ARD groups, 79 patients (83.2%) had intravenous ARD, 6 patients (6.3%) with oral and 10 patients (10.5%) had subcutaneous injection. The baseline characteristics of the patients included in the study are listed in (Table 3.1).

Table 3.1: Characteristics of patients diagnosed with MRONJ.

Variable	Category	Number of patients (%) (n=95)
Age (years)	Mean	69.9 ± 8.7 years
Gender		
	Male	39 (41.1)
	Female	56 (58.9)
Primary cause		
	Breast cancer	35 (36.8)
	Prostate cancer	24 (25.3)
	Multiple myeloma	10 (10.5)
	Osteoporosis	13 (13.7)
	Lung cancer	4 (4.2)
	Other (Colon, Systemic Mastocytosis, Renal, Bladder, Thyroid, Endometrium)	9 (9.5)
Comorbidities		
	Diabetes Mellitus	17 (17.9)
	Cardiovascular disease	29 (30.5)
	Chemotherapy	57 (60)
	Irradiation (body)	51 (53.7)
	Steroid intake	28 (29.5)
	Antiangiogenic drugs	2 (2.1)
	Smoking	28 (29.5)
Antiresorptive drug (ARD)		
Bisphosphonate:		85 (89.5)
	Zoledronate	58 (61.1)
	Pamidronate	3 (3.2)
	Ibandronate	2 (2.1)
	Combination	22 (23.1)
Denosumab		10 (10.5)
Route of administration		
	Intravenous	79 (83.2)
	Oral	6 (6.3)
	Subcutaneous	10 (10.5)

Initial presentation of the lesion was only one case referred to stage 0 (1.1%) with no bone exposure but non-specific signs and symptoms of MRONJ. Fifteen patients (15.8%) were categorized as stage 1 where bone was exposed in the absence of pain and clinical signs of infection. The majority of cases (n=59; 62.1%),) were classified as stage 2 based on exposed necrotic bone in the maxillofacial region accompanied by pain or signs of infection. Twenty patients (21.1%). were presented with stage 3 lesions with complications such as pathological fracture, extra-oral fistula formation, extension of the lesion to the inferior border of the mandible or to the floor of the maxillary sinus. Most of MRONJ lesions were located in the mandible (n=55; 57.9%), 25 patients (26.3%) had maxillary lesions and 15 patients (15.8%) had involvement of the maxilla and mandible. Characteristics of MRONJ lesions are presented in (Table 3.2).

Table 3.2: Characteristics of MRONJ lesions.

Characteristics	Number of patients (%)
Staging of MRONJ	
Stage 0	1 (1.1)
Stage 1	15 (15.8)
Stage 2	59 (62.1)
Stage 3	20 (21.1)
Clinical presentation	
Pain	81 (85.3)
Exposed bone	70 (73.7)
Disturbance in wound healing	55 (57.9)
Inflammation	54 (56.8)
Pus	39 (41.1)
Pathological fracture	9 (9.5)
Swelling	55 (57.9)
Fistula	35 (36.8)
Sinus involvement	13 (13.7)
Histopathological Features	
Necrotic bone	94(98.9)
Inflammatory infiltrate	87(91.6)
Bacterial colonization	67(70.5)
Location	
Mandible	55 (57.9)
Maxilla	25 (26.3)
Both	15 (15.8)
Triggering events	
Extractions	56 (58.9)
Dentoalveolar surgery	15 (15.8)
Denture sore	4 (4.2)
Periodontal treatment	7 (7.4)
Spontaneous	13 (13.7)

The posterior teeth specially the first and second molars were the most affected teeth by MRONJ than the anterior teeth. The frequency of MRONJ in teeth of each quadrant is represented in (Figure 3.2).

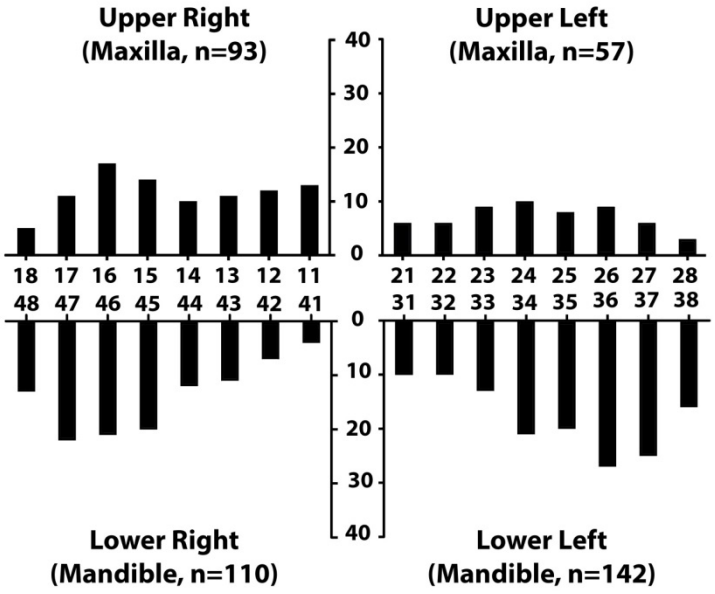


Figure 3.2: Distribution of teeth involved in MRONJ.
Distribution of teeth involved in MRONJ at the different quadrants of maxilla and mandible where n is the number of teeth involved in each quadrant.

Regarding the onset of MRONJ, the most frequent signs and symptoms were: pain in 81 patients (85.3%), exposed bone in 70 patients (73.7%), disturbance in wound healing in 55 patients (57.9%), inflammation in 54 patients (56.8%), pus in 39 patients (41.1%), pathological fracture in 9 patients (9.5%), swelling in 55 patients (57.9%), fistula in 35 patients (36.8%) and sinus involvement in 13 patients (13.7%). The lesions were stratified into lesions with a known triggering event or spontaneous development of MRONJ. The most common events prior to the development of MRONJ lesions were extraction in 56 patients (58.9%), dentoalveolar surgery in 15 patients (15.8%), denture sores in 4 patients (4.2%), periodontal treatment in 7 patients (7.4%) and lesions developed spontaneously in 13 patients (13.7%). Histopathological examination of the bone specimens revealed typical picture of MRONJ lesions where nearly all the patients showed an active inflammatory process with

necrotic bone (n=94, 98.9%), inflammatory cell infiltrate (n=87, 91.6%) and bacterial colonization (n=67, 70.5%). The characteristics of MRONJ lesions are illustrated in Table 2.

Ninety five patients had undergone microbiological culture tests. However, only 55 patients had undergone PCR for actinomyces. Based on bone culture results, the most common microorganism were both actinomyces and mixed oral flora (n=23, 24.2%) each then enterobacter group (n=19, 20%), streptococci (n=18, 18.9%), miscellaneous microorganisms (n=13, 13.6%), candida (n=9, 9.4%) and finally enterococcus (n=5, 5.2%) (Figure 3.3).

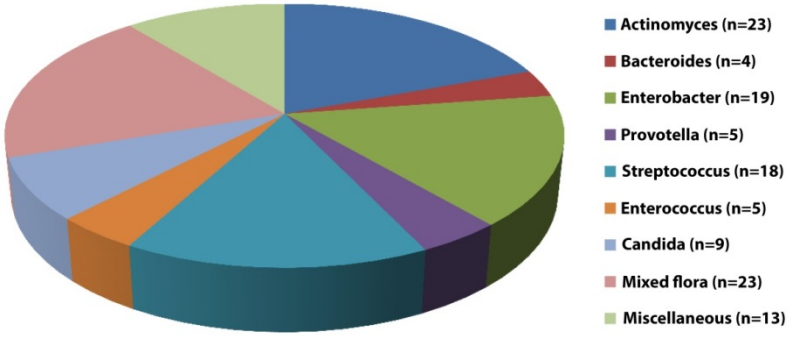


Figure 3.3: Pie-chart of micro-organisms in MRONJ.
Pie charts showing distribution of microorganism in bone sample of MRONJ lesions from 2008 to 2014.

As actinomyces were the most commonly found microorganisms, we therefore performed PCR to confirm the presence of actinomyces. Of the 55 patients, 53 (96.4%) were PCR and culture positive and 35 (63.6%) were positive only for PCR but negative for actinomyces culture. The results are shown in (Table 3.3).

Table 3.3: PCR results of MRONJ bone samples.

Culture	**PCR (n, %)**	
(n=55)	***Positive***	***Negative***
Positive	18(32.7)	0(0)
Negative	35(63.6)	2(3.6)
Total	53(96.4)	2(3.6)

DISCUSSION

The main objective of this study was to identify microorganisms manifested in MRONJ with special attention to actinomyces using microbiological cultures and PCR which might be useful in assisting surgeons in making proper decisions on the treatment modality of the disease based on the hypothesis that infection maybe the most important factor negatively influencing the onset and progression of MRONJ.

MRONJ can reduce the patient's quality of life and may produce significant morbidity due to impairment of chewing, swallowing and speaking as well as deterioration of facial aesthetics. Thus, it is of tremendous importance to treat those patients to adequately eliminate pain, control infection of soft and hard tissue and eradicate bone exposure [184].

From the results of our study, it was proved that actinomyces were highly prevalent in MRONJ patients by microbiological culture which was consistent with an earlier study on MRONJ bone samples [174]. A previous study on a pathological specimen of MRONJ lesion showed that the lesions were composed of areas with active inflammatory cells with acellular necrotic debris and bone resorption [185]. The histopathological findings of the bone samples in our study were similar.

The terminology MRONJ had been well recognised worldwide nowadays due to the increase in the prevalence of the disease. The pathogenesis of the disease raised many questions regarding the potential mechanisms underlying the pathophysiology [186]. Several mechanisms had also been proposed as: i) over suppression of bone turnover, ii) a response to infection, iii) immunomodulation, iv) ischemia due to the antiangiogenetic effects of BPs, v) soft tissue toxicity. Arguably, all theories could play a role in the pathogenesis of BRONJ. However, none of them was able to explain why the jawbone is the exclusive target [18, 173]. However, microbial infection in the pathogenesis of MRONJ is debatable and is not fully elucidated with few publications referring to the importance of infection as a prime

component in the multifactorial disease [31, 173, 181]. In our study, we have confirmed the presence of actinomyces in the bone samples but it is not clearly known whether osteonecrosis occurs first and then infection of the necrotic lesion or infected lesion undergoes osteonecrosis [187, 188]. There are some evidences showing that infection is necessary for osteonecrosis with formation of a bacterial biofilm in the lesion [18, 189, 190] as the oral cavity is occupied by hundreds of bacterial species existing as mixed biofilm. When the patient immunity is decreased, those microorganisms show opportunistic infection as actinomyces which are dominant pathogenic microorganisms detected at MRONJ by histopathological studies [191].

From our results, we confirmed that PCR using 16S rRNA was useful in identifying actinomyces directly from bone samples. PCR targeting the 16S rRNA gene of the actinomyces is highly conserved within species of the same genus and is thus considered the new standard for classification and identification of bacteria as well as a reliable method for the distinction of species that are difficult to cultivate [192, 193]. PCR is superior to microbiological cultures in diagnosis of oral actinomyces as being highly sensitive and rapidly detecting actinomyces either dead or alive. Another advantage is that it quantifies DNA rather than viable organisms. However, culturing methods cannot detect non-viable bacteria [194]. Previous studies have used different molecular methodologies to identify and differentiate actinomyces from oral samples after anaerobic cultivation, including PCR-RFLP, chromosomal DNA fingerprinting, 16S rRNA gene sequencing and oligonucleotide–DNA hybridization using universal primers or oligonucleotide probes [195-197].

Fifty-three (96.4%) of the 55 bone samples reacted positively with the universal primer pair designed for actinomyces suggesting their presence. These results show that PCR targeting the 16S rRNA region can be used to detect actinomyces in MRONJ bone samples.

Microbiological cultures were used as a traditional technique to identify actinomyces from bone samples. Anaerobic culturing was done in all 95 samples. However, these results were confirmed by PCR for 55 bone samples. The positive PCR results of the bone samples that were negative to culture were attributed to the high sensitivity of the PCR compared to culture methods, the way of transporting the specimens to the laboratory, death of some actinomyces during culturing and the inhibition of growth of actinomyces by the presence of other organisms affecting their ability to grow in culture. However, DNA from dead organisms can still be detected by PCR as explained by another study [194].

From our results, MRONJ occurred in the mandible twice as likely to be affected as in the maxilla which was in agreement with previous studies [198, 199]. Age older than 65 years was found to be a risk factor for MRONJ. Some studies recognized no statistically significant correlation between ageing and MRONJ [200] whereas others have included advanced age as a potential co-factor [201]. Correlations between MRONJ and comorbidities as diabetes mellitus, cardiovascular disease, chemotherapy or steroid intake have been discussed. These comorbidities affect bone remodelling by microvascular ischemia and compromised wound healing as well as impaired osteoblastic differentiation and function and the additional immunosuppressive and antiangiogenic effects [35, 202]. The great majority of MRONJ occur in females. The reason for the female dominance seems to be due to the higher number of breast cancer patients compared with prostate cancer patients and the greater prevalence of osteoporosis in females than in men [201]. MRONJ has been reported in patients with malignancies, particularly in those with breast and prostate cancer. [203] The profile of patients affected by this complication seems to show a similar pattern in our study. The majority of patients presented with MRONJ were at stages II which is comparable to findings in other studies [137, 151]. The classic clinical presentation of MRONJ is bone exposure with signs of infection, swelling and a purulent discharge [204]. Our study has corroborated that

MRONJ is more frequent in subjects on intravenous bisphosphonates as reported elsewhere [140, 161]. The cumulative risk of developing MRONJ was significantly greater in patients receiving zoledronic acid.

Although no consensus has been reached regarding the mechanism of MRONJ, in the present study, MRONJ developed either spontaneously or due to dentoalveolar reasons as tooth extraction, periodontal disease and denture trauma. Previous studies had shown that dental treatment is a risk factor for developing MRONJ [135]. In contrast, some studies had proved that tooth extraction and dentoalveolar surgical procedures aimed at treating and curing local infections leading to decreased risk for the development of MRONJ [205-207]. local infections were treated and overcome by the removal of infected teeth and suspicious bony lesions, and by antibiotic treatment and mucosal coverage of the extraction wounds, protecting the extraction sockets from bacterial ingrowth after extraction [206].

One limitation of this study was that there was no control group of untreated MRONJ patients. In addition, no non-MRONJ patients were characterized for bacterial species. The number of patients was reduced from 150 to 95 due to the incomplete records or absence of histopathological, microbiological or PCR diagnosis.

CONCLUSION

The pathogenesis of MRONJ had raised many questions regarding the potential mechanisms underlying the pathophysiology with special attention to the role of microbial infection. Actinomyces were the most frequent microorganisms in the disease. However, this does not necessarily lead to the pathogenic role. PCR was found to be the most reliable method for the detection of these microorganisms.

4. PUBLICATION IV

TREATMENT STRATEGIES AND OUTCOMES OF BISPHOSPHONATE RELATED OSTEONECROSIS OF JAW (BRONJ) WITH CHARACTERIZATION OF PATIENTS: A SYSTEMATIC REVIEW

Fliefel R, Tröltzsch M, Kühnisch J, Ehrenfeld M, Otto S. Int J Oral Maxillofac Surg. 2015;44(5):568-85.

ABSTRACT

The aim of this systematic review was to answer the question: What are the treatments available for bisphosphonate-related osteonecrosis of the jaws (BRONJ) and their outcomes? A literature search of PubMed, Cochrane Library, and Web of Science databases was conducted in accordance with the PRISMA statement, search phrases were ('jaw osteonecrosis' OR 'bisphosphonate-related osteonecrosis' OR 'bisphosphonate osteonecrosis') AND ('treatment' OR 'outcomes'). Ninety-seven articles published between 2003 and February 2014 were reviewed. The studies reported 4879 cases of BRONJ. The mean age of the patients was 66.5±4.7 years. The male to female ratio was 1:2. The mean duration of bisphosphonate (BP) administration was 38.2±15.7 months. The quality of the publications was good, with some moderate and poor. Minimally invasive surgical treatment was the treatment most used. Medical treatment was also used. Adjunctive treatments included laser, growth factors, hyperbaric oxygen and ozone. The articles provided a broad range of outcome variables to assess the treatment of BRONJ and the outcomes of each treatment. Considerable heterogeneity was found regarding study design, sample size, and treatment modalities. Clinical trials with larger samples are required to provide sufficient information for each treatment modality to predict the outcomes of each treatment.

INTRODUCTION

Bisphosphonates (BPs) are a class of drugs [208] used across a wide range of disciplines including endocrinology, oncology, orthopaedics and dentistry [209]. They are commonly prescribed for bone diseases[208] as in osteoporosis, Paget's disease of bone, hypercalcemia of malignancy, osteolytic bone metastases and osteolytic lesions of multiple myeloma [210, 211]. Their use has resulted in a statistically significant reduction in skeletal complications, including pathologic fractures, spinal cord compression, hypercalcemia of malignant disease and the need for subsequent radiotherapy or surgery to bone [212-214].

BPs are synthetic analogues of the naturally occurring pyrophosphate molecule that may be broadly classified on the basis of whether or not they contain a nitrogen atom, with nitrogen-containing bisphosphonates (N-BPs) being more potent than non-N-BPs [215]. They differ one from another in the substitution of the active side chains on their phosphorous-carbon phosphorous structural backbone.

BPs mechanism of action is the inhibition of bone resorption by suppressing osteoclast activation and inducing osteoclast apoptosis [216, 217]. The efficacy of BP has been established in several studies [218-221]. However, the use of bisphosphonates may have side effects[222].Bisphosphonate related osteonecrosis of the Jaw (BRONJ) has been characterized as a main side effect of bisphosphonate therapy [223-225].The first descriptions of BRONJ were in 2003 [226-228]. Since then, numerous reports have been published for the development of osteonecrosis of the jaw in patients treated with bisphosphonates [134, 229-240].

BRONJ lesions may remain silent till the occurrence of outcoming events such as [241] invasive dental procedures, infections, mechanical trauma to the jawbone as well as concomitant use of immunosuppressive and chemotherapy drugs [242, 243]. According to recent position paper by the American association of Oral and Maxillofacial Surgeons

(AAOMS) , risk factors for the development of BRONJ can be grouped as drug-related, local, demographic and systematic, genetic and preventive [29]. The clinical manifestation of BRONJ may vary from having necrotic bone exposure ranging from a few millimetres to larger areas, which can be asymptomatic for weeks, months, or years [244],simple swellings of soft tissues, abscesses to more complex cases presenting with fistulas and diffuse pain [245].

There are two major theories regarding the pathophysiology of BRONJ. One is the osteoclast-based, ‘‘inside-out,’’ theory, in which inhibition of osteoclastic activity and marked suppression of bone turnover, together with spread of physiologic micro-damage and possibly local infection, leads to bone death within the jaw, with subsequent exposure. As such, the bone exposure would be a late event. The second, ‘‘outside-in,’’ theory suggests a break in the oral mucosa leads to ingress of bacteria and local infection which, coupled with poor bone remodelling leads to bone death. BRONJ may result from a combination of these two mechanisms and hypovascularity also may play an important role [246, 247]. Although there have been reports with no obvious co-morbidity factors. It is reasonable to believe that co-factors play a relevant role in the development of these lesions [230, 248].

Management of BRONJ has centred on efforts to eliminate or reduce severity of symptoms, to slow or prevent the progression of disease and to eradicate diseased bone[249]. There is currently no gold standard for the treatment of BRONJ. Several treatment options have been described in relation to the AAOMS staging of (BRONJ) [250]. No agreement on a surgical versus non-surgical approach to therapy has been reached in the treatment of BRONJ[136, 251-253]. Some recommendations focus on prevention and a conservative approach [134, 226, 232, 254].

Treatment strategies are administering antibiotics, oral antibacterial mouth rinse, stop of BPs if possible, pain control, surgical debridement or resection for long-term palliation of

infection and pain[230, 254] , sequential removal of sequestra and extensive involvement may necessitate large area of debridement to include segmental mandibulectomy and partial maxillectomy [230], mandibular reconstruction with the fibula flap [255], cover the exposed areas with tissue flaps [226]. Hyperbaric oxygen therapy, fluorescence-guided bone resection, and low-intensity laser therapy have been also studied as therapeutic tools [143, 253, 256, 257].

Other treatment modalities that increase bone wound healing using growth and differentiation factors are being studied [258, 259], or transplantation of intralesional autologous bone marrow stem cell [260]. Recently, Teriparatide (N-terminal 34 amino acids of recombinant human parathyroid hormone) was reported for medical treatment of BRONJ[261]. Pentoxifylline and α-tocopherol in addition to antimicrobial therapy decreased area of bone exposure and symptoms in BRONJ patients [262]. The use of ozone in combination with antibiotics and surgery for patients with exposed bone lesions was also subject to clinical investigation and found to resolve pain, secretions, and halitosis [263].

Therefore, the main objective of this study were to conduct systematic review of literature to determine (a) available treatment strategies for BRONJ describing (b) the outcome variables measured by each treatment modality,(c) success of the treatment expressed by the outcome.

MATERIALS AND METHODS

All the included studies were subjected to a Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [264]. PRISMA consists of a 27 item checklist and a four-phase flow diagram that relates to the title, abstract, introduction, methods, results, and discussion sections of articles and funding. They were developed based on recommendations on what should be included in an accurate and complete report of systematic reviews and meta-analyses.

The systematic search included the time period 2003, the year of the initial description of BRONJ to 28th of February 2014. All publications identified in the literature search were retrieved from online journals and selected on the basis of the inclusion criteria.

Inclusion Criteria

(a) Academic publications; the review included any published studies (cross-sectional surveys, cohort and case-control studies), clinical trials, case series and retrospective studies (b) in English language confirming diagnosis of BRONJ by AAOMS (American Association of Oral and Maxillofacial Surgeons) or ASBMR (American Society of Bone and Mineral Research); (c) studies on humans ;(d) Participants of any age and gender with clinical diagnosis of BRONJ; (e) any form of treatment; (f) outcomes variables should be mentioned in the publication; (g) outcome of the treatment.

Exclusion Criteria

(a) Single case reports of BRONJ (b) Experimental laboratory studies (c) Case series with less than 5 patients (d) literature reviews, Letters, editorials, PhD theses and abstracts were excluded.

Disease Definition

The disease definition as proposed by AAOMS and ASBMR included the persistence of exposed necrotic bone in the oral cavity for 8 weeks, despite adequate treatment, in a patient with current or previous history of bisphosphonate use, without local evidence of malignancy and no prior radiotherapy to the affected region [140, 231, 245, 265] .

A clinical staging system has been proposed to classify patients with established BRONJ with appropriate treatment for each stage [231, 245, 265, 266] (Table 4.1).

Table 4.1: Staging and treatment of bisphosphonate-related osteonecrosis of the jaw (BRONJ) according to AAOMS

BRONJ Stage	Clinical Conditions	Treatment Strategies
At risk	No apparent necrotic bone in patients who have been treated with either oral or IV bisphosphonates.	No treatment indicated. Patient education.
Stage 0	No clinical evidence of necrotic bone, but non-specific clinical findings and symptoms.	Systemic management, including the use of pain medication and antibiotics.
Stage 1	Exposed and necrotic bone in asymptomatic patients without evidence of infection	Oral anti-bacterial mouth rinse. Clinical follow-up on a quarterly basis. Patient education and review of indications for continued BP use.
Stage 2	Exposed and necrotic bone associated with infection as evidenced by pain and erythema in region of exposed bone with or without purulent drainage.	Symptomatic treatment with oral antibiotics Oral anti-bacterial mouth rinse Pain control Superficial debridement to relieve soft tissue irritation
Stage 3	Exposed necrotic bone in patients with pain and erythema and one or more of the following: exposed and necrotic bone extending beyond the region of alveolar bone, such as inferior border and ramus in the mandible, maxillary sinus or zygoma in the maxilla, resulting in pathologic fracture, extra-oral fistula, oral antral/oral nasal communication, or osteolysis extending to the inferior border of the mandible or to the maxillary sinus floor	Oral anti-bacterial mouth rinse Antibiotic therapy and pain control Debridement/surgical resection for prolonged relieve of pain and infection

Electronic database search:

Three databases – PubMed, Cochrane Library, and Web of Science– were electronically searched. The heading sequence ("jaw osteonecrosis" OR "bisphosphonate-related osteonecrosis" OR "bisphosphonate osteonecrosis") AND ("treatment" OR "outcomes") were searched as text word. The results of the database searches were combined and duplicate articles were excluded. All references were gathered and screened for eligibility.

First round search

Abstracts were reviewed and all articles containing the keywords were retained. Articles that were not in English were excluded. Complete versions were then obtained for all the articles that that met the inclusion criteria.

Second round search and evaluation

A manual search was done of the reference lists of all the articles retained after the first round for appropriate studies relevant to the review topic. A search for unpublished literature was not performed. Literature reviews and systematic reviews also were considered with the

objective of identifying cases already reported. All the articles were fully read for final selection.

Third round search

Each of the publications included in this round was critically appraised for assessment of validity and the following data were extracted from the accepted articles onto a standardized spreadsheet: Reference & year, Study design, Number of patients in study, Mean age of patients, Gender of patients, Location of the lesions, Primary cause of the BRONJ, Type of BPs used, Route of administration of BP used, Range of duration of use of BP Triggering factors, Co-morbidities, Treatment methods, Outcome variables measured, follow up period, outcomes of the different treatments.

Statistical Analysis

The duration of BP exposure was defined as time in months from the date of first BP infusion administered to the last recorded infusion.

A qualitative data analysis was performed with the aim of summarizing the results of the included studies. The mean age of patients with ONJ and the ratio of male to female patients were calculated to determine whether any particular stratum had a greater predisposition to develop ONJ than another. The existence of potential risk factors for ONJ was examined; the mean dose and range; the treatment duration; and the proportions of patients receiving immunosuppressant therapy (eg. corticosteroids) or other comorbidities or a history of dental trauma, infection, or surgical procedures.

The quality of accepted publications was assessed based on a modification of the ASBMR [140] by reporting of 12 parameters for all patients diagnosed with BRONJ: age, sex, primary cause of the disease, name of bisphosphonate, duration, mode of administration, affected site, medical history (concomitant medications, comorbidities), triggering factors, treatment , outcome variable measured and treatment outcome. The quality of each publication was

classified as good (10-12 variables reported), moderate (5-9 variables), or poor (1-4 variables).

RESULTS

Literature search results

The results of the literature search are presented by flow chart showing study selection according to the PRISMA statement (2009) [264].The initial search strategy yielded 1355 titles/abstracts from the databases analysed: 1085 from PubMed, 235 from Web of Science, 35 from Cochrane Library and 5 additional articles were identified through a hand search of relevant reference lists, bringing the number of accepted articles to 1360. After title and abstract screening, and/or paper analyses, 300 potentially relevant were accepted for article retrieval and full-text review. 200 papers were included in the qualitative synthesis and finally 103 papers were excluded after a preliminary review due to non-compliance with the inclusion/exclusion criteria, lack of outcome, not related to our proposed research question or irrelevancy (Figure 4.1).

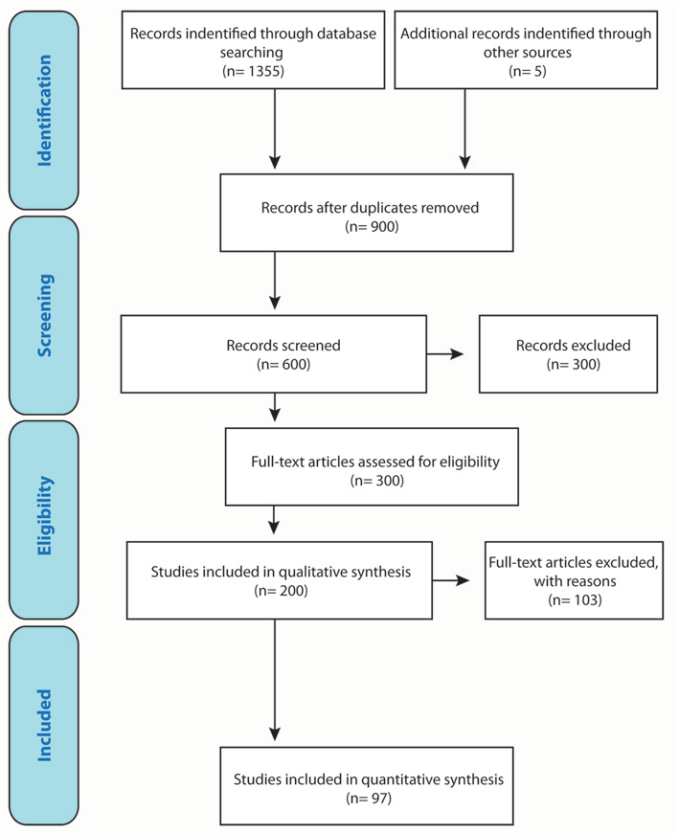


Figure 4.1: Flow chart of the study.
Flow chart of the search strategy and study selection used in this systematic review.

The remaining 97 articles were included in the final review describing 4879 cases of BRONJ. The quality of the publications were between poor, moderate and good where 79 publications were classified as good (81.5%) and 16 publications were classified as moderate (16.4%) and 2 publications as poor (2.1%). Of the 97 accepted publications, 35 (36.1%) were case series [134, 136, 138, 153, 176, 255, 256, 262, 267-293], 3 (3.1%) were clinical trials [294-296] , 18 (18.5%) prospective [130, 131, 133, 144, 158, 162, 199, 297-307], 37 (38.2%) retrospective studies [132, 137, 164, 165, 198, 202, 224, 241, 249, 308-335] and 4 (4.1%) clinical reports [336-339] (Table 4.2)

Table 4.2: Summary of the publications for the systematic review with the study design, total number of patients, mean age of patients in years, administration time of BRONJ in months and treatment modalities.

REFERENCE	STUDY DESIGN	NUMBER OF PTS	AGE	ADMIN TIME FOR BP(MONTHS)	QUALITY OF PUBLICATION	TREATMENT
Thumbigere-Math 2009 [198]	Retro	26	64	45.8	G	
Thumbigere-Math 2012[308]	Retro	18	60	44.3	G	
Anavi-Lev 2013 [293]	CS	52	70.7	40	G	
Holzinger 2013 [297]	Prosp	88	N/P	N/P	M	
Saussez 2009 [315]	Retro	34	62	34.5	G	
Montebugnoli 2007[136]	CS	16	61.2	17.9	G	**Medical and**
Dannemann 2006 [270]	CS	14	65	N/P	G	**Minimal invasive**
Beninati 2013 [298]	Prosp	51	68	41	G	**Surgery**
Alons 2009 [316]	Retro	7	66.9	55.2	G	
Lazarovici 2009 [176]	CS	101	63.5	48.5	G	
Junquera 2009 [273]	CS	21	65.1	25	G	
Stanton 2009 [318]	Retro	33	64.5	N/P	G	
Estilo 2008 [319]	Retro	28	N/P	34.1	G	
Dimitrakopoulos 2006 [320]	Retro	11	61	6	G	
Fortuna 2012[299]	Prosp	26	68.4	23.3	G	
Abu Id 2008 [322]	Retro	78	65.6	12	G	
Pozzi 2007 [323]	Retro	35	70	36	G	
Williamson 2010 [300]	Prosp	40	64	N/P	G	
Longobardi 2007 [276]	CS	18	55	42.3	G	
Wutzl 2008 [303]	Prosp	58	68.3	35.5	G	
O'Ryan & Lo 2012 [202]	Retro	30	77	52.8	G	
Scoletta 2010 [306]	Prosp	37	68	25.5	G	
Nomura 2013 [287]	CS	13	71.2	29.6	G	
Jabbour 2012 [288]	CS	14	69	37.5	G	
Mücke 2011 [144]	Prosp	108	68.5	N/P	M	
Mortensen 2007 [269]	CS	7	66	N/P	G	
O'Ryan 2009 [137]	Retro	59	61.4	N/P	G	**Medical, Minimal**
Elad 2006 [337]	CR	57	62.7	N/P	G	**invasive and Major**
Stockmann 2010 [131]	Prosp	50	69.5	31	G	**Surgery**
Ibrahim 2008 [224]	Retro	8	66.5	14.6	G	
Kim 2012 [335]	Retro	21	64.3	30	G	
Yarom 2007 [317]	Retro	11	69.7	49.2	G	
Hong 2010 [327]	Retro	24	72.1	43.1	G	
Lerman 2013 [340]	Retro	120	63	36	G	
Maurer 2011 [326]	Retro	21	69	47.4	G	
Hansen 2013 [339]	CR	37	N/P	N/P	P	
Hoefert 2011 [312]	Retro	47	66.1	41.9	G	
Marx 2005 [134]	CS	119	N/P	N/P	G	
Van den Wyngaert 2009 [338]	CR	33	58	27	G	**Medical**
Moretti 2011 [301]	Prosp	34	69.0	39	G	
Alsehimy 2014 [302]	Prosp	96	66.5	N/P	G	
Lazarovici 2010 [285]	CS	27	70	N/P	G	
Nicolatou-Galitis 2011[341]	Prosp	63	63.6	37.1	G	
Epstein 2010 [262]	CS	6	75	74.6	G	
Vescovi 2011 [342]	Retro	567	67.2	N/P	G	
Graziani 2012[165]	Retro	347	67	23	G	**Minimal invasive**
Mercer 2013 [321]	Retro	91	69.8	60	G	**Surgery**
kos 2010 [324]	Retro	18	67.0	34.9	G	
Wutzl 2006 [278]	CS	17	64.8	32	G	
Ferlito 2012[292]	CS	94	66	24	M	
Schubert 2012 [307]	Prosp	258	N/P	N/P	M	

Retro: retrospective CS: Case Series, Prosp: Prospective, RCT: Random Clinical Trial, CR: Clinical Report N/P: not reported G: Good M: Moderate P: Poor

Table 4.2: Summary of the publications for the systematic review with the study design, total number of patients, mean age of patients in years, administration time of BRONJ in months and treatment modalities.

REFERENCE	STUDY DESIGN	NUMBER OF PTS	AGE	ADMIN TIME FOR BP(MONTHS)	QUALITY OF PUBLICATION	TREATMENT
Rugani 2010[267]	CS	5	75.4	36	G	
Romeo 2011 [268]	CS	12	62	N/P	M	
Angiero 2009 [310]	Retro	49	69.7	14.8	G	
Stübinger 2009 [336]	CR	8	59.1	53	G	
Vescovi 2014[311]	Retro	63	N/P	N/P	M	
Vescovi 2012 [271]	CS	151	66.6	48.2	G	
Vescovi 2007 [274]	CS	19	71	N/P	M	
Manfredi 2011 [325]	Retro	25	70.4	55.9	G	
Atalay 2011 [331]	Retro	20	55.4	32.4	G	**Laser**
Scoletta 2010 [304]	Prosp	20	71.3	42.9	G	
Rugani 2013 [343]	CS	12	63.9	N/P	M	
Vescovi 2010 [333]	Retro	91	67	N/P	M	
Vescovi 2008 [289]	CS	28	70.3	N/P	M	
Martins 2012 [309]	Retro	22	58.09	24.68	G	
Curi 2011 [272]	CS	25	60.7	N/P	G	
Mozzati 2012 [328]	Retro	32	69.7	37	G	**Growth factor**
Bocanegra-Perez 2012 [305]	Prosp	8	66.3	1	G	**(PRP or BMP2)**
Coviello 2012 [291]	CS	7	75.57	66	G	
Cicciu 2012 [282]	CS	20	N/P	N/P	P	
Ripamonti 2011[294]	RCT	10	65	N/P	M	
Agrillo 2007 [275]	CS	58	64	N/P	M	**Ozone**
Ripamonti 2012 [296]	RCT	24	62.5	N/P	G	
Agrillo 2012 [241]	Retro	131	60	N/P	G	
Boonyapakorn 2008 [199]	Prosp	22	61.1	N/P	G	
Urade 2011 [329]	Retro	263	68.1	N/P	G	**Discontinuation**
Park 2010 [279]	CS	5	72.6	79.2	G	**of BP**
Watters 2013 [138]	CS	109	64	N/P	G	
Wilde 2011 [332]	Retro	24	N/P	N/P	G	
Chiu 2010 [277]	CS	12	69.7	67.2	G	
Freiberger 2012 [295]	RCT	22	66.1	N/P	M	**Hyperbaric**
Freiberger 2007[256]	CS	16	N/P	18	M	**Oxygen**
Kwon 2012 [281]	CS	6	77.5	55.2	G	
Narvaez 2013 [286]	CS	7	72	55.2	G	**Teriparatide**
KM Kim 2014 [334]	Retro	15	77.1	45.6	G	
Pautke 2011 [130]	prosp	15	63.2	44.4	G	
Fleisher 2008 [153]	CS	10	N/P	N/P	M	**Guided debridment**
Seth 2010 [314]	retro	11	61.3	N/P	G	
Carlson & Basile 2009 [133]	prosp	82	N/P	N/P	M	
Badros 2006 [330]	retro	22	61	N/P	G	
Bedgoni 2011[162]	prosp	30	66	N/P	G	**Major Surgery**
Jacobsen 2012 [164]	retro	110	67	N/P	G	
Voss 2012 [132]	retro	21	68.5	40.1	G	
Hanasono 2013 [290]	CS	13	66.6	N/P	G	
Nocini 2009[255]	CS	7	61	N/P	G	
Lemound 2012 [284]	CS	20	68	34.8	G	
Blus 2013 [283]	CS	8	71.3	32	G	
Total		**4879**	**66.5±4.7**	**38.2±15.7**		

Age and Gender

A total of 4879 patients were identified and treated in the 97 publications with a mean age of 66.5 ± 4.7years. In the 4481 cases in which the sex distribution was reported, 1471 were male patients (32.8 %) and 3010 were female patients (67.2 %) with a female predilection in the ratio of 2:1 among all reported cases.

BRONJ characteristics

Eighty nine publications described the site of BRONJ in 4627 patients receiving bisphosphonates while only 8 publications [262, 286, 292, 293, 295, 296, 304, 339] , the site were not reported. BRONJ lesions were located most commonly in the mandible in 3011 patient (65.1%), followed by the maxilla in 1320 patients (28.5 %) or both jaws in 296 patients (6.4 %).

Primary cause of Disease

Bisphosphonate therapy was started in 4602 cases for the following indications: 1434 cases multiple myeloma (31.2%), 1359 cases breast cancer (29.5%), 903 cases in osteoporosis (19.7%) ,442 cases prostate cancer (9.6%), , 116 cases in metastasis (2.5%) and 348 cases in other cancers (7.6%) including lung, renal and bladder carcinoma in the review. Most patients (60.7 %) had multiple myeloma or metastatic breast cancer.

Characteristics of Bisphosphonate Treatment

The bisphosphonate prescribed was specified for all 4118 patients with BRONJ. Overall, 2427 (58.9%) patients received zoledronate, 571(13.9%) patients received pamidronate, 523 (12.7%) patients received alendronate, 128 (3.1%) patients received ibandronate, 469 patients received a combination of bisphosphonates.

Bisphosphonate treatment was principally intravenous (IV) in 3245 patients (83.2%) while 656 patients (16.8%) received oral bisphosphonates.

Duration of Treatment

There was variability in the duration of BP therapy ranging from 1 to 79.2 months with mean duration of BP therapy 38.2 ± 15.7.

Triggering factors and Comorbidities

The most important triggering factors for the development of BRONJ were described in 3198 cases in the included articles, whereby tooth extraction was the principal cause in 1974 patients (61.7%), trauma from manipulation of dental implants in 123 cases (3.9 %). A history of dental surgery was reported for 230 patients (7.2 %); 159 cases (5.0 %) were reported in periodontal diseases and prosthesis-induced trauma in 237 cases (7.4 %). A large proportion of BRONJ lesions appeared spontaneously in 475 patients (14.8%).

With regard to concomitant diseases and medication, 2674 patients had comorbidities: diabetes mellitus was observed in 298 patients (11.2%) , 225 (8.4%) patients were hypertensive, 1062 (39.7%) patients were under chemotherapy, 215 (8.0%) patients were smoking, 108 (4.0%) patients had thrombocoagulopathies, 658 (24.6%) were taking corticosteroids and 108 (4.1%) were free from any concomitant diseases. The incidence of BRONJ was associated with chemotherapy (39.7%) of the patients compared to corticosteroid therapy (24.6%). Characteristics of patients diagnosed with BRONJ in the included articles are shown in (Table 4.3).

Table 4.3: Characteristics of patients diagnosed with BRONJ.

Characteristics	Details	Number	Percentage (%)
Gender	Male	1471	32.8
	Female	3010	67.2
Location	Maxilla	1320	28.5
	Mandible	3011	65.1
	Both	296	6.4
Primary cause of the disease	Multiple Myeloma	1434	31.1
	Breast cancer	1359	29.5
	osteoporosis	903	19.7
	Prostate cancer	442	9.6
	Other cancers	348	7.6
	Metastasis	116	2.5
Type of BP adminstrated	Zoledronate	2427	58.9
	Pamidronate	571	13.9
	Alendronate	523	12.7
	Ibandronate	128	3.1
	Combination	469	11.4
Route of administration of BP	IV	3245	83.2
	Oral	656	16.8
Triggering factors	Extraction	1974	61.7
	Dental implant	123	3.9
	Dental surgery	230	7.2
	Periodontal disease	159	5.0
	Prosthetic trauma	237	7.4
	Spontaneous	475	14.8
Comorbidities	Diabetes	298	11.2
	Corticosteroids	658	24.6
	Hypertension	225	8.4
	Thrombosis	108	4.0
	Smoking	215	8.0
	Chemotherapy	1062	39.7
	None	108	4.1

Management of Osteonecrosis of the Jaw with the outcome of each treatment

Regarding the management of the BRONJ lesions, the studies showed discontinuation of BP administration (5.1%) in addition to treatment either by medical therapy (50%) or minimal invasive surgical therapy (45.9%) whereas (22.4%) of patients underwent major surgical procedures, such as segmental resection of the jaw bones.

Various adjunctive treatments such as hyperbaric oxygen therapy, laser therapy, ozone therapy, Teriparatide, Fluorescence guided debridement, treatment with growth factors (PRP or BMP 2), Piezotherapy had also being mentioned.

Medical treatment of BRONJ was reported in 49 publications [131, 134, 136, 137, 144, 158, 176, 198, 199, 202, 224, 249, 262, 269, 270, 273, 276, 279, 285, 287, 288, 293, 297-303, 306, 308, 310, 312, 315-323, 325-327, 329, 332, 335, 337, 338] and minimal invasive surgical

treatment in 44 publications.[131, 134, 136, 137, 144, 165, 176, 199, 202, 224, 249, 269, 270, 273, 276, 278, 287, 288, 292, 297-300, 303, 306, 307, 313, 315-324, 327, 329, 335, 337, 339]. Major Surgical intervention was delivered to 22 publications [131-133, 137, 144, 162, 164, 224, 249, 269, 290, 314, 317, 325-327, 329, 330, 332, 335, 337, 339] including use of surgical flaps in 2 publications [255, 284] and Piezotherapy [283]. Laser therapy was reported in 13 publications [267, 268, 271, 274, 280, 289, 304, 309-311, 325, 331, 333, 336] , ozone therapy in 4 publications [241, 275, 294, 296], Platelet rich plasma in 5 publications [272, 291, 305, 309, 328] and BMP2 [282], hyperbaric oxygen in 3 publications [256, 277, 295], teriparatide in 3 publications [281, 286, 334]. Fluorescence or tetracycline guided debridement was reported in 2 publications [130, 153].

715 patients were treated by medical and minimal invasive surgical treatment, 422 patients were treated by medical, minimal invasive and major surgical treatment, 286 patients were treated by medical treatment only, 767 patients were treated by minimal invasive surgical treatment, 252 patients were treated by major surgical treatment, 25 patients were treated by guided debridement, 322 patients were treated by laser treatment, 92 patients were treated by growth factors treatment, 161 patients were treated by major surgical treatment, 361 patients stopped BP treatment in addition to other treatment modalities, 45 patients were treated by hyperbaric oxygen, 27 patients were treated by teriparatide (Table 4.4).

The outcome of the treatment was classified as: ***complete healing*** (CH)—complete regrowth of oral mucosa over previously exposed bone; ***partial healing*** (PH)—either a decrease in lesion size (largest linear dimension) or the number of lesions and/or cessation of pain or signs of infection; ***stable disease***—no improvement in clinical signs or symptoms; or progressive disease—increase in the size or number of lesions or increased pain and severity of infection; ***Regressive disease***—decrease in the size or number of lesions or decreased pain

and severity of infection and a negligible or ***no healing*** (NH) when there was no sign of improvement.

The outcomes of the treatment modalities of the BRONJ were assessed in 3475 patients. Outcome of the different treatment modalities were compared as following (Table 4.4).

Medical and Minimal invasive surgical treatment

715 patients were treated by medical and conservative surgical treatment; 278 patients (38.9%) showed CH, 125 patients (17.5%) showed PH, 94 patients (13.1%) had stable lesions, 52 patients (7.3%) had progressive lesions, 64 patients (9%) had regressive lesions, only 5 patients (0.7%) had recurrent lesions,97 patients (13.6%) had NH lesions.

Medical, Minimal invasive and Major Surgical treatment

422 patients were treated by medical, conservative and surgical treatment; 169 patients (40%) showed CH, 105 patients (24.9%) showed PH, 34 patients (8.1%) had stable lesions, 19 patients (4.5%) had progressive lesions, 5 patients (1.2%) had regressive lesions, 47 patients (11.1%) had recurrent lesions, 43 patients (10.2%) had NH lesions.

Medical treatment only

286 patients were treated by medical treatment; 129 patients (45.1%) showed CH, 52 patients (18.2%) showed PH, 23 patients (8%) had stable lesions, 8 patients (2.7%) had progressive lesions, 52 patients (18.2%) had regressive lesions, 20 patients (6.9%) had recurrent lesions, 2 patients (0.7%) had NH lesions.

Minimal invasive surgical treatment

767 patients were treated by conservative surgical treatment; 301 patients (39.2%) showed CH, zero patients (0%) showed PH, 152 patients (19.8%) had stable lesions, 61 patients (8%) had progressive lesions, 231 patients (30.1%) had regressive lesions, 0 patients (0%) had recurrent lesions, 22 patients (2.9%) had NH lesions.

Major Surgical treatment

252 patients were treated by surgical treatment; 207 patients (82.1%) showed CH, 11 patients (4.4%) showed PH, 8 patients (3.2%) had stable lesions, 5 patients (2%) had progressive

lesions, zero patients (0%) had regressive lesions, 11 patients (4.4%) had recurrent lesions, 10 patients (4%) had NH lesions.

Guided Debridement treatment

25 patients were treated by guided debridement; 12 patients (48%) showed CH, 10 patients (40%) showed PH, zero patients (0%) had stable lesions, 1 patient (4%) had progressive lesions, zero patients (0%) had regressive lesions, zero patients (0%) had recurrent lesions, 2 patients (8%) had NH lesions.

Laser treatment

322 patients were treated by laser treatment; 146 patients (45.3%) showed CH, 18 patients (5.6%) showed PH, 81 patients (25.2%) had stable lesions, 5 patients (1.6%) had progressive lesions, 33 patients (10.2%) had regressive lesions, 2 patients (0.6%) had recurrent lesions, 37 patients (11.5%) had NH lesions.

Growth factor (PRP & BMP2) treatment

92 patients were treated by growth factors treatment; 75 patients (81.5%) showed CH, 2 patients (2.2%) showed PH, 6 patients (6.5%) had stable lesions, zero patients (0%) had progressive lesions, 8 patients (8.7%) had regressive lesions, 1 patient (1.1%) had recurrent lesions and zero patients (0%) had NH lesions.

Ozone treatment

161 patients were treated by surgical treatment; 93 patients (57.8%) showed CH, 27 patients (16.8%) showed PH, 5 patients (3.1%) had stable lesions, zero patients (0%) had progressive lesions, 28 patients (17.4%) had regressive lesions, zero patients (0%) had recurrent lesions, 8 patients (5%) had NH lesions.

Discontinuation of BP treatment in addition to other treatment modalities

361 patients stopped BP treatment; 127 patients (35.2%) showed CH, 27 patients (7.5%) showed PH, 142 patients (39.3%) had stable lesions, 50 patients (13.9%) had progressive lesions, 3 patients (0.8%) had regressive lesions, 5 patients (1.4%) had recurrent lesions, 7 patients (1.9%) had NH lesions.

Hyperbaric oxygen treatment

45 patients were treated by hyperbaric oxygen; 12 patients (26.7%) showed CH, 8 patients (17.8%) showed PH, 2 patients (4.4%) had stable lesions, 6 patients (13.3%) had progressive lesions, 17 patients (37.8%) had regressive lesions, zero patients (0%) had recurrent lesions and zero (0%) patients had NH lesions.

Teriparatide treatment

27 patients were treated by teriparatide; 22 patients (81.5%) showed CH, 5 patients (18.5%) showed PH, zero patients (0%) had stable lesions, zero patients (0%) had progressive lesions, zero patients (0%) had regressive lesions, zero patients (0%) had recurrent lesions and zero patients (0%) had NH lesions.

Table 4.4: Outcome of each treatment modality.

Treatment	Outcome Number of patients (%)							Total Number of patients (%)
	CH	PH	St	Pr	Rgr	Rec	NH	
Medical and minimal invasive surgery	278(38.9)	125(17.5)	94(13.1)	52(7.3)	64(9)	5(0.7)	97(13.6)	715(21.0)
Medical, minimal invasive and major surgery	169(40)	105(24.9)	34(8.1)	19(4.5)	5(1.2)	47(11.1)	43(10.2)	422(12.1)
Medical treatment	129(45.1)	52(18.2)	23(8)	8(2.7)	52(18.2)	20(6.9)	2(0.7)	286(8.2)
Minimal invasive surgery	301(39.2)	0(0)	152(19.8)	61(8)	231(30.1)	0(0)	22(2.9)	767(22.0)
Major surgery	207(82.1)	11(4.4)	8(3.2)	5(2)	0(0)	11(4.4)	10(4)	252(7.3)
Guided debridement	12(48)	10(40)	0(0)	1(4)	0(0)	0(0)	2(8)	25(0.7)
Laser therapy	146(45.3)	18(5.6)	81(25.2)	5(1.6)	33(10.2)	2(0.6)	37(11.5)	322(9.2)
Growth factors	75(81.5)	2(2.2)	6(6.5)	0(0)	8(8.7)	1(1.1)	0(0)	92(2.6)
Ozone therapy	93(57.8)	27(16.8)	5(3.1)	0(0)	28(17.4)	0(0)	8(5)	161(4.6)
Discontinuation of Bisphosphonates	127(35.2)	27(7.5)	142(39.3)	50(13.9)	3(0.8)	5(1.4)	7(1.9)	361(10.3)
Hyperbaric oxygen	12(26.7)	8(17.8)	2(4.4)	6(13.3)	17(37.8)	0(0)	0(0)	45(1.2)
Teriparatide	22(81.5)	5(18.5)	0(0)	0(0)	0(0)	0(0)	0(0)	27(0.8)
Total	1571(45.2)	390(11.2)	547(15.8)	207(5.9)	441(12.7)	91(2.6)	228(6.6)	3475

CH: Complete healing, PH: Partial healing, St: Stable lesion, Pr: Progressive lesion, Rgr: Regressive lesion, Rec: recurrent lesion NH: Non healing lesion.

Follow-up and Treatment Outcome

After the initial BRONJ treatment, follow-up periods reported only in 80 publications ranged from 4 weeks to 50 months with a mean of 12.9 ± 9.9

Outcome Measures

A total of 7outcome variables that were used in the studies were identified.

The most frequently measured outcome was mucosal healing occurring in 47 publications (48%). The bone exposure was the next most frequently used (n=30, 30.6%), followed by pain (n=31, 31.6%) then change in signs and symptoms (n=28, 28.6%), improvement of stage (n=14, 14.3%), reduction in lesion size and number (n=12, 12.2%) and finally infection control (n=7, 7.1%). The treatment of BRONJ and the outcome variables measured with the mean follow up of each treatment are summarized in (Table 4.5)

Table 4.5: Summary of treatment modalities and the outcome variables measured with the mean follow up of each treatment

Treatment	Outcome variables measured	Follow-up (months)
Medical and Minimal invasive	Improved signs and symptoms, decrease in lesion size and number, elimination of pain, reduction in soft and hard tissue inflammation ,no bone exposure or bone exposure less than 1-2 mm, no suppuration, improvement of stage, persistence of fistula, cessation of pus and extra-oral manifestations, mucosal coverage, radiographic success(cessation of bony destruction),presence or recurrence of infection, BRONJ at stage 0	11.1±6.6
Medical, Minimal Invasive and Major Surgery	closure of oroantral, fistula, Stage improvement, healing of the lesion, extension of exposed bone areas, bone exposure , decrease of pain, healing of mucosa, improved signs and symptoms, asymptomatic lesions, patients free from symptoms, recurrence of BRONJ, recurrence of sinusitis	11.6±5.2
Medical	no fistula, reduction of exposed bone, reduction of the pain, closure of the mucosal defect, persistence of exposed bone or progressive necrosis, reduction of the size of the lesion, size of necrotic lesions, resolution of BRONJ manifestations, cessation of pus or purulent secretion, mucosal inflammation, signs and symptoms improvement	16.4±5.2
Minimal Invasive Surgery	improvement of the stage (transition to a less severe stage), deterioration of wound healing, recurrence rate of wound dehiscence, closure of lesion, pain reduction, complete healing of soft tissue, signs of inflammation, exposed bone, no symptoms of infection for a minimum of 3 months period.	6.4±3.6
Laser	efficiency of surgical laser application, pain reduction, infection control, mucosal healing, no signs and symptoms, healing evaluated radiographically, complete removal of visible necrotic bone, absence of new exposed bone near surgical area, no signs of infection, stage improvement, size of the lesion, oedema, visual analogue score of pain, presence of pus, fistulas and halitosis, bone exposure	10.7±9.7
Growth factor (PRP or BMP2)	intact and healed mucosa, no exposed necrotic bone, no sign of infection or fistula, absence of pain, no radiographic signs of residual infection or evidence of, bone sequestration, Bleeding	18.2±18.3
Ozone	spontaneous explusion or sequestrum of necrotic bone to be removed surgically, healed and re-epithelialized mucosa, presence or absence of oral mucosa redness around the lesion area, pethechiae or bleeding, pain intensity, diminishing of symptoms	9.9±5.5
Discontinuation of BP	healing of the mucosa, Pain relief, Bleeding, stage improvement, resolution of symptoms, presence or absence of exposed necrotic bone, radiographic evidences of BRONJ, no fistulas, absence of swelling	27.8±29.2
Hyperbaric Oxygen (HBO)	clinical evidence of symptom relief, pain reduction, absence of sequestrum, oral lesion size and number, regrowth of oral mucosa over exposed bone	20±5.7
Teriparatide	change of the biochemical markers(osteocalcin and c terminal telopeptide cross link type I collagen), clinical and radiographic healing, improvement of BRONJ stage	4.5±2.1
Major Surgery	osseous union judged clinically and radiographically without signs of residual infection, or exposed bone at the time of evaluation, post-operative complication, infection, recurrence of BRONJ, oral pain, exposed bone, mucosal healing, percentage of flap survival, percentage of complications at the donor and recipient site, symptoms free	18±5.2
Guided Debridment	closure of mucosa, exposed bone, symptoms free	1.5±0.7

DISCUSSION

The aim of this systematic review is to summarize the literature concerning the patients receiving bisphosphonates, treatments of BRONJ and outcomes of these treatments. There was high clinical heterogeneity among the studies included, which was unsurprising given the differing interventions used and the considerable variations in techniques applied and

combinations or delivery of interventions. Differences in the search periods may explain the higher prevalence of BRONJ in the present review.

There are some limitations with respect to the search strategy. It is possible that eligible studies were missed despite the extended search. Also excluded was the grey literature for the reason that basic information such as authorship, publication date, or publishing body may not be discerned with certainty. This review did not include searches of EMBASE, SCOPUS, or abstracts from dental, maxillofacial, and surgical conferences which may also have contributed to underestimation of the number of reported BRONJ cases. It's obvious in this systematic review the continuously increasing number of BRONJ cases since its first appearance.

The occurrence of BRONJ appears to be related to cumulative dose, duration of treatment and type of bisphosphonate [140, 344-347] where a positive correlation occurs with higher doses, longer duration of therapy and nitrogen-containing BPs.

Earlier studies have reported that the type of BP may play a role in BRONJ development, particularly the nitrogen containing BP like pamidronate and zoledronate with higher risk with zoledronate followed by pamidronate. [134, 135, 201, 230, 245, 344, 345, 348-350] The cumulative hazard of developing BRONJ is significantly greater with zoledronate treatment than with pamidronate or pamidronate plus zoledronate.[201] [135] due to the more potent inhibitory effect on bone turnover rate and stronger anti-resorptive activity of zoledronate compared with pamidronate. Zoledronate is 10 to 100 times more potent than pamidronate. [351] Consistent with these studies, we noted that most patients in the publications (58.9%) had received zoledronate only or pamidronate (13.9%) or zoledronate plus pamidronate (11.4%).

The mean duration of BP treatment was 38.2±15.7 months. It is a crucial factor for the development of BRONJ (192). It has been suggested that development of BRONJ requires a

long period of exposure (23). As reported in the literature, the risk of developing BRONJ is related to the therapy duration and the risk seems to be higher after 3 years of treatment in association with clinical risk factors [253]. Recently, Lo et al [352] reported a higher prevalence of BRONJ (0.21%) in patients treated with these drugs for more than 4 years, in comparison with those treated less than 2.5 years.

Current data suggests that IV BPs are much more frequently associated with BRONJ than oral BPs [201, 253, 353]. This has led to the development of different management strategies for patients on oral or IV BPs. This was in accordance with our search that revealed 83.2% of BRONJ lesions were developed from IV BPs. The results confirm data from other studies indicating that the prevalence of BRONJ is much lower in patients on oral BPs than in patients treated with intravenous BPs.[354]

A greater incidence of BRONJ has been reported in patients with malignancies particularly in those with multiple myeloma and breast cancer. [135, 201, 344, 348] Our results agree with these reports stating that BRONJ was more frequently noted in patients with multiple myeloma and breast cancer compared with prostate cancer, lung cancer, renal cell carcinoma and other neoplasm group.

With regard to a history of invasive dental treatment, 61.7 % of the patients had undergone a dental extraction before development of BRONJ. This finding is consistent with the review by Badros et al, which reported a significant association between the occurrence of BRONJ and age and a history of dental extraction in patients with multiple myeloma treated with intravenous bisphosphonates. [330] In agreement with published reports, tooth extraction in this review was associated with the development of BRONJ. [134, 225, 345, 355-357]According to the systematic review publications, BRONJ was found to be spontaneous in 14.8%. Our findings correspond with those of the authors reporting a higher percentage of so called spontaneous cases varying from 14.1% to 60%. [138, 160, 199, 202, 225, 228, 230,

236, 245, 253, 271, 308, 313, 319, 358-362] This may be due to the fact that it is difficult to establish the initiating factor in some patients.

Correlations between the occurrence of BRONJ and specific co-medication such as corticosteroids or chemotherapy have been discussed. [363], [354] [246, 364] These treatments may also increase the vulnerability of the oral mucosa and reduce its nutritive supply. [134, 246, 361] Of the patients, 39.7% were under chemotherapy. Moreover 24.6% used corticosteroids. In fact, corticosteroids and some other chemotherapy medications possess an anti-angiogenic effect by inhibiting the vascular endothelial growth factor (VEGF) and the fibroblast growth factor (FGF).[134, 246, 361, 365]

There is a considerable discussion in the literature whether aging plays a significant role in BRONJ development. Some studies found no statistically significant correlation between aging and BRONJ. [200, 366] Some authors include advanced age as a BRONJ co-factor [201, 279, 367], which could be related to the physiological effects of aging, including inflammatory issues [368], immune dysfunction [369], reduction of the blood flow and the remodelling ability [370, 371] , and increased oxidative stress[372]. In fact, these features are all implicated with BRONJ pathogenesis and could explain why this disease is not reported in young patients, even with other risk factors associated [373].Some authors reported a positive correlation between gender and BRONJ [279]. It has been speculated that oestrogen therapy may play a role in this correlation, since hormonal reposition has been associated with an increased risk of BRONJ[374].

Controversial aspects have also been discussed regarding gender as a BRONJ co-factor. Some studies found no statistically significant correlation between gender and BRONJ [201, 367] .Therefore, we observed that the large proportion of female patients from the studies [208, 248, 253, 279, 317, 325, 375-380] can represent only a coincidence, since women take oral BPs more frequently than males, especially because rheumatoid arthritis and osteoporosis are

more common in women [381]. In accordance with other series reported in the literature [359], the present review shows a high prevalence of BRONJ among women (67.2%).

BRONJ affects the mandible more often than the maxilla. There was a mandible/maxilla involvement ratio of 2/1, which could be attributed to the decreased vascularity of the mandible and to the existing local conditions, distribution that was similar to results reported also by other authors [306, 318, 382]. Only the mandible and maxilla appear to be susceptible, highlighting their unique nature compared with other parts of the skeleton. The jaws are the only bones in the human body that are in frequent contact with the outside world and are subject to repeated micro trauma through the presence of teeth and the forces of mastication; moreover the turnover of alveolar bone is 10-fold greater than in the long bones [250]. BRONJ occurred more often in the mandible (59%) than in the maxilla (27%), as was reported by Marx et al [134]. A possible explanation of osteonecrosis, especially in the mandible might be the anti-angiogenic effect of bisphosphonate [383-386] and anatomic and physiologic feature of mandibular bone that would increase the risk of osteonecrotic pathology [387]. This action would result in a direct induction of avascular necrosis of tissue repair and may interrupt intraosseous circulation and blood flow of the jaw [236]. Furthermore, bisphosphonate can also inhibit endothelial cell function [386] and increase the rate of apoptosis [384], leading to a decrease in capillary-tube formation [388].

The management of BRONJ is still a controversial topic. Several treatment protocols have been proposed, but there is no general consensus for many crucial questions, such as whether or not performing surgery is beneficial [268]. Some authors reported that BPs discontinuation for a variable period (one to six months) before and after interventions favoured the surgical outcome [303, 389] emphasising a possible anti-angiogenetic effect on the soft tissues around the necrosis and the removal of this effect may have a role in healing. There may also be

psychological aspects; patients may be stressed by the idea of taking drugs that could have an adverse effect on the bones.

Our results suggested that minimal invasive surgical treatment was the most commonly used method for the management of the BRONJ in which 767 patients were managed using sequestrectomy, curettage, debridement or smoothening of bone. These results were in agreement with Alons et al [316] who treated 7 patients with sequestrectomy and curettage of the defect with a minimum of periosteal deflection. Mitsimponas et al [390] reported a complete success rate of 53% in a patient group with different surgical procedures, including bone smoothing, incision and drainage, ulcer excision and closure after debridement. Eckert et al [391] demonstrated a 58% success rate in 24 operated patients. The surgical concept included resection of the necrotic bone and a stable soft tissue closure. Millesi et al [392] treated 55 patients with sequestrectomy, debridement, or partial resection with or without osteosynthesis after 6 months and found an overall complete success in 50%.

Carlson et al [133] reported high cure rates and improved stages of disease after surgery. Carlson states that performing segmental resection of the mandible and partial maxillectomies with the intention of achieving vital bone margins are of crucial importance in the management of BRONJ. According to Otto et al [393], surgery might be the only curative treatment in refractory disease. In these studies the authors favour radical surgery. The observation of the efficacy of resection for BRONJ has recently been reported in the dental literature (56, 214, 250).

Medical treatment is favoured by the AAOMS position paper whose authors state that surgery should be deferred as long as possible [250]. Van den Wyngaert et al. [338] and Scoletta et al.[306] stated that a medical treatment of BRONJ leads to mucosal healing in 50% of cases.

However, the healing rate of BRONJ lesions in the studied group was also significantly associated with the stage of BRONJ at presentation, with lower healing rates observed for

high stages [338].Font et al recommended in their BRONJ update a long-term antibiotic regime and CHX 3 or 4 times a day. Aggressive surgical therapies were not considered; moreover, an inadequate healing with a lack of mucosal closing was confirmed [394].

Growth factor application can be considered a challenge because of improving the soft and hard tissues healing. Acting like chemotactic agents, they stimulate angiogenesis, migration, proliferation, and differentiation of stem cells from the surrounding mesenchymal tissues into bone-forming cells in an area of injury [330, 395]. A new therapy of BRONJ based on rhBMP-2 application had been discussed and showed how growth factor application involves an increase in soft tissue healing [282].Some studies have reported treatment of refractory cases of BRONJ with bone resection followed by topical application of PRP [258, 259, 396] in which PRP is an autologous concentration of human platelets and a source of different protein growth factors. Protein growth factors such as platelet-derived growth factor, transforming growth factor-β, vascular endothelial growth factor, and epidermal growth factor-β are polypeptides released from the platelets when they are activated and can induce paracrine effects on stimulated cells. [397-399]

Recurrent BRONJ lesions could be managed successfully by the surgical use of the laser. These results are similar to those presented by Stübinger et al [336] and Vescovi et al [333] who used an Er:YAG laser for the bony debridement. Also ozone is effective on avascular necrosis-related pathologies by stimulating and/or preserving the endogen antioxidant system and by blocking the xantine/ xantine oxidase pathway, active in free radical synthesis [400-402]; by activating blood circulation, increasing red blood cells and hemoglobin concentration [403], enhancing diapedesis and phagocytosis, and stimulating the mononucleate phagocytic system [403-405].

The proposed rationale behind the beneficial effects of hyperbaric oxygen (HBO) therapy in BRONJ is increased wound healing, reduction of oedema and inflammation, stem cell

mobilization, and moderation of the suppression of bone turnover by BP [406]. Recent studies have revealed that HBO therapy also generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) that affect the signalling process critical to wound healing [406, 407] . HBO therapy also has a possibility to improve inflammation and infection around necrotic tissues by increasing blood vessels, oxygen concentration, and antibiotic levels in patients with BRONJ [406, 407].

Teriparatide (TP), a recombinant human parathyroid hormone (PTH) , is an osteoanabolic agent that has stimulatory effects on osteoblasts and subsequently osteoclasts and increases bone turnover by promoting bone formation with a positive balancing in bone metabolism [408, 409]. TP regulates bone resorption by increasing osteoclastic activity [410]. Therefore, Teriparatide is known to have quick and strong stimulatory effects on bone remodelling, even in the face of previous exposure to bisphosphonates [411-415]. The use of TP on refractory BRONJ lesions was first defined by Harper and Fung [416] who observed soft-tissue healing in a patient with a 3 month TP administration. Additionally, in a case study, Ohbayashi et al [417] demonstrated bone regeneration 6 months after TP therapy in a refractory BRONJ patient. Ma et al [415] showed that TP reverses the inhibitory effects of anti-resorptive drugs such as BPs in vivo. The BPs suppress osteoclastic activity by inducing apoptosis of these cells and cause them to detach from the bone surface [418]. Moreover of the adjunctive treatments was the fluorescence- guided bone resection that was introduced in the surgical therapy of BRONJ to determine the extent of the surgical debridement [143, 154].

It is difficult to compare the outcome of different BRONJ therapies for 2 mutually non-exclusive reasons: First, the definition of therapy success has not been universally defined, and in particular studies favouring medical therapy regimens often consider maintaining the status as success. Second, only a few studies have, to date, compared the therapy outcome of medical and surgical treatment in a controlled clinical manner [136].

The key factors for successful treatment have not been clearly identified yet. There are several aspects that are likely to influence the success of surgery and can cause progression of disease [267]. Comparing different studies about therapeutic success in BRONJ is made difficult by different definitions of success [312].

Ruggiero and Drew [419] considered preservation of quality of life by controlling pain, managing infection and preventing the development of new areas of necrosis as a treatment goal. Taking this into consideration, a relief of symptoms may very well be a “success” for the oncologic patient [316]. Vescovi et al [156] defined “clinical success” as a positive result (e.g., transition from a higher stage to a lower stage, complete mucosal healing) or a minimum time span of 3 months without clinical symptoms. With regard to the definition of BRONJ [354] , “clinical success” principally should include absence of pain and other symptoms of oral infection, lack of oral or cutaneous fistulas, and an intact mucosal cover over formerly exposed bone.[257]

Comparison between outcomes of different therapies is complicated because of the inclusion of patients taking different bisphosphonates and doing so in an uncontrolled clinical manner [272].

Treatment outcome is considered a success when oral mucosal healing is maintained without bone exposure or infection and there is acceptable radiographic healing for a 12-month period after surgery. Therefore, following patients for at least 1 year postoperatively may be indicated to disclose the possibility of recurrence of disease [272] which was in accordance with our results from the publications that showed a mean follow up period of 12.87 ± 9.88 months.

Data on treatment outcome of ONJ in the literature are vague and scarce. Marx et al [134] reported that 90% of the patients functioned free of pain under continuous antibiotic treatment, but they did not specify the type of response (CR, PR, or NR). Mavrokokki et al

[345] reported that 70% of the patients were classified as ongoing cases and that 30% had been resolved, but there were no details regarding PR and NR. Abu-Id et al [322] recently published a multicentre study from Germany, Austria, and Switzerland based on questionnaires of 78 ONJ patients. They reported that 60% of their 78 patients were treated with minor invasive surgical procedures or medical treatment with local disinfectants and antibiotics. The remaining patients were treated radically by means of bone resection up to viable bone. Of the patients who were treated medically, 38% were classified as responsive, as were 86% of the patients who were treated radically.

CONCLUSION

Mucosal coverage is the main goal of BRONJ treatments to prevent secondary infection. BRONJ management remains controversial, and there is no definitive standard of care for this disease. Nonsurgical, conservative and minimally invasive treatment regimen of BRONJ is considered useful for controlling the disease leading to predictable good results in cases of low and medium-potency BRONJ. Further research especially for high-potency BRONJ (refractory Stage 3 lesions) is indicated. BRONJ might be approached also by new adjunctive treatments such as ozone therapy or hyperbaric oxygen or growth factors in order to ensure the optimal patient treatment protocol. The application of adjunctive treatments is an opinion-based approach rather than evidence-based one. Controlled studies or clinical trials should be followed to evaluate these adjunctive treatments for the BRONJ patients.

5. PUBLICATION V

GENE THERAPY FOR BONE DEFECTS IN ORAL AND MAXILLOFACIAL SURGERY: A SYSTEMATIC REVIEW AND META-ANALYSIS

Riham Fliefel, Jan Kühnisch, Michael Ehrenfeld, Sven Otto. Under review in Stem cells and Development 2016.

ABSTRACT

Craniofacial bone defects are challenging problems for maxillofacial surgeons over the years. With the development of cell and molecular biology, gene therapy is a breaking new technology with the aim of regenerating tissues by acting as a delivery system for therapeutic genes in the craniofacial region rather than treating genetic disorders. A systematic review was conducted summarizing the articles reporting gene therapy in maxillofacial surgery to answer the question: Was gene therapy successfully applied to regenerate bone in the maxillofacial region? Electronic searching of online databases was performed in addition to hand-search of the references of the included articles. No language or time restrictions were enforced. Meta-analysis was done to assess significant bone formation after delivery of gene material in the surgically induced maxillofacial defects. The search identified 2081 articles of which 57 were included with 1726 animals. Bone morphogenetic proteins (BMPs) were commonly used proteins for gene therapy. Viral vectors were the universally used vectors. Sprague-Dawley rats were the frequently used animal model in experimental studies. The quality of the articles ranged from excellent to average. Meta-analysis results performed on 21 articles showed that defects favoured bone formation by gene therapy. Funnel plot showed symmetry with the absence of publication bias. Gene therapy is on the top list of innovative strategies that developed in the last 10 years with the hope of developing a simple chairside protocol in the near future combining improvement of gene delivery as well as knowledge of the molecular basis of oral and maxillofacial structures.

ABBREVIATIONS

μCT: Micro computed tomography
911 helper: human embryonic retinoblasts
AAV: Adeno-associated virus,
ALP: Alkaline phosphatase
b-FGF: Basic fibroblast growth factor
BMD: Bone mineral density
BMP-2: Bone morphogenetic protein 2
BMP-7: Bone morphogenetic protein 7
CHA: Coral hydroxyapatite
CMPC: Calcium magnesium phosphate cement
EGFP: Enhanced green fluorescence protein
FACS: Fluorescence-activated cell sorting
FEA: Finite element analysis
HA/TCP: Hydroxyapatite/beta-tricalcium phosphate
HGF: Hepatocyte growth factor,
HVJ: Hemagglutinating virus of Japan
IGF 1: Insulin growth factor
LMP-3: LIM mineralization protein 3,
MKP-1: Mitogen-activated protein kinase phosphatase 1
MBG: Mesoporous bioglass
N/R: Not reported
NGF-β: Nerve growth factor beta
NNB: Natural non-organic bone
OF: Orthodontic force
OSX: Osterix
OSTEOBONE: Calcium silicon phosphorus
pOBs: Periosteal derived osteoblasts
PBS: Phosphate buffered saline
PDGF-A: Platelet derived growth factor A
PDLSCs: Periodontal stem cells
PFU: Plaque forming unit
Pg-LPS: lipopolysaccharide mediated bone loss
RANKL: Receptor activator of nuclear factor kappa-B ligand
RUNX2: Runt-related transcription factor 2
SEM: Scanning electron microscope
TM: Tooth movement
TRAP: Tartrate resistance acid phosphatase
TU: Transduction units
VEGF: Vascular endothelial growth factor
WEHI 164: mouse skin fibroblast
JM 109: Escherichia Coli
β-TCP: Beta-tricalcium phosphate
293FT: human embryonic kidney cells with the SV40 large T antigen
ADSCs: Adipose derived stem cells
AV: Adenovirus
BGC: Bioactive glass ceramic
BMMSCs: Bone marrow mesenchymal stem cells
BMP-4: Bone morphogenetic protein 4
BMP-9: Bone morphogenetic protein 9
CFSE: Carboxyfluorescein diacetate succinimidyl ester
CRE8: Cre-expressing 293 cells
DPSCs: Dental pulp stem cells
ERR: External root resorption
ELISA: Enzyme linked immunosorbent assay
GAM: Gene activated matrix
HA/COL: Hydroxyapatite/ Collagen
HA/PA: Hydroxyapatite/polyamide
HEK293: human embryonic kidney 293 cell line
HIF-1*α*: Hypoxia-inducible factor-1 alpha
iPSCs: Induced pluripotent stem cells
IFU: Infectious units per ml
LacZ: β-galactosidase
Luc: Firefly luciferase
MOI: multiplicity of infection
mSS: Premineralized silk fibroin protein scaffolds
NB: Nano-bubbles
NIH3T3: mouse embryo fibroblast
NOD/SCID mice: Non-obese/severe combined immunodeficient
OPG: Osteoprotegrin
PCR: Polymerase chain reaction
PDGF-B: Platelet derived growth factor B
PDLA: Poly D, L-lactide
PF127: Pluronic F127
PG13: mouse embryonic fibroblast
PLGA: Poly lactic co glycolic acid
RSV: respiratory syncytial virus
SDF: Syngeneic dermal fibroblasts
TGF-b: Transforming growth factor beta
TNFR: Tumour necrosis factor alpha receptor
TSG-6: Tumour necrosis factor alpha-stimulated gene-6
US: Ultra-sound
WB: Western Blot

INTRODUCTION

Craniofacial anomalies and bone defects resulting from bone loss due to trauma, reconstructive surgery, neoplasia, congenital defects, infection or periodontal disease present a difficult and challenging problem for maxillofacial surgeons and scientists over the years with the goal of restoring facial form, function and occlusion. Conventional therapies are directed toward maxillofacial surgery, the use of prostheses or bone grafts. However, the effectiveness of these techniques is constrained by donor site morbidity, high cost and insufficient tissue resources. Recently, it had been agreed on the urgent need for new strategies for craniofacial reconstruction to improve bone regeneration with complete healing of the defects regardless of size [420-422]. As an alternative to the traditional techniques, "tissue engineering" has developed as a new and promising multi-disciplinary technique in the field of maxillofacial reconstruction and surgery [423].

With the development of cell and molecular biology, DNA-based technology had appeared as a promising method to meet challenges of tissue engineering in different applications. The genetic principle is either applied individually or together with tissue engineering to be known as gene-enhanced tissue engineering that regenerates lost tissue by local delivery of cells that have been genetically-modified to deliver signalling factors at DNA-level [424]. To date, gene therapy is the leading technology in medicine providing hope for those individuals that are suffering genetic disorders.

Gene therapy is known to be transferring genetic materials or functioning gene to replace a damaged one inducing individual's own cells to produce a therapeutic agent to improve the clinical outcome. It has several advantages over traditional treatments as the expression in host cells lasts longer for weeks to years than pharmaceutical compounds or recombinant protein which range from several hours to days. It reduces technical challenges associated

with ex-vivo protein expression and purification. Finally, the delivery of genetic sequences could mimic the natural biologic healing response [425, 426].

There have been a couple of advances in gene therapy relevant to dentistry since 1995. When applying the gene therapy principles, the maxillofacial region has significant advantages compared to other sites in the body, including easy access and observability. Potential applications for gene-based tissue engineering therapies in the oral and maxillofacial complex include treatment of salivary gland diseases, autoimmune diseases, cancerous and precancerous lesions, pain, caries, dermatological disorders, delivery of growth factors for periodontal regeneration, pulp capping/dentin regeneration, treatment of malignant neoplasms of the head and neck, bone regeneration for bone grafting of large osseous defects in dental and craniofacial reconstruction and articular cartilage repair [427, 428].

Although gene therapy was originally accepted as a means of treating heritable genetic disorders, its application in the craniofacial region is more often directed at regenerating tissues by acting as a delivery system for therapeutic genes promoting healing directly to cells within the defect or by genetically engineering mesenchymal stem cell progenitors to produce factors prior to implantation resulting in higher and more constant levels of protein production [2, 44, 429].

Thus, we have conducted a systematic review summarizing the articles reporting trials of gene therapy worldwide in the field of oral and maxillofacial surgery.

MATERIAL AND METHODS

This study was registered in SYRCLE (SYstematic Review Centre for Laboratory animal Experimentation) systematic review protocol for animal intervention studies (www.syrcle.nl). The guidelines for reporting systematic reviews and meta-analyses of animal studies was proposed by Peters et al [430] that are akin to the PRISMA guidelines for the reporting of

systematic reviews and meta-analyses of healthcare interventions in human clinical studies [431].

Review questions

The following PICO question was mainly addressed: Was gene therapy successfully applied to regenerate bone or heal defects in the oral and maxillofacial region?

Search strategy and selection criteria

A systematic review of the literature was performed to provide an overview of published articles describing gene therapy in the field of Oral and Maxillofacial Surgery. Medical databases were searched to 18th December 2015. The data search included a combination of the following keywords: ‘‘Gene therapy’’ ‘‘AND’’ ‘‘Maxillofacial surgery’’ ‘‘OR’’ ‘‘Gene therapy’’ ‘‘AND’’ ‘‘Bone tissue engineering’’, ‘‘Genetic Engineering’’ ‘‘AND’’ ‘‘Maxillofacial bone’’, ‘‘Gene therapy’’ ‘‘AND’’ “Distraction Osteogenesis” ‘‘OR’’ ‘‘Gene therapy’’ ‘‘AND’’ “Alveolar bone” ‘‘OR’’ ‘‘Gene therapy’’ ‘‘AND’’ “Periodontal tissue” ‘‘OR’’ ‘‘Gene therapy’’ ‘‘AND’’ “Temporomandibular joint”. All the possible combinations of these words were explored. Medical subject headings (MeSH terms) without subheading restrictions was used and the heading sequence was ‘‘Gene therapy’’ ‘‘AND’’ ‘‘Dentistry’’.

In addition, we performed hand-search to the references of the included articles, papers of interest and related systematic or non-systematic reviews. The International Journal of Oral and Maxillofacial Surgery, Journal of Craniomaxillofacial Surgery, Gene therapy, Molecular therapy and Human gene therapy journals were also screened to identify possible references not reported elsewhere. No language or time restrictions were enforced. Relevant full publications and meeting abstracts were identified by electronic searching of three online databases (PubMed, Cochrane library and Web of Knowledge). After the identification of articles in the databases, the articles were imported into Endnote X7 software (Thompson Reuters, Philadelphia, PA, USA) to store, manage search results and remove duplicates

regardless of whether the studies are eventually included or excluded in the systematic review. Titles and abstracts identified were screened resulting in a number of seemingly relevant studies for the systematic review. The abstracts of the articles were then reviewed and the full text was obtained for those articles with apparent relevance. The identified articles were selected based on the inclusion criteria and exclusion criteria.

Inclusion criteria

(1) Relevant data on Gene therapy, (2) Animal studies, (3) Defects performed in the Oral and Maxillofacial region, (4) Any language.

Exclusion criteria

(1) In vitro studies, (2) Gene therapy in bones other than maxillofacial, (3) Calvarial bones defects, (4) Review articles, (5) letters to the editor, editorials, poster or oral presentations or articles with only abstract, (6) Oral cancer or soft tissue lesions, (7) Studies based on the use of only growth factors or cell-based therapies.

To improve the sensitivity of the relevant studies, each publication identified in the electronic search were assessed independently by two independent reviewers (RF and SO) to make a decision on inclusion/exclusion criteria or data extraction and quality of the articles with differences resolved by discussion.

Data extraction

All information was extracted using a standardized data form created in Excel. Data extracted included: 1) Author, 2) Year, 3) Journal, 4) Country, 5) Language, 6) therapeutic gene, 7) Vector, 8) Control gene, 9) Virus Titres (Concentration), 10) Cell lines for generation of virus, 11) Experiment design, 12) Disease model, 13) Site, 14) Animal Model, 15) Sample size, 16) Defect size, 17) Carrier/Scaffold, 18) Gene delivery route, 19) Stem cells source, 20) Experimental groups, 21) Cell concentration to be used in the defect, 22) Analysis methods with main endpoint results.

Data was extracted from either text or tables in the results section of the included studies. Data that was presented as graphs was extracted electronically using WebPlotDigitizer software, version: 3.9 (WebPlotDigitizer, US, http://arohatgi.info/WebPlotDigitizer, 2015).

Methodological quality assessment

The quality assessment of all the included studies in this systematic review was performed based on ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines [432] and evaluated based on a predefined grading system [433] applied to the following items: (1) Title, (2) Abstract/Summary, (3) Introduction/Background, (4) Introduction/ Primary and secondary objectives, (5) Methods/Ethical statement, (6) Methods/Study design, (7) Methods/Experimental procedure, (8) Methods/Experimental Animals, (9) Methods/Housing and husbandry, (10) Methods/Sample size, (11) Methods/Allocation animals to experimental groups, (12) Methods/Experimental outcomes, (13) Methods/Statistical methods, (14) Results/Baseline data, (15) Results/Numbers analysed, (16) Results/Outcomes and estimation, (17) Results/Adverse events, (18) Discussion/Interpretation and scientific implications, (19) Discussion/Generalisability and translation, (20) Discussion/ Funding.

Risk of bias assessment

Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) risk of bias tool was applied to assess the internal validity of the included studies using RevMan software (version 5.3) [434, 435]. A modified 7-point-item check list was used to assess the risk of bias, including: (1) published in a peer-reviewed journal; (2) random allocation to treatment or control; (3) treatment allocation concealment; (4) blinded assessment of outcome; (5) reporting of a sample size calculation; (6) statement of compliance with animal welfare regulations and (7) statement of potential conflict of interest. Each trial was assessed by two independent observers (RF and SO) and any differences resolved by discussion.

Outcome Measure

The primary outcome measure for this meta-analysis was significant new bone formation by histology (% of area and % of volume) or radiograph (bone volume fraction) between the experimental and control group.

Statistical Analysis

A qualitative data analysis was performed with the aim of summarizing the results of the studies included. Meta-analyses as well as forest and funnel plots were conducted using RevMan software (Review Manager [RevMan] Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). Bone formation was assessed as continuous outcome variables by inverse variance (IV) method and recorded as the standardized mean difference (SMD) with 95% confidence interval (CI). The effect size of the SMD was classified as follows: 0.2 represents a small effect, 0.5 a moderate effect, and 0.8 a large effect [436]. The I^2 indicating heterogeneity and Cochran's Q statistical test were calculated; a value of I^2: 0% to 40% might not be important, 30% to 60% may represent moderate heterogeneity; 50% to 90% may represent substantial heterogeneity and 75% to 100% shows considerable heterogeneity [437]. A weighted fixed-effect model was used to estimate the overall effect size. Results with a P-value of < 0.0001 were considered indicative of statistical significance. Potential publication bias was explored using funnel plot generated using RevMan.

RESULTS

Search results

The search identified a total of 2081 references from the different databases and hand search: PubMed (n= 2000), Web of science (n= 63), Cochrane library (n= 7), hand-search (n=11). After duplicates removal via Endnote duplicate function, 1509 articles were screened for titles/abstracts and resulted in only 148 studies for full-text evaluation with the exclusion of

1361 articles that were irrelevant to the topic or review articles. Further screening resulted in a total of 57 studies which were considered eligible for the systematic review and fulfilled the final selection criteria. **Figure 5.1** illustrates the search flow and the identification of eligible studies.

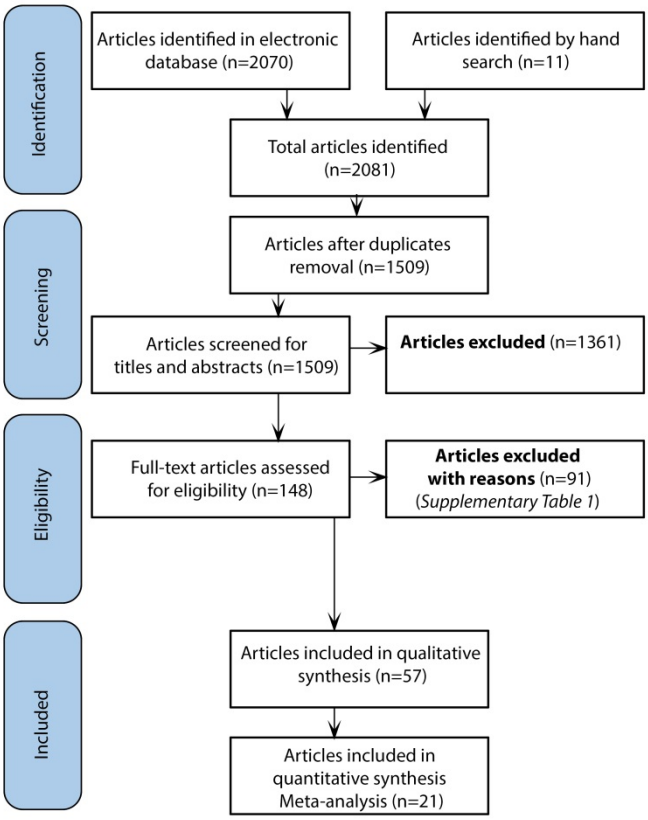


Figure 5.1: Flow-chart of the process of literature search and studies included in the review.

Study characteristics

The articles analysed were published between 1999 and 2015. Most of the studies were conducted in USA [438-448] and China [378, 449-477]. However, few studies were conducted in Taiwan [478], Japan [479, 480], Spain [481], Germany [482], Italy [483], Korea [484] or as a collaboration between two countries [471, 485-492]. Almost all articles (91.3%) were published in English [378, 438-457, 459-463, 465-469, 471-475, 477-487, 489-493] while only 5 articles (8.7%) [458, 464, 467, 470, 476] were published in Chinese. Bone morphogenetic proteins (BMPs) were the most commonly used proteins for gene therapy (n=28, 49.1%) [439, 440, 443, 445, 446, 451, 452, 454, 457-459, 463, 464, 466, 467, 470-474, 478, 482, 484-489] followed by Platelet-derived growth factors (PDGF; n=6, 10.5%) [438, 441, 442, 447, 471, 472], while the remaining 23 articles (40.4%) were using various proteins as: Enhanced green fluorescent protein (EGFP) [450, 469, 479, 490], Tumour

necrosis factor alpha receptor (TNFR) [444], Hepatocyte growth factor (HGF) [449], Receptor activator of nuclear factor kappa-B ligand (RANKL) [480, 494], Basic fibroblast growth factor (b-FGF) [453, 465], β-galactosidase (LacZ) [493], Osterix (OSX) [455, 456], LIM mineralization protein 3 (LMP-3) [483], Vastatin [378], Vascular endothelial growth factor (VEGF) [460], Osteoprotegrin (OPG) [461, 475, 476, 492], Runt-related transcription factor 2 (RUNX2) [462], Nerve growth factor beta (NGF-β) [468], Tumour necrosis factor alpha-stimulated gene-6 (TSG-6) [491], Mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) [448] and Hypoxia-inducible factor-1 alpha (HIF-1α) [477].

In 30 articles (52.6%) [438-442, 445-449, 452-454, 457, 459, 462, 463, 470-474, 478, 482, 483, 485, 488, 489, 493, 495], adenovirus was the universally used vector. However, other vectors were used as: plasmid (n=12, 21%) [443, 451, 455, 464-467, 475, 476, 479, 486, 496], adeno-associated virus (n=4, 7%) [378, 444, 450, 460], hemagglutinating virus of Japan (HJV; n=3, 5.3%) [480, 481, 492], liposome (n=2, 3.5%) [482, 487], lentivirus (n= 5, 8.8%) [461, 468, 469, 477, 491] and retrovirus (n=1, 1.8%) [490]. For the control genes, Green fluorescent protein (GFP) were the most abundant control in 20 articles (35.1%) [378, 446, 449, 451, 453-455, 457, 460, 462, 465, 468, 470, 472-474, 477, 483, 485, 486, 489, 496] followed by β-galactosidase (LacZ) in 9 articles (15.8%) [439, 440, 448, 452, 459, 478, 482, 485, 488] and Luciferase (Luc) in 6 articles (10.5%) [438, 441, 442, 445, 447, 479] respectively. However, in 22 articles (38.6%), the control gene was not reported. Seven different packaging cell lines were used for replication of the viruses: HEK293 (human embryonic kidney 293 cell line) [378, 440-442, 444, 446, 448-450, 460-463, 470, 472, 473, 484, 485, 493], 293FT (human embryonic kidney cells with the SV40 large T antigen) [469], WEHI 164 (mouse skin fibroblast) [457], NIH3T3 (mouse embryo fibroblast) [480, 481], CRE8 (Cre-expressing 293 cells) [483], 911 helper (human embryonic retinoblasts (HER) [482] and PG13 (mouse embryonic fibroblast) [490]. The experiments were performed either

as in vitro/ in vivo in 38 articles (66.7%) [378, 440, 446-449, 452-454, 457, 459-463, 465-467, 469, 471-478, 480-486, 488-491] or were completely in vivo studies in 19 articles (33.3%) [438, 439, 441-445, 450, 451, 455, 456, 458, 464, 468, 470, 479, 487, 492, 493]. **Table 5.1** presents the characteristic of the included studies.

Table 5.1: Summary of essential features of all studies included in the systematic review

Author	Year	Journal	Country	Language	Therapeutic Gene	Vector	Control	Virus Titres (Concentration)	Cell lines for generation of virus	Experiment design
Abramson [438]	2007	Eur Cell Mater	USA	English	PDGF-B	AV	Luc	N/R	N/R	In vivo
Alden [439]	2000	J Craniofac Surg	USA	English	BMP-2/BMP-9	AV	LacZ	5×10^{7} particle/µl	N/R	In vivo
Ashinoff [440]	2004	Ann Plast Surg	USA	English	BMP-2	AV	LacZ	N/R	HEK293	In vitro/In vivo
Chang [441]	2009	Hum Gene Ther	USA	English	PDGF-B	AV	Luc	N/R	HEK293	In vivo
Chang [442]	2010	Gene Ther	USA	English	PDGF-B	AV	Luc	N/R	HEK293	In vivo
Chang [485]	2003	Gene Ther	Taiwan/USA	English	BMP-2	AV	LacZ	N/R	HEK293	In vitro/In vivo
Chen [479]	2009	J Dent Res	Japan	English	EGFP	Plasmid	Luc	N/R	JM 109	In vivo
Chen[443]	2007	Plast Reconstr Surg	USA	English	BMP-4	Plasmid	N/R	N/R	N/R	In vivo
Chen[478]	2008	Gene Ther	Taiwan	English	BMP-2	AV	LacZ	50 MOI	N/R	In vitro/In vivo
Cirelli [444]	2009	Gene Ther	USA	English	TNFR	AAV	N/R	$5\text{-}20\times10^{12}$ DRP/ml	HEK293	In vivo
Cao[449]	2015	Stem Cell Res Ther	China	English	HGF	AV	GFP	50-400 MOI	HEK293	In vitro/In vivo
Dai [450]	2007	Front Biosci	China	English	EGFP	AAV	N/R	N/R	HEK293	In vivo
Dunn [445]	2005	Mol Ther	USA	English	BMP-7	AV	Luc	N/R	N/R	In vivo
Hu [451]	2007	J Orthop Res	China	English	BMP-7	Plasmid	GFP	N/R	N/R	In vivo
Iglesias-Linares [481]	2011	Orthod Craniofac Res	Spain	English	RANKL	HVJ	N/R	N/R	NIH3T3	In vitro/In vivo
Jiang [486]	2006	Int J Oral Maxillofac Surg	China/Canada	English	BMP-4	Plasmid	GFP	N/R	JM 109	In vitro/In vivo
Jiang [454]	2009	Clin Oral Implants Res	China	English	BMP-2	AV	GFP	50 PFU/cell (MOI)	N/R	In vitro/In vivo
Jiang [452]	2009	Biomaterials	China	English	BMP-2	AV	LacZ	80 PFU/cell (MOI)	N/R	In vitro/In vivo
Jiang [453]	2010	Bone	China	English	b-FGF	AV	GFP	N/R	N/R	In vitro/In vivo
Jin [446]	2003	J Periodontol	USA	English	BMP-7/Noggin	AV	GFP	200 PFU/cell (MOI)	HEK293	In vitro/In vivo
Jin [447]	2004	Mol Ther	USA	English	PDGF-B /PDGF-A	AV	Luc	200 PFU/cell (MOI)	N/R	In vitro/In vivo
Kanzaki [480]	2006	Gene Ther	Japan	English	RANKL	HVJ	N/R	N/R	NIH3T3	In vitro/In vivo
Kroczek [487]	2010	J Craniomaxillofac Surg	Germany Netherlands	English	BMP-2	Liposome	N/R	N/R	N/R	In vivo
Kuboki [493]	1999	Arch Oral Biol	Japan	English	LacZ	AV	N/R	N/R	HEK293	In vivo
Lai [455]	2014	J Zhejiang Univ Sci B	China	English	OSX	Plasmid	GFP	N/R	N/R	In vivo
Lai [456]	2011	Oral Surg Oral Med Oral Pathol Oral Radiol Endod	China	English	OSX	Plasmid	N/R	N/R	N/R	In vivo
Lattanzi [483]	2008	Gene Ther	Italy	English	LMP-3	AV	GFP	N/R	CRE8	In vitro/In vivo
Li [457]	2010	J Biomed Mater Res A	China	English	BMP-7	AV	GFP	N/R	WEHI 164	In vitro/In vivo
Li [458]	2010	Zhonghua Yi Xue Za Zhi	China	Chinese	BMP-7	N/R	N/R	N/R	N/R	In vivo
Li [378]	2009	Arch Oral Biol	China	English	Vastatin	AAV	GFP	5×10^{3}, 1×10^{4}-5×10^{4} PFU/cell (MOI)	HEK293	In vitro/In vivo
Long [459]	2011	Oral Surg Oral Med Oral Pathol Oral Radiol Endod	China	English	BMP-2	AV	LacZ	100 PFU/cell (MOI)	N/R	In vitro/In vivo

Table 5.1(cont.): Summary of essential features of all studies included in the systematic review

Author	Year	Journal	Country	Language	Therapeutic Gene	Vector	Control	Virus Titres (Concentration)	Cell lines for generation of virus	Experiment design
Park J [482]	2003	Gene Ther	Germany	English	BMP-2	AV Liposome	LacZ	1-3×10^{10} PFU/ml	911 helper	In vitro/In vivo
Park S [484]	2015	J Biomed Mater Res A	Korea	English	BMP-2	AV	N/R	100 PFU/cell (MOI)	HEK293	In vitro/In vivo
Rabie [460]	2007	Gene Ther	China	English	VEGF	AAV	GFP	N/R	HEK293	In vitro/In vivo
Steinhardt [488]	2008	Tissue Eng Part A	Israel/USA	English	BMP-2	AV	LacZ	3000 PFU/cell	N/R	In vitro/In vivo
Su [461]	2015	Stem Cell Res Ther	China	English	OPG	Lentivirus	N/R	1.5×10^{6} TU/ml	HEK293	In vitro/In vivo
Sun [489]	2010	Arch Oral Biol	China/USA	English	BMP-2	AV	GFP	50 PFU/cell	N/R	In vitro/In vivo
Sun [463]	2013	J Oral Maxillofac Surg	China	English	BMP-2	AV	N/R	N/R	HEK293	In vitro/In vivo
Sun [462]	2014	J Orthop Res	China	English	Runx2	AV	GFP	5×10^{9} PFU/ml Runx2 2×10^{10} PFU/ml GFP	HEK293	In vitro/In vivo
Sun [464]	2007	Shanghai Kou Qiang Yi Xue	China	Chinese	BMP-2	Plasmid	N/R	N/R	N/R	In vivo
Tan [465]	2009	Cytotherapy	China	English	b-FGF	Plasmid	GFP	N/R	N/R	In vitro/In vivo
Tang [466]	2008	Cell Biol Int	China	English	BMP-2	Plasmid	N/R	N/R	N/R	In vitro/In vivo
Tang [467]	2006	Zhonghua Kou Qiang Yi Xue Za Zhi	China	Chinese	BMP-2	Plasmid	N/R	N/R	N/R	In vitro/In vivo
Wang [468]	2015	Br J Oral Maxillofac Surg	China	English	NGF-β	Lentivirus	GFP	N/R	N/R	In vivo
Wei [490]	2013	Stem Cells Dev	USA/China	English	EGFP	Retrovirus	N/R	N/R	PG13	In vitro/In vivo
Wen [469]	2012	Arch Oral Biol	China	English	EGFP	Lentivirus	N/R	N/R	293FT	In vitro/In vivo
Yang [491]	2014	PLoS One	USA/China	English	TSG-6	Lentivirus	N/R	N/R	N/R	In vitro/In vivo
Ye [470]	2006	Shanghai Kou Qiang Yi Xue	China	Chinese	BMP-2	AV	GFP	100 MOI	HEK293	In vivo
Yu [448]	2011	Gene Ther	USA	English	MKP-1	AV	LacZ	N/R	HEK293	In vitro/In vivo
Zhang [473]	2007	Biomaterials	China	English	BMP-7	AV	GFP	2×10^{10} particles/ml	HEK293	In vitro/In vivo
Zhang [472]	2009	J Control Release	China	English	BMP-7/PDGF-B	AV	GFP	2×10^{10} particles/ml	HEK293	In vitro/In vivo
Zhang [471]	2015	J Clin Periodontol	Switzerland China	English	BMP-7/PDGF-B	AV	N/R	1.4×10^{10} PFU/ml	N/R	In vitro/In vivo
Zhao [474]	2010	Oral Dis	China	English	BMP-2	AV	GFP	80 PFU/cell (MOI)	N/R	In vitro/In vivo
Zhao [492]	2012	Orthod Craniofac Res	China/Japan	English	OPG	HVJ	N/R	N/R	N/R	In vivo
Zhou [476]	2010	Hua Xi Kou Qiang Yi Xue Za Zhi	China	Chinese	OPG	Plasmid	N/R	N/R	N/R	In vitro/In vivo
Zhou [475]	2012	Int J Periodontics Restorative Dent	China	English	OPG	Plasmid	N/R	N/R	N/R	In vitro/In vivo
Zou [477]	2012	PLoS One	China	English	HIF-1α	Lentivirus	GFP	7 MOI	N/R	In vitro/In vivo

Alveolar bone defects with or without dental implant were the prevalent model used for gene therapy in 20 articles (35.1%) [438, 439, 442, 443, 445, 452, 457, 461, 463, 466, 467, 472-474, 477, 482, 483, 485, 486, 488], periodontal disease with or without alveolar bone involvement (n=17, 29.8%) [441, 444, 446-449, 458, 464, 465, 469, 471, 475, 476, 478, 479, 484, 491] followed by distraction osteogenesis (n=9, 15.8%) [440, 451, 453, 455, 456, 459, 462, 468, 487], temporomandibular joint (n=4, 7%) [378, 450, 460, 493], orthodontic tooth movement (n= 3, 5.2%) [480, 481, 492], sinus floor elevation (n=2, 3.5%) [454, 489], tooth restoration with bio-root regeneration (n=1, 1.8%) [490] and central fissures (cleft) (n=1, 1.8%) [470]. Most of the defects were in the mandible (n=39, 68.4%) [378, 439-441, 446, 447, 450-453, 455-470, 472-477, 482-484, 486-489, 493] while 16 articles (28%) [438, 442-445, 448, 454, 471, 478-481, 485, 489, 491, 492] showed that the defects were created in the maxilla. One article was reported in both jaws (1.8%) [449] and the location was missing in one article (1.8%) [490]. The posterior mandible (premolar-molar area) was the most frequent region. However, some studies did the experiments in the anterior region.

Sprague-Dawley rats were the frequently used animal model in experimental studies of gene therapy (n=17, 29.8%) [378, 438-442, 444, 445, 447, 448, 450, 451, 460, 466, 467, 469, 491] followed by Wistar rats in 6 studies (10.5%) [479-483, 492], Lewis Fisher in 3 studies (5.3%) [446, 452, 474] and ginue-pigs or mice in one article each (n=2, 3.5%) each [488, 493]. White New Zealand rabbits were also used as a small animal model for the studies (n=14, 24.6%) [453-457, 459, 461-463, 468, 470, 478, 486, 489]. For large animals models, dogs and pigs were commonly used in 11 (19.3%) [443, 458, 464, 465, 471-473, 475-477, 482] and 4 (7%) studies respectively [449, 485, 487, 490]. Sample size ranged between 4 and 24 for large animal models. However, for small animal models, the sample size ranged between 11 and 144 animals.

Two different defect shapes were identified: circular (n=9, 15.8%) [439, 452, 464-467, 474, 482, 488], rectangular (n=22, 38.6%) [441, 443, 445-447, 449, 454, 457, 461, 463, 469, 471-473, 475-478, 483, 485, 486, 489] and 26 studies (45.6%) [378, 438, 440, 442, 444, 448, 450, 451, 453, 455, 456, 458-460, 462, 468, 470, 479, 480, 484, 487, 490-494] did not mention the shape of the defect. Variable diameters were recognised for the circular defects ranging from 1 to 6 mm. The rectangular defects were characterised by heterogeneity of dimensions.

Gene delivery route was ex-vivo in 35 articles (61.4%) [446, 449-459, 461-463, 465-470, 474-478, 482-490], in-vivo in 21 articles (36.8%) [378, 438-445, 447, 448, 460, 464, 471-473, 479-481, 492, 493] and both in only one article (1.8%) [491]. For in-vivo gene delivery route (direct injection or GAM), physiological saline, collagen gels or lipid bubbles were used to deliver the genetically modified cells or material to the defect. The scaffolds used for seeding of the cells differed in each study. The used scaffolds were: beta-tricalcium phosphate **(**β-TCP) [454, 461, 474], Bioactive glass ceramic **(**BGC) [463], Coral hydroxyapatite (CHA) [466, 467], Hydroxyapatite/ Collagen (HA/COL) [483, 484], Hydroxyapatite/ beta-tricalcium phosphate **(**HA/TCP) [490], Premineralized silk fibroin protein scaffolds (mSS) [452], Natural non-organic bone (NNB) [486], Mesoporous bioglass/silk fibrin (MBG) [471], hydroxyapatite/polyamide (HA/PA) [457], Pluronic F127 (PF127) [478], Poly D, L-lactide (PDLA) [443], Poly lactic co glycolic acid (PLGA) [475, 476], Calcium magnesium phosphate cement (CMPC) [477] and Calcium silicon phosphorus (OsteoBone) [489]. Regarding the source of transfected/transduced stem cells, mesenchymal stem cells (MSCs) were used in 25 experiments (43.8%) either from bone marrow or adipose tissue or induced pluripotent [451-459, 465-468, 474-478, 482, 486-489, 491], while different type of cells such as syngeneic dermal fibroblasts (SDFs) [446, 483], dental pulp stem cells (DPSCs) [449, 490], periodontal derived stem cells (PDLSCs) [461, 463, 469, 472, 473, 484, 490] and periosteal derived osteoblast cells (pOBs) [470] were used in other studies with different

concentration of the cells. All the experiments had been divided into different study groups for comparing the efficiency of gene therapy in the disease model. **Table 5.2** shows the extracted data from the included studies with reference to the disease model and animals used.

Table 5.2: Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Abramson [438]	Alveolar bone defect with dental implant	Maxilla (bilateral: first molars)	Male Sprague Dawley rats	16	N/R	2.6% collagen gel	In-vivo (GAM)	----------	High dose Low dose Collagen alone Untreated control	8×10^{11} particles/ml 8×10^{10} particles/ml
Alden [439]	Alveolar bone defect	Mandible (bilateral: angle)	Sprague Dawley rats	13	4 mm circular	Physiological saline	In-vivo (local injection)	----------	BMP 2 BMP 9 LacZ	3.75×10^{8} particles/7.5 µl
Ashinoff [440]	Distraction Osteogenesis	Mandible (Right side: body)	Male Sprague Dawley rats	54	N/R	N/R	In-vivo (local injection)	----------	Untreated control BMP2 LacZ	1×10^{10} IFU
Chang [441]	Periodontal alveolar bone defect	Mandible (Buccal plate:1st and 2nd molars roots)	Sprague Dawley rats	144	3×2×1 mm^3	2.6% collagen gel	In-vivo (GAM)	----------	High dose Low dose Collagen alone	5.5×10^{8} PFU/ml 5.5×10^{9} PFU/ml in 20µl collagen
Chang [442]	Alveolar bone defect with dental implant	Maxilla (Bilateral: first molars)	Male Sprague Dawley rats	100	N/R	2.6% collagen gel	In-vivo (GAM)	----------	High dose Low dose Luc rhPDGF-BB Collagen alone	5.5×10^{8} PFU/ml 5.5×10^{9} PFU/ml
Chang [485]	Alveolar bone defect	Maxilla (Bilateral: infraorbital rim)	Female miniature swine	20	3×1.2 cm^2	Collagen Type I	Ex-vivo	----------	BMP2 LacZ	N/R
Chen [479]	Periodontal Disease	Maxilla (labial PDL: incisors)	Male Wistar rats	29	N/R	Lipid bubbles	In-vivo (GAM)	----------	DNA DNA/US DNA/NB DNA/US/NB	N/R
Chen [443]	Alveolar bone defect	Maxilla (Bilateral: anterior)	Foxhound dogs	N/R	2 cm	PDLA	In-vivo (GAM)	----------	BMP-4 scaffold Autograft Scaffold only Blank control	N/R
Chen [478]	Periodontal alveolar bone defect	Maxilla (Bilateral: incisors)	Male New Zealand White rabbits	12	15×7×5 mm^3	PF127	Ex-vivo	BMMSCs	BMP-2 transfected MSCs/PF127 Bgal transfected MSCs/PF127 Untransfected MSCs/PF127 PF127 only	50×10^{6}cell/ml
Cirelli [444]	Periodontal Disease	Maxilla (Bilateral: palatal gingival tissue between molars)	Male Sprague Dawley rats	45	N/R	Physiological saline	In-vivo (local injection)	----------	Vehicle Pg-LPS TNFR:Fc TNFR:Fc + Pg-LPS	1×10^{11}DRP/100ml

Table 5.2 (cont.): Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Cao [449]	Periodontal Disease	Maxilla&Mandible (First molars)	Male Wuzhishan mini-pigs	20	5×7×3mm^3	Physiological saline	Ex-vivo	DPSCs	DPSCs HGF-DPSCs DPSCs sheet HGF-DPSCs sheet Blank control	1×10^{7}cells/0.6 ml
Dai [450]	Tempromandibular joint (Mandibular Condylar growth)	Mandibular condyles	Female Sprague Dawley rats	60	N/R	N/R	Ex-vivo	----------	EGFP PBS only	2×10^{11} genome copies/50 µl
Dunn [445]	Alveolar bone defect with dental implant	Maxilla (First molars)	Sprague–Dawley rats	44	2×1 mm^2	2.6% collagen gel	In-vivo (GAM)	----------	Luc BMP 7	2.5×10^{11} particles
Hu [451]	Distraction Osteogenesis	Mandible (Right side)	Male Sprague-Dawley rats	44	N/R	Physiological saline	Ex-vivo	BMMSCs	BMP 7 EGFP-N1 physiological saline	1×10^6 cell/0.15 ml
Iglesias-Linares [481]	Orthodontic tooth movement	Maxilla (Bilateral:Second molars)	Wistar rats	72	N/R	Solution	In-vivo (local injection)	----------	TM force + PBS (R&L) TM force + Corticotomy (R)/TM force + Flap surgery(L) TM force + RANKL (R)/TM force + Plasmid without RANKL insert (L) Cortictomy (R)/Flap Surgery (L) RANKL (R)/Plasmid without RANKL insert (L)	N/R
Jiang [486]	Alveolar bone defect	Mandible (Bilateral)	Female New Zealand White rabbits	14	15×6 mm^2	NNB	Ex-vivo	BMMSCs	NNB/EGFP-BMP 4 NNB/EGFP NNB/untransfected bMSCs NNB alone Blank control	50×10^6 cell/scaffold
Jiang [454]	Sinus floor elevation	Maxilla (Bilateral)	Male New Zealand rabbits	20	13×3×5 mm^3	β-TCP	Ex-vivo	BMMSCs	β-TCP alone Untransduced bMSCs/ β-TCP EGFP–bMSCs/ β-TCP BMP-2–bMSCs/ β-TCP	2×10^7 cell/scaffold
Jiang [452]	Alveolar bone defect	Mandible (Ascending ramus)	Male Fisher 344 rats	24	5mm circular	mSS	Ex-vivo	BMMSCs	mSS/bMSCs transduced BMP 2 mSS/bMSCs transduced LacZ mSS/bMSCs mSS alone	2×10^7 cell/scaffold
Jiang [453]	Distraction Osteogenesis	Mandible (Right side: between 1st premolar and mental foramen)	Male New Zealand rabbits	42	N/R	Physiological saline	Ex-vivo	BMMSCs	b-FGF transfected MSCs in physiological saline, EGFP transfected MSCs in physiological saline. Physiological saline	1×10^{7}cell/0.15 ml

Table 5.2 (cont.): Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Jin [446]	Periodontal alveolar bone defect	Mandible (Bilateral: mandibular 1st and 2nd molar:buccal root PDL)	Lewis rats	25	0.3×0.2 cm^2	Gelatin sponge	Ex-vivo	SDFs	GFP control-treated Noggin-treated BMP 7	1×10^6 cell/scaffold
Jin [447]	Periodontal alveolar bone defect	Mandible (Bilateral buccal plate of 1st and 2nd molars)	Sprague–Dawley rats	30	0.3×0.2 cm^2	2.6% collagen gel	In-vivo (GAM)	----------	Luc PDGF-B PDGF-A Collagen matrix alone	2.5×10^{11} viral particles(PN)/ml
Kanzaki [480]	Orthodontic tooth movement	Maxilla (Right 1st molar of OF group)	Male Wistar rats	25	N/R	Vector solution	In-vivo (local injection)	----------	Control group OF group with or without RANKL Mock group	N/R
Kroczek [487]	Distraction Osteogenesis	Mandible (Right side)	Female Goettingen mini-pigs	24	N/R	Aqueous solution	Ex-vivo	BMMSCs	BMP 2 group BMP 7 group TGF-b group IGF 1 group Liposome vector group No induction group	4×10^5cell/dish
Kuboki [493]	Tempromandibular joint	Mandibular condyles (Bilateral)	Hartley guinea-pigs	16	N/R	Physiological saline	In-vivo (local injection)	----------	Gene Placebo Control	4.8×10^7 PFU/cell
Lai [455]	Distraction Osteogenesis	Mandible (Right side)	Male New Zealand rabbits	44	N/R	Physiological saline	Ex-vivo	ADSCs	transfected ADSCs EGFP-N1transfected ADSCs physiological saline only	1×10^7cell/0.2 ml
Lai [456]	Distraction Osteogenesis	Mandible (Left side: anterior to 1st molar)	Male New Zealand rabbits	44	N/R	Physiological saline	Ex-vivo	BMMSCs	transfected BMMSCs, autologous BMMSCs physiological saline only	1×10^7cell/0.2 ml
Lattanzi [483]	Alveolar bone defect	Mandible (behind the root of the incisor)	Wistar rats	36	5×5 mm^2	HA/COL	Ex-vivo	SDFs	LMP-3 transduced SDF on HA/COL Untransduced SDF on HA/COL HA/COL scaffold without cells Control group EGFP	N/R
Li [457]	Alveolar bone defect	Mandible (Bilateral)	New Zealand rabbits	44	12×8 mm^2	HA/PA	Ex-vivo	BMMSCs	Scaffold seeded with BMP 7 transduced MSCs Scaffolds seeded with osteogenically cultured MSCs. Pure HA/PA scaffolds	2×10^6cell/scaffold
Li [458]	Periodontal Disease	Mandible (Bilateral:premolar teeth)	Adult Beagle dogs	5	N/R	collagen membrane	Ex-vivo	BMMSCs	Pure collagen membrane Collagen membrane / transfected cells Collagen membrane / untransfected cells	1×10^7 cell/scaffold

Table 5.2 (cont.): Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Li [378]	Tempromandibular joint	Mandibular condyles (Bilateral)	Female Sprague–Dawley rats	30	N/R	N/R	In-vivo (local injection)	----------	Vastatin EGFP	2×10^{11} genome copies/50µl
Long [459]	Distraction Osteogenesis	Mandible (Right side: between anterior teeth and 1st premolar)	Male Japanese rabbits	36	N/R	Physiological saline	Ex-vivo	BMMSCs	Distraction 0.8 mm/d Distraction of 2.4mm/d with MSCs transfected with lacZ Distraction of 2.4 mm/d with MSCs transfected with BMP-2.	1×10^{7} cell/ ml
Park J [482]	Alveolar bone defect	Mandible (Left ramus)	Wistar rats	56	6mm circular	Collagen sponge	Ex-vivo	BMMSCs	BMP-2-infected BMSC LacZ-infected BMSC Untreated BMSC Empty collagen sponges	1×10^{6} cell/scaffol
Park S [484]	Periodontal alveolar bone defect with dental implant (Peri-implantitis wound)	Mandible (Bilateral: premolars and 1st molar)	Adult Beagle dogs	6	N/R	HA/COL hydrogel	Ex-vivo	PDLSCs	HA with collagen gel (control group,) HA with collagen gel/ PDLSCs HA with collagen gel/BMP2/PDLSC	N/R
Rabie [460]	Tempromandibular joint	Mandibular condyles (Bilateral)	Female Sprague–Dawley rats	90	N/R	Physiological saline	In-vivo (local injection)	----------	VEGF EGFP PBS	2×10^{11} genome copies/50µl
Steinhardt [488]	Alveolar bone defect	Mandible (Right side)	NOD/SCID mice	N/R	1mm circular	Collagen sponge	Ex-vivo	BMMSCs	MSC-BMP2 MSC-lacZ Control group (no implant)	5×10^{6} cell/scaffol
Su [461]	Alveolar bone defect	Mandible (Left side: alveolar bone of incisors)	Male New Zealand rabbits	20	$5\times10\times4$ mm^3	β-TCP	Ex-vivo	PDLSCs	Control β-TCP PDLSCs/β-TCP OPG-PDLSCs/β-TCP	5×10^{6} cell/scaffol
Sun [489]	Sinus floor elevation	Maxilla (Bilateral)	Male New Zealand rabbits	8	$13\times3\times5$ mm^3	OsteoBone	Ex-vivo	BMMSCs	BMP-2-infected BMSC/Scaffold EGFP-infected BMSC/Scaffold	2×10^{7} cell/scaffol
Sun [463]	Alveolar bone defect	Mandible (Bilateral)	Adult New Zealand rabbits	18	10×6 mm^2	BGC	Ex-vivo	PDLSCs	BMP-2–modified tissue-engineered bone Unmodified tissue-engineered bone Single BGC graft Defects without any implantation	2×10^{7} cell/scaffol
Sun [462]	Distraction Osteogenesis	Mandible (Right side: anterior to 1st premolar)	Female New Zealand rabbits	90	N/R	Physiological saline	Ex-vivo	ADSCs	Runx2 transfected ADSCs GFP-transfected ADSCs	1×10^{7} cell/ml

Table 5.2 (cont.): Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Sun [464]	Periodontal alveolar bone defect	Mandible (Bilateral: premolars)	Adult beagle dogs	6	5mm	Collagen sponge	In-vivo (GAM)	----------	BMP-2 plasmid group BMP-2 group PBS	N/R
Tan [465]	Periodontal Disease	Mandible (Bilateral: 1st, 2nd and 3rd premolars)	Male beagle dogs	4	5mm vertical	Sodium alginate	Ex-vivo	BMMSCs	bFGF transfected BMSCs Untransfected BMSCs	2×10^7 cell
Tang [466]	Alveolar bone defect	Mandible (Left ramus)	Female Sprague Dawley rats	40	4mm circular	CHA	Ex-vivo	BMMSCs	Control groups: empty defect CHA/autologous transfected BMP-2 CHA/untreated autologous BMSCs	5×10^6 cell/scaffold
Tang [467]	Alveolar bone defect	Mandible (Ramus)	Female Sprague-Dawley rats	24	4mm circular	CHA	Ex-vivo	BMMSCs	Control groups: left untreated BMSCs that transfected with BMP-2	5×10^9 cell/scaffold
Wang [468]	Distraction Osteogenesis	Mandible (Bilateral)	Male New Zealand rabbits	20	N/R	Physiological saline	Ex-vivo	BMMSCs	MSC transduced with NGF-b Control: EGFP	5×10^6 cell/0.1ml
Wei [490]	Tooth restoration/Bio-Root regeneration	N/R	Inbred miniature pigs	18	N/R	HA/TCP	Ex-vivo	DPSCs PDLSCs	HA/TCP Autologous Vc-induced PDLSCs in HA/TCP/DPSC Allogeneic Vc-induced PDLSCs in HA/TCP/DPSC	1×10^6 cell/scaffold
Wen [469]	Periodontal alveolar bone defect	Mandible (Right 1st molars)	Sprague-Dawley rats	6	1×3 mm^2	Collagen gel	Ex-vivo	PDLSCs	eGFP transfected PDLSCs untransfected PDLSCs Empty defect	5×10^5 cell
Yang [491]	Periodontal Disease	Maxilla (Bilateral: 1st molar)	Female Sprague–Dawley rats	30	N/R	**systemic:** culture media **Local:** Matrigel	Ex-vivo/ In vivo (systemic)	iPSC-derived MSCs	Healthy control Untreated periodontitis iPSC-MSCs-treated periodontitis iPSC- MSCs/TSG-6-treated periodontitis	5×10^6 cell/200µl media L: 1×10^6 cell/20µl gel
Ye [470]	Central fissures	Mandible	New Zealand rabbits	45	N/R	Bioglass	Ex-vivo	pOBs	BMP-2 transfected POBs/bioglass EGFP transfected POBs/bioglass Untransfected POBs/bioglass Bioglass only Blankcontrol	2×10^7 cell/scaffold
Yu [448]	Periodontal Disease	Maxilla (1st and 2nd molars)	Male Sprague–Dawley rats	51	N/R	HEPES	In-vivo (local injection)	----------	MKP-1 LacZ HEPES- buffered saline	1×10^9 PFU
Zhang [473]	Alveolar bone defect with dental implant	Mandible (Bilateral: Premolar region)	Adult hybrid dogs	9	$6\times5\times4$ mm^3	Chitosan/ Collagen	In-vivo (GAM)	PDLSCs	Pure scaffold Scaffolds with BMP7 Scaffolds with Easy1	1×10^7 cell/scaffold
Zhang [472]	Alveolar bone defect with dental implant	Mandible (Bilateral: Premolar region)	Adult hybrid dogs	6	$6\times5\times4$ mm^3	Chitosan/ Collagen	In-vivo (GAM)	PDLSCs	Scaffolds with Easy1: control Scaffolds with BMP 7 Scaffolds with PDGF-B Scaffolds with BMP-7/PDGF-B	1×10^7 cell/scaffold

Table 5.2 (cont.): Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Zhang [471]	Periodontal Disease	Maxilla (2^{nd} & 3^{rd} premolars)	Male beagle dogs	5	5×5 mm^2	MBG/silk fibrin	In-vivo (GAM)	----------	Control non-filled defects scaffold alone PDGF-B scaffold BMP7 scaffold PDGF-B + BMP7 scaffold	5×10^5 cell/scaffold
Zhao [474]	Alveolar bone defect	Mandible (Bilateral: ramus)	Male Fisher 344 rats	11	5mm circular	β-TCP	Ex-vivo	BMMSCs	b-TCP alone b-TCP with untreated bMSCs b-TCP with bMSCs transduced with EGFP b-TCP with bMSCs transduced with BMP-2	2×10^7 cell/scaffold
Zhao [492]	Orthodontic tooth movement	Maxilla (Right 1st molars)	Male Wister rats	18	N/R	Vector solution	In-vivo (local injection)	----------	OPG transfection group Mock vector transfection group Control group	N/R
Zhou [476]	Periodontal alveolar bone defect	Mandible (Bilateral: premolars)	Male purebred beagle dogs	4	4×4×3 mm^3	PLGA	Ex-vivo	BMMSCs	BMSCs/OPG-PLGA BMSCS-PLGA	1×10^6 cell/scaffold
Zhou [475]	Periodontal alveolar bone defect	Mandible (Bilateral: premolars)	Male purebred beagle dogs	4	4x4x3 mm^3	PLGA	Ex-vivo	BMMSCs	BMSCs/OPG-PLGA BMSCS-PLGA PLGA Negative control: root planing only	1×10^6 cell/scaffold
Zou [477]	Alveolar bone defect with dental implant	Mandible (Bilateral: premolars region)	Adult male labrador retriever dogs	5	6×5×4 mm^3	CMPC	Ex-vivo	BMMSCs	Blank CMPC CMPC/BMSCs/GFP CMPC/BMSCs/HIF CMPC/BMSCs/cHIF	2×10^5 cell/scaffold

Different analysis methods were used for either the in-vitro or in-vivo experiments as: Western blot (n=12, 21%) [440, 446, 448, 461, 462, 465, 475-478, 485, 486], In-situ hybridization (n=6, 10.5%) [450, 460, 463, 466, 467, 482], PCR (n=27, 47.3%) [378, 438, 441, 442, 444, 446, 447, 450, 453, 454, 457, 460-462, 464, 465, 469, 471-474, 477, 482, 483, 488, 491, 493], Bioluminescence (n=5, 8.7%) [438, 441, 445, 447, 479], µCT (n= 20, 35%) [439, 442, 444, 448, 449, 452, 453, 459, 462, 465, 471, 474, 477, 478, 483, 485, 487, 488, 492], [490], Histology (n=48, 84.2%) [439-443, 445-449, 451-458, 461-466, 468-471, 473-490, 492, 493], Staining (n=16, 28%) [440, 448, 449, 452, 454, 461, 466, 469, 474, 478, 482, 483, 485, 487, 488, 493], Radiograph (n=18, 31.5%) [440, 443, 444, 451-453, 455-457, 459, 462, 466, 470, 474, 475, 477, 482, 490], Histomorphometry (n=22, 38.5%) [440, 443, 445-447, 451, 452, 454, 457, 458, 461, 463, 466, 468, 470, 471, 473, 474, 477, 486, 489, 490], SEM (n=14, 24.5%) [442, 445, 451, 457, 461, 466, 471, 473, 474, 476, 477, 486, 490, 491], Biomechanical analysis (n=8, 14%) [442, 453, 457, 459, 462, 470, 485, 490], Immunohistochemistry (n=25, 43.8%) [378, 446-448, 451-453, 456, 457, 460, 461, 466, 467, 469, 475-477, 480, 482, 484, 485, 487, 489, 490, 492], Confocal microscopy (n=7, 12.2%) [452, 461, 472, 473, 479, 482, 489], Bone resorption assay (n=2, 3.5%) [480, 481], ALP activity (n=7, 12.2%) [446, 461, 463, 469, 471, 473, 486], Immunofluorescence (n=15, 26.3%) [378, 453, 455-457, 460, 461, 465, 469, 474, 481, 483, 486, 488-490], FACS (n=12, 21%) [378, 446, 449, 460, 465, 469, 477, 478, 482, 488, 490, 491], TRAP (n=3, 5.2%) [444, 460, 491], Cell proliferation (n=8, 14%) [446, 457, 461, 463, 465, 469, 471, 484] or ELISA (n=16, 28%) [444, 448, 449, 452, 459, 460, 463, 471-473, 478, 482, 484, 488, 489, 491]. **Table 5.3** summarizes the endpoint results of the main analytical methods used for the experiments either in vitro or in vivo.

Table 5.3: Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Abramson [438]		PDGFB demonstrated more mineralized tissue at 4 weeks than 2 weeks. Viral copies in blood and organs not significantly different between treated and untreated rats at all time points			
Alden [439]				3D CT showed marked osteogenesis and bony healing in BMP-2 and BMP-9 treatment groups while control did not show notable healing.	Slight healing of the defect in control. However, BMP-2 and BMP-9 showed marked bony regeneration across the defect site. BMP-2-treated defect demonstrated almost complete regeneration of the mandible indistinguishable from the normal mandible.
Ashinoff [440]				Increased radio-density in BMP-2-treated animals with increased new bone formation compared to control	
Chang [441]		Viral vector of PDGFB was detected within the first week in DNA and gradually decreased to undetectable levels after 2 weeks.	Luc/collagen showed high level in animals receiving high-dose Luc compared with low-dose.		Two weeks after surgery, nearly complete bone bridging of the alveolar bone in both PDGF-B groups whereas limited bridging in collagen-only animals. At 35 days, bone had completely bridged all of the defect area.
Chang [442]		Absence of PDGF-B in bloodstream.		µCT showed higher bone volume fraction in PDGF-B and rhPDGF-BB groups than low dose PDGF-B and Luc groups.	Bone was noted at coronal margin in Luc group and thicker bone trabeculae were evident in all PDGF-treated specimens. At day 14, near-complete defect fill was noted for all PDGF groups
Chang [485]				3D CT revealed complete repair of defects implanted with BMP-2. However, small islands of bone formation were observed in the βgal. Immunohistochemistry results revealed positive staining in BMP-2 cell constructs.	cancellous bone formation at defects implanted with BMP-2. Visible bone formation was noted at defect site implanted with BMP-2 cell constructs while βgal control had islands of bone formation with variable thickness and marked notching in the infraorbital rim.
Chen [479]			At day 1 after treatments: DNA+NB and DNA+US treatments were as low as with DNA alone treatment. Ultrasonication after DNA + NB injection significantly increased luciferase activity. Rats with removed gingivae exhibited weak luciferase activity in labial tissues of maxillary incisors		Histology showed no haemorrhage or inflammation, while fluorescence images showed EGFP expression mostly confined to labial gingival tissues of maxillary incisors

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	**PCR**	**Bioluminescence**	**Radiograph (plain or µCT)**	**Histology/Immunohistochemistry**
Chen [443]				At week 4, rhBMP-4 and autograft-treated groups showed a significant increase in bone regeneration when compared with the defect-only group and the scaffold only groups. No tooth eruption was seen at the 4-week time point in any of the four groups. New bone could be differentiated from grafted bone. By week 12, the entire defect had been filled.	The difference between new and grafted bone could not be seen. All four groups exhibited tooth eruption at 12 weeks.
Chen [478]	Showed that the adenovirus mediated BMP-2 gene was positively expressed and processed in MSCs of the defect.			3D CT showed that BMP-2 group had the highest mean regenerated bone volume and there were no significant differences between the other three groups.	6 weeks, BMP-2 group exhibited greater amounts of new bone formation. The newly regenerated trabecular bone was covered by a thick layer of osteoid and osteoblasts with continual bone-forming activity. The β-gal group exhibited woven bone formation, from the apical aspect of the defect to the middle of the root. The PF127 group displayed minimal amounts of bone formation at the apical third
Cirelli [444]	TNFR:Fc protein 4 weeks before Pg-LPS delivery showed high level which were sustained during 8-week experimental period compared to Pg-LPS, vehicle or no treatment.	High expression of IL-6, IL-10, RANKL and OPG observed at 4 weeks in Pg-LPS-exposed animals, but not in TNRF:Fc.		2Dand 3D µCT of maxillae showed linear bone loss. Significant alveolar bone destruction was observed in Pg-LPS group continuously over 8 weeks. Administration of TNFR:Fc prevented linear bone resorption during entire study compared with Pg-LPS only treated group.	An intense inflammatory cell infiltrate observed in subepithelial connective tissue and surrounding alveolar bone of periodontia of Pg-LPS- animals but a significantly less intense inflammatory reaction was observed in TNFR:Fc+Pg-LPS animals. Control animals did not show evidence of inflammatory cell infiltrates.
Cao [449]	Increased expression of HGF in transfected MSCs.			3D CT indicated limited bone formation in the control group. In contrast, marked bone regeneration occurred in the hDPSC, HGF-hDPSC, hDPSC sheet and HGF-hDPSC sheet groups. The heights of newly regenerated bone were significantly higher in all treatment groups compared with control group. The bone volumes in all treatment groups were significantly larger than the volume in the control group.	At week 12, new periodontal tissue regeneration within the periodontal defects was significantly less pronounced in the control group compared with the regeneration in the treatment groups. Alveolar bone regeneration was also more pronounced in the HGF-hDPSC, hDPSC sheet group and HGF-hDPSC sheet group than in the control group. The percentages of periodontal bone in the hDPSC injection, HGF-hDPSC injection, hDPSC sheet, and HGFhDPSC sheet groups were significantly higher than that of the control group.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Dai [450]		PCR of eGFP in heart, kidney, spleen and liver, mRNA was not detected reducing the prospects of systemic adverse effects. RT-PCR of transgene expression in the mandibular condyle revealed constant expression throughout the experiment. At day 21, there was a substantial increase in transgene expression.			
Dunn [445]			Showed sustained release of the gene product. All implants displayed the localized nature of expression in the near vicinity of the oral implants. The gene was expressed strongly for the first few days with peak expression at day 4 then declined by 2–5 weeks.		BMP-7-treated defects displayed tissue consistent with early osteoid formation throughout the defect area. Ad/Luc group exhibited normal bone healing, with most specimens showing minimal bone formation at the defect borders. At 28 days, bone formation was heightened both at the defect margins and along the dental implant surface in Ad/BMP-7-treated sites.
Hu [451]		Confirmed transcription of BMP-7 in transfected MSCs in contrast with negative signal in MSCs transfected with N1.		Radiodensity of callus in group A at 2 weeks was greater than in group B which was higher than group C. After 6 weeks of healing, more mineralization of distraction zone was seen in all three groups, but group A had greater radiodensity.	Immunocytochemistry showed BMP-7 expression in transfected MSCs while MSCs transfected with N1 exhibited negative signals. Bone regeneration in the distraction gaps was intramembranous ossification. At 2 weeks, positive signals for BMP-7 were found in the distraction zones in all three groups. Strong BMP-7 expression of was observed in group A, moderate in group B, and weak in group C. At 6 weeks, very weak BMP-7 positive staining was seen and a similar pattern and intensity was noted among the three groups.
Iglesias-Linares [481]					TM force groups with corticotomy or RANKL transfection showed a larger bone resorption area than control groups. Transfection group under orthodontic force maintained a higher bone resorption rate than corticotomy group under force throughout the experiment.
Jiang [486]					No inflammation or giant cell-type reaction was observed in any of the groups in immunohistochemistry.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	**PCR**	**Bioluminescence**	**Radiograph (plain or µCT)**	**Histology/Immunohistochemistry**
Jiang [454]					At week 2, newly formed trabeculae were found in the four groups. Just a slight newly formed bone was observed in group A; however, more bone area was found in group B and group C. In group D, a larger area of newly formed bone was found not only in the periphery but also in the centre of the space. At week 8, newly formed bone area increased in all four groups.
Jiang [452]	BMSCs transduced with BMP-2 produced higher levels of BMP-2 during the entire culture period as compared with LacZ and untransduced MSCs using ELISA	Upregulation of collagen type I in MSCs transduced with BMP-2. Runx2 showed moderate upregulation. Osteopontin showed sustained marked upregulation. Osteocalcin showed a steep increase. Osteogenic markers in LacZ transduced bMSCs remained at basal levels.		A larger defined radio-opaque new bone formation and mineralization was observed in BMP-2- transduced bMSCs group when compared to the LacZ and untransduced groups.	Increased bone formation in BMP-2-transduced bMSCs implants, less bone formation in LacZ or untransduced bMSCs-seeded scaffolds and no obvious bone formation was found in scaffold alone defects using histological sections. Immunohistochemistry displayed intensive BMP-2 staining in both bone matrix and surrounding fibroblastic-like tissue for BMP-2-transduced bMSCs whereas in LacZ bMSCs and untransduced bMSCs groups, BMP-2 staining was present but much weaker. No obvious positive staining was detected in the scaffold alone group.
Jiang [453]		bFGF was at a highest level at day 7 in bFGF transfected MSCs and sustained at high level in the next 3 weeks. Negative signal of bFGF was detected in MSCs or MSCs transfected with EGFP.		At 8 weeks, radiodensity of distracted callus in group was higher than those in groups A and B while radiodensity in group B was higher than in group A. µCT showed that the lingual cortical bone was formed well than the buccal cortical bone in all groups.	Immunohistochemistry showed bFGF expressed in bFGF transfected MSCs while negative signals in MSCs transfected with EGFP. Histology revealed newly formed trabeculae in all groups.
Jin [446]					Expression of BMP-7 and noggin was undetectable by 10 and 35 days after surgery by immunohistochemistry. Minimal to no osteogenesis was seen in GFP and noggin groups at early time point. Defects treated with BMP-7 demonstrated cartilage and limited areas of bone in the majority of the defects. At 35 days extensive bone formation was seen in most of the defects treated by BMP-7 while minimal osteogenesis and cementogenesis and lack of fibre insertion was noted in GFP and noggin groups.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Jin [447]		PCR showed expression of PDGF-B in PDGF-B transduced SDFs but not in cells transduced by luc or PDGF-A or cells without any adenovirus transduction.	The highest was at day 1 post-gene delivery and decreased at days 4-7. At 14-28 days postgene transfer, luciferase decreased compared to day 1.		Immunostaining was performed at days 3, 7and 14. In PDGF-B-treated group, greater numbers of positively stained cells on the surfaces of the alveolar bone and denuded tooth roots as well as the tissues surrounding the collagen matrix containing PDGF-B compared to other treatments at both days 3 and 7. At 3 days after treatment, no significant evidence of bone or cementum formation in any of the treatment groups and very few cells invaded into the adenovirus collagen implant.
Kanzaki [480]					No severe inflammations in periodontal tissue on repeated local RANKL gene transfer. Strong RANKL protein expression in the periodontium after 2 or 4 days from RANKL gene transfer. Very few RANKL protein expressions in the periodontium after 6 days from RANKL gene transfer. The number of osteoclasts was high at day 2 after RANKL gene transfer. The number of osteoclasts was reduced time dependently.
Kroczek [487]				Some differences between early and late period of consolidation in relation to the osteoinductive substance applied. The central distraction zone had no ossification. Induction with TGF-b revealed crystallization spots dispersed homogenously over the central distraction zone. Osteoinduction with BMP-7 showed consolidation of the central distraction zone after 1 week with a small gap in the central distraction zone. In the late consolidation period, the gap was bridged by fine bone trabeculae. Induction with BMP-2 resulted in an accelerated, dense new bone formation.	Lamination of the distracted bone areas adjacent to the osteotomy sites with longitudinally orientated columns of lamellar bone. The bone trabeculae showed osteoid deposition and early mineralization along their sides. The process of bone formation resembled more an intramembranous than chondroid ossification mode. Induction with TGF-b resulted in bone formation similar to one without induction. Positive immunostaining of BMP-2 was observed in distracted callus in all groups. Cellular elements with increased BMP-2 expression were found both in the distraction zone and in the consolidated osseous area close to the osteotomy region. A reduced BMP-2 expression was found in the central distraction zones.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Kuboki [493]		The absence of LacZ in liver, kidney, heart, and brain in LacZ- or control group. In TMJ of LacZ-injected animals, expression of LacZ was detected and not detected in the joints of control group.			There was no observable difference between the virus-injected and the PBS-injected joints. The frontal section of the mandibular joint 1 week after LacZ injection clearly showed that articular surface-lining cells were stained blue.
Lai [455]				Radiodensity of distraction areas in group A was higher than that in groups B and C at Weeks 2 and 6 after the distraction procedure.	Bone cells in the distracted areas were stretched along in the direction of the distraction. At 2 week, the two fragments of mandibles in all groups were filled with newly formed bone trabeculae. Similar results were seen in groups B and C, but much denser and thicker bone trabecules were observed in the distracted areas in group A than in group B and group C. At 6 weeks, the distraction gaps of the mandible were full of newly generated bone in all three groups.
Lai [456]				Radiograph of a distracted mandible at 2 weeks showed that callus appeared to be greater in group A when compared with group B which was higher than group C.	Bone regeneration in distraction gaps was intramembranous ossification. At 2 weeks, the new bone trabeculae formation began bridging in the 3 groups. More thick and dense trabecules were seen in the distraction gaps in group A than group B and C. At 6 weeks, the gaps were filled with newly formed bone in all groups. At 2 weeks, immunohistochemistry of BSP showed areas of fibrous connective tissue within the gaps and were mainly detected in the cellular components of fibroblast like cells, preosteoblasts, and osteoblasts in all 3 groups. Cells in group A showed greater amount and more intense staining for BSP within the gaps than group B which is more than group C.
Lattanzi [483]		Efficient LMP-3 expression 24 and 48 h. qPCR demonstrated that LMP-3 in transduced cells slightly increased in a time-dependent manner.		All rats treated with LMP-3 transduced SDFs showed positive X-rays at 8 and 12 weeks after surgery. No radiological evidences of bone formation could be demonstrated in three out of four animals at the earliest time point and in animals treated with scaffold alone or with non-transduced cells. 3DµCT revealed the successful repair of the defects implanted with LMP-3 cell constructs, which occurred in a time-related manner until 12 weeks after implantation. No bone formation was observed in the control group.	All rats treated with LMP-3 transduced SDFs showed positive histology at 8 and 12 weeks. No histological evidences of bone formation could be demonstrated in three out of four animals at the earliest time point and in animals treated with scaffold alone or with non-transduced cells.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Li [457]		BMP-7 was expressed in BMP-7 transfected MSCs while MSCs transfected with N1 exhibited negative signals.		Radiodensity in group A was higher than in group B and C. At 8 weeks, increasing mineralization in the implants was seen in group A than the other two groups.	Immunocytochemistry showed BMP-7 was expressed in BMP-7 transfected MSCs while N1 transfected MSCs exhibited negative signals. Immunocytochemistry of ALP and collagen I in group A was stronger than in group B. New bone formation was found in the implanted area in all three groups. At 4 weeks, the interface zone was surrounded by primitive mesenchymal cells differentiated into osteoblasts and new bone matrix was progressively deposited and became ossified. At week 4 and 8, all the parameters were significantly higher in group A than in group B than in group C. However, no significant difference in these parameters was found among three groups at week 16.
Li [458]					The percentage of new alveolar area in transfected and non-transfected BMSC were significantly higher than the control and there was also significant difference between two experimental groups. The percentage of new cementum length in two experimental groups was significantly higher than the control but there was no significant difference between two BMSCs groups.
Li [378]		Vastatin was only found in the experimental group. There were no transcripts detectable in the control group.			Positive signals in immunostaining at day 7 while absence of signals in control group. Expression of Vastatin was the highest on day 7, decreased from day 14 to day 60. The expression was in the proliferative and chondroblast layers on day 7. On day 14, Vastatin expressed in chondrocyte and pre-hypertrophic chondrocyte layers. The expression moved to the pre-hypertrophic chondrocyte and hypertrophic chondrocyte layers on day 21. On day 30, the expression moved deeper to hypertrophic chondrocyte layer. Only minor e xpression could be found in the deep hypertrophic chondrocyte layer on day 60.
Long [459]	BMP-2 levels were significantly higher in BMP-2 transfected MSCs compared with lacZ–transfected MSCs			The distraction gaps in group B rabbits did not show ideal new bone formation at week 2 while group A and C showed partial. The distraction gap in group A and C animals showed more mature new bone formation and higher radiopacity at week 4 compared with week 2. At week 8, radiograph of group A and C were almost identical to each other. µCT showed little new bone formation in the distraction gaps of group B animals at week 2. However, in groups A and C, new bone tissue was gradually mineralized from the centre to the margin in the distraction gap. More trabecular bone was mineralized at week 4 in group C than in group A. Groups A and C looked similar at week 8.	

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	**PCR**	**Bioluminescence**	**Radiograph (plain or µCT)**	**Histology/Immunohistochemistry**
Park J [482]					Immunocytochemistry of osteocalcin showed mineralization in genetically modified BMSC but rarely in control group. In both gene transfer groups, the amount of osteocalcin increased similarly. At 4 weeks endochondral bone formation occurred in the gene transfer groups and in the control; however, the amount of newly formed bone in the control was much less than in genetically modified BMSC. Treatment of defects with BMP-2-infected BMSC resulted in nearly complete bony healing within 4 weeks after the transplantation.
Park S [484]	BMP-2 expression level in the BMP2/ PDLSCs was significantly higher than in non-transduced PDLSCs. BMP-2 expression increased for 7 days and decreased until day 21.			The bone was lost at 4 months after the induction of experimental peri-implantitis in radiographs.	The bone labelling experiments demonstrated that new bone formation and re-osseointegration in the BMP2/PDLSC group occurred along the implant surface until week 8. PDLSC group showed less newly formed bone than BMP2/PDLSC group. The control group showed a limited amount of new bone formation around the peri-implantitis defects.
Rabie [460]	VEGF delivered group was higher than those two control groups from day 21 to day 60. VEGF expressed from mandibular condyle was significantly increased from day 14 and lasted during the whole time periods. On day 30, VEGF expression was more than in control group.	the expression of VEGF in condylar cartilage at day 7 and the maximum level at 21 days consistent with the result of in situ hybridization.			Immunohistochemistry confirmed increased VEGF expression in VEGF delivered condyle and positive signal in nearly all layers of condyle at day 30. VEGF expression was limited to the hypertrophic layer in control groups. The length and width of the condylar head increased significantly. The length of the condylar process significantly increased. Collagen type II was positive in chondroblast and hypertrophic layer. In control groups, collagen type II and type X positive layer decreased with age. However, after VEGF delivery, the collagen type II positive layer was significantly increased at day 21, compared to eGFP and PBS injection.
Steinhardt [488]		High levels of BMP2 in the cells but the protein expression levels were very similar.		Almost fully regenerated defect after 8 weeks. Minimal regeneration was observed after 8 weeks in control group infected with lacZ.	Masson trichrome staining revealed formation of new bone tissue and almost complete healing of the defect implanted with MSC-hBMP2. Minimal amount of new bone tissue was evident but no complete regeneration in lacZ or no implant.
Su [461]		Increased OPG level in hOPG transfected cells compared with non-transfected cells.			Toluidine blue staining showed no bone regeneration detected at the alveolar bone control group. A small amount of new bone could be seen in the β-TCP group with some osteoid formation in the periphery and centre of β-TCP scaffold. PDLSCs/β-TCP group showed more new alveolar bone formation, with numerous small bone trabeculae interconnected with each other.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	**PCR**	**Bioluminescence**	**Radiograph (plain or µCT)**	**Histology/Immunohistochemistry**
Sun [489]	High BMP-2 in MSCs transduced with BMP-2 as compared with EGFP.				BMP-2 immunocytochemistry showed high staining in BMP-2 infected MSCs than that in control and EGFP infected cells. In BMP-2-MSCs/scaffold and EGFP-MSCs/scaffold, more newly formed trabeculae were found dose to the parent bony wall and lifted membrane. At 4 weeks after implantation, newly formed bone area in the entire augmented area was larger than that at 2 weeks.
Sun [463]	High concentration of BMP-2 in the supernatant of cultured cells. There was no BMP-2 detected in uninfected cells during the entire time course.				More new bone tissue was found in the peripheral part of the grafted defects than in the central part. The central part of the grafts showed that the amount of bone in groups A and B was significantly larger than in group C. In the unfilled controls, there was more fibrous connective tissue formed in the defects after 12 weeks and no full bone healing was found.
Sun [462]		q-PCR showed the higher expression of Runx2 in Runx- transfected ADSCs than GFP transfected ADSCs and controls.		At week 9, radiograph of Groups A2 and D2 showed mature bone formation. µCT indicated the formation of new bone in Groups A2 and D2 than in the other two groups. Little new bone formation was observed in the distraction gaps of Groups B2 and C2.	The distraction gaps in specimens from Groups A2 and D2 were filled primarily with fibrous tissue and tiny trabeculae at week 3. By 6 weeks, more new bone tissue was formed with thicker and wider trabeculae.
Sun [464]					At 8 weeks, a complete osseous healing occurred and dense new periodontal ligament fibers rich in blood vessels were observed in BMP-2 group and rhBMP-2 group whereas fewer new bone occurred and sparse collagen fibers aligned irregularly were observed in the blank control group. The height of new bone and cementum were significantly greater in the two experimental group than in the blank control group.
Tan [465]				New bone formation in the two groups but the density of the newly formed bone in the bFGF-modified BMSC group was higher than that in BMSC-alone. µCT showed extensive new bone apposition in continuity with the trabecular host bone structure in the bFGF-modified BMSC transplantation group and BMSC-alone transplantation group.	Both groups exhibited periodontal regeneration, including newly formed cementum, periodontal ligament and bone. The newly formed bone and periodontal ligament in sites receiving bFGF-modified BMSC were greater than those receiving BMSC alone.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Tang [466]				Radiographs confirmed that implanted BMSCs expressing BMP-2 promoted bone formation..	BMP-2 expression was detected by immunohistochemistry in transfected cells but not in the untreated BMSCs. Bone formation was observed on the composites seeded with transfected BMSCs expressing BMP-2 and the group implanted with CHA seeded with untreated BMSCs but the negative control implants did not induce bone formation. At 4 weeks the bone defects that were treated with transfected BMSCs showed formation of mature bone matrix with a trabecular pattern at the defect margin. At week 8, the defect was nearly completely closed and the newly formed mature bone had a typical trabecular pattern.
Tang [467]					New bone formation was found at the margin of the defect treated with the BMSC modified by hBMP-2 gene transfer at 4 weeks and appeared mature 8. However, the amount of newly formed bone was much less with some adipose tissue at defect margins 8 weeks in control group.
Wang [468]	Secretion of NGF from the transduced MSC which increased to day 7.				Control group had signs of nerve degeneration with few regenerating nerve fibres whereas in experimental group there were abundant regenerating nerve fibres.
Wei [490]				Six months after transplantation, bone-like tissue formation was observed in HA/TCP group with no obvious boundary between the newly regenerated tissue and bone as well as HA/TCP/ DPSC/PDLSC sheet implant formed a hard root structure and a clear PDL space was found between the implant and surrounding bony tissue. µCT demonstrated that there was no obvious hard root structure and PDL space in HA/TCP group whereas a visible root structure and PDL space-like areas in HA/TCP/DPSC/PDLSC sheet group.	PDLSCs sheet had two or three layers and uniformly spread as a two dimensional tissue structure. Immunostaining for vimentin was positive. Fibronectin and type I collagen were present in the harvested PDLSC sheet.
Wen [469]		At 7 days, the expression levels of COL-1 and RUNX2 in PDLSCS were higher than those in eGFP-PDLSCs; the expression levels of ALP and OPN eGFP-PDLSCs were similar to those in PDLSCs.			6 weeks after surgery new regenerated bone, newly formed cementum and periodontal ligament were observed in group A and B. Strong expression of GFP and OPN was observed in the newly formed bone and cementum in the experimental group.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	**PCR**	**Bioluminescence**	**Radiograph (plain or µCT)**	**Histology/Immunohistochemistry**
Yang [491]	The production of proinflammatory cytokines was also significantly decreased in serum samples.	Increased TSG-6 expression in transfected iPSC-MSCs whereas low TSG-6 expression in untransfected iPSC-MSCs. Systemic administration of iPSC-MSCs and iPSC-MSCs/ TSG-6 reduced periodontal inflammation.			The infiltration of inflammatory cells in the periodontal tissues was markedly decreased in iPSC-MSCs/TSG-6 group.
Ye [470]				Higher bone density was found in the rabbit mandibular central fissures of group I 4 to 8 weeks after implantation.	Much more new bony callus in group I than in other groups.
Yu [448]				Cells transduced with MKP-1 exhibited reduced bone resorption after LPS stimulation compared with LacZ or HEPES control.	There were no significant inflammatory cells and few multinucleated osteoclasts on the alveolar bone surface in the periodontal tissues injected with PBS. In contrast, there were significantly more inflammatory cells more fibroblasts and more multinucleated osteoclasts in the periodontal tissues injected with LPS. Immunohistological staining revealed that MKP-1 was present in the periodontal tissues of rats injected with MKP-1 but undetectable in control groups of rats.
Zhang [473]	The maximum concentration of BMP7 in the culture media was detected after 6–9 days incubation and then followed by a moderate decline.	Significant differences in expression levels of OPN and BSP when HPLCs were cultured in BMP7 scaffolds.			The new bone formation of Group 2 was significantly greater than other groups at 4 and 8weeks. BMP7 group significantly increased the percentage bone defect fill in the defects compared to other groups.
Zhang [472]	HPLCs incubated in Group 3 produced higher level PDGF-B and produced higher level BMP7 in Group 2 during the entire culture period. There was no significant difference in the production of PDGF between groups 3 and 4. Similar results were noted in BMP7 secreted by Group 2 and Group 4.	Osteopontin and Type I collagen values of the PDGF-B expressing scaffolds were significantly greater than that of the control. The significant differences were observed in the mRNA expression levels of osteopontin, bone sialoprotein and Type I collagen when the HPLCs were cultured in combination scaffolds compared with BMP-7 or PDGF-B expressing scaffolds.			The new bone formation of the BMP-7 expressing scaffolds and the combination were significantly greater than that of the control at 4 and 8 weeks.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	**PCR**	**Bioluminescence**	**Radiograph (plain or µCT)**	**Histology/Immunohistochemistry**
Zhang [471]	By 7 days, over fourfold significant increases in PDGF-B and BMP7 was observed. The addition of adPDGF-B significantly increased cell recruitment approximately eight times more than control scaffolds and over six times higher than BMP7 scaffolds.	In all scaffolds containing BMP7 or PDGF-B+BMP7, mRNA levels of each gene was significantly increased. The scaffolds containing adPDGF-B alone was only able to significantly upregulate mRNA levels of COL1.		Control defects demonstrated little tissue formation with regeneration of periodontal tissues. Defects filled with scaffolds alone regenerated little new periodontal tissues. Compared to scaffolds/PDGF-B. In contrast, scaffolds containing BMP7 demonstrated greater new bone formation. Scaffolds with PDGF-B and BMP7 demonstrated qualitative features similar to those of native periodontal structures.	Control defects demonstrated little tissue formation with regeneration of periodontal tissues below 20% for cementum, alveolar bone and PDL. Defects filled with scaffolds alone regenerated less new periodontal tissues. Scaffolds containing PDGF-B demonstrated new formation of PDL. In contrast, scaffolds containing BMP7 demonstrated greater new bone formation. Scaffolds with PDGF-B and BMP7 demonstrated qualitative features similar to those of native periodontal structures.
Zhao [474]		OPN and OCN from BMP-2-transduced MSCs showed only a slight increase relative to GFP-transduced MSCs. At 9 days of culture, OPN dramatically increased in BMP-2-transduced MSCs compared with GFP-transduced MSCs.		Radiopacities at the defect sites inβ-TCP alone group, untreated MSCs⁄β-TCP group and GFP-transduced MSCs⁄β-TCP group. µCT showed that bone formation was less for defects filled with untreated MSCs⁄β-TCP and GFP-transduced MSCs⁄β-TCP but still advanced when compared with the implantation of β-TCP alone. Substantial new bone formation was observed after 8 weeks in the critical size defects which received BMP-2-transduced MSCs⁄β-TCP construct.	mall amount of irregularly arranged woven bone tissue at the centre pores of β-TCP scaffold and fibrous connective tissue was still frequently observed. In the defects filled with implantation of BMP-2-transduced MSCs⁄β-TCP construct, mature newly formed bone tissue with few fibrous connective tissues infiltration was observed in the β-TCP pores at both centre and marginal area. Bone marrow also largely formed accompanied with the bony ingrowth.
Zhao [492]				BMD and BVF were significantly increased in the OPG transfection group compared to the control and mock groups.	The amount of ERR in the three groups was minimal and no significant differences among the three groups at the first two time points. By the last day of orthodontic tooth movement, the volume of ERR in all three groups was significantly increased. After 2 weeks of retention, the volume of ERR in all three groups was significantly decreased especially in OPG transfection group. In the control and mock groups, there was significantly more ERR by the last day of retention. Immunohistochemistry showed that OPG protein expression was facilitated in the periodontium when was injected in the OPG transfection group.

Table 5.3 (cont.): Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Zhou [476]					After 6 weeks, the height of new alveolar bone and cementum and the formation of new connective tissue were significantly greater in the experimental group than in the control groups.
Zhou [475]				New bone formation was observed in the defect. The height of the newly formed bone was more than that of the original bone crest and there was close fusion between the old and new bone. In the cell control group and scaffold control group, the height of the newly formed bone was not as good as that in the experimental group. In the negative control, there was virtually no new bone formation.	Immunohistochemistry showed that the expression of OPG protein in the BMSCs OPG group was higher than that in the control group. Significantly more tissue regeneration for the scaffolds with BMSCs OPG was noted compared with the other groups.
Zou [477]		HIF-1a mRNA and protein expression was upregulated in the target gene groups compared with the control group.		Scaffolds implanted in the correct position and tightly contacted the implant. In the HIF-1a expressing groups, new bone formation and osseointegration were superior to the GFP, CMPC and blank groups as measured by bone density and the bone contact ratio of dental implants. µCT showed that the new bone formation in the HIF and cHIF groups was greater than that in the other groups at 12 weeks.	Higher in HIF group than CMPC group, the blank group or the GFP group but less than the percentage in the cHIF group. BIC in each target gene groups was significantly higher than the control groups and no significant difference was observed between the CMPC group and the blank group. There were significant differences in bone density between the cHIF or HIF group and each control group but no significant difference was seen among the three control groups.

Methodological quality assessment of included articles

The items included in the assessment of the quality of the articles are summarized in **Table 5.4.**

Table 5.4: Categories and grading used to assess the quality of the selected studies

Item	Description	Grade
1	**Title**	0 = inaccurate/not concise 1 = accurate and concise
2	**Abstract** Summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study	0 = clearly inaccurate 1 = possibly accurate 2 = clearly accurate
3	**Introduction** Background-objectives, experimental approach and rationale, relevance to human biology	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
4	**Introduction** Objectives-primary and secondary	0 = not clear 1 = clear
5	**Methods** Ethical statement-nature of the review permission, relevant licenses, national and institutional guidelines for the care and use of animals	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
6	**Methods** Study design-number of experimental and control groups, any steps taken to minimize bias (i.e., allocation concealment, randomization, blinding)	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
7	**Methods** Experimental procedure-precise details (i.e., how, when, where, why)	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
8	**Methods** Experimental animals-species, strain, sex, developmental stage, weight, source of animals	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
9	**Methods** Housing and husbandry-conditions and welfare-related assessment interventions (i.e., type of cage, bedding material, number of cage companions, light/dark cycle, temperature, access to food and water)	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
10	**Methods** Sample size-total number of animals used in each experimental group, details of calculation methods	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate
11	**Methods** Allocation of animals to experimental groups-randomization or matching, order in which animals were treated or assessed	0 = no 1 = yes
12	**Methods** Experimental outcomes-definition of primary and secondary outcomes	0 = no 1 = unclear/not complete 2 = yes
13	**Methods** Statistical methods-details and unit of analysis	0 = no 1 = unclear/not complete 2 = yes
14	**Results** Baseline data characteristics and health status of animals	0 = no 1 = yes
15	**Results** Number analysed-absolute numbers in each group included in each analysis, explanation for exclusion	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate
16	**Results** Outcomes and estimation-results for each analysis with a measure of precision, as standard error or confidence interval	0 = no 1 = unclear/not complete 2 = yes
17	**Results** Adverse events-details and notifications for reduction	0 = no 1 = unclear/not complete 2 = yes
18	**Discussion** Interpretation/scientific implications-study limitations including animal model, implications for the 3Rs	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate
19	**Discussion** Generalizability/translation-relevance to human biology	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate
20	**Discussion** Funding-sources, role of the funders	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate

The quality of finally selected studies was assessed by different categories [432, 433]. A relationship was driven between the Quality Score/Maximum Score by dividing the maximum score by category to the total score (T=36). Three possible quality coefficients: was conducted: 0.8–1 Excellent, 0.5–0.8 Average, <0.5 Poor as reported elsewhere [432, 433, 497]. In the included articles, 21 articles were excellent articles fulfilling nearly all the criteria of the ARRIVE guidelines with coefficients 0.8-1. Thirty-five articles were qualified as average articles with coefficients 0.5-0.8 and only one article was categorized as being of poor quality with coefficients <0.5. All the titles of the manuscripts were accurate. The abstracts were clearly accurate in 24 articles (42.1%) and possibly accurate in 30 articles (52.6%) and clearly inaccurate in 3 articles (5.3%). Introduction (background, objectives, experimental approach and rationale, relevance to human biology) was clear and sufficient in all the articles. Introduction (Objectives-primary and secondary) was clear in nearly all the articles (n=56, 98.2%) while only one article was not clear (n=1, 1.8%). The methods (Ethical statement-nature of the review permission, relevant licenses, national and institutional guidelines for the care and use of animals) were clearly sufficient in 50 articles (87.7%), possibly sufficient in one article (1.8%) and clearly insufficient in 6 articles (10.5%). The methods (study design-number of experimental and control groups, any steps taken to minimize bias, that is, allocation concealment, randomization and blinding) were possibly sufficient in 12 articles (21%) and clearly sufficient in 45 articles (78.9%). The methods (experimental procedure-precise details, that is, how, when, where, why) were clearly sufficient in 34 article (59.6%), possibly sufficient in 22 articles (38.6%) and clearly insufficient in one article (1.8%) of the manuscripts. The methods (experimental animals-species, strain, sex, maturity, weight, source of animals) were possibly sufficient in 25 articles (43.8%) and clearly sufficient in 32 articles (56.1%). The methods (housing and animal-husbandry and welfare-related assessment interventions, that is, type of cage, bedding

material, number of cage companions, light/dark cycle, temperature, access to food and water) were clearly insufficient in 41 articles (72%) and possibly sufficient in 16 articles (28%). The methods (sample size-total number of animals used in each experimental group, details of calculation methods) were clearly adequate in 53 articles (93%), possibly adequate in 3 article (5.2%) and clearly inadequate in one article (1.8%). The methods (allocation of animals to experimental groups-randomization or matching, order in which animals were treated or assessed) were expressed in 52 articles (91.2%) and were not expressed in five article (8.8%). The methods (experimental outcomes-definition of primary and secondary outcomes) were unclear/incomplete in 6 articles (10.5%) and absent in one article (1.8%) while 50 articles (87.7%) showed complete outcomes. The methods (statistical methods-details and unit of analysis) were missing in 7 articles (12.3%) and were provided in 50 articles (87.7%).

The results (baseline data characteristics and health status of animals) were not provided in 33 articles (57.9%) and were provided in 24 articles (42.1%). Results (number analysed-absolute numbers in each group included in each analysis, explanation for exclusion) were clearly inadequate in 4 articles (7%), possibly adequate in 44 articles (79%) and clearly adequate in only 8 articles (14%). Results (outcomes and estimation results for each analysis with a measure of precision, as standard error or confidence interval) were not complete in 17 articles (30%) and complete in 40 articles (70%). Results (adverse events details and notifications for reduction) were missing in 6 articles (10.5%), not complete in 33 articles (58%) and clearly accurate in 18 article (31.5%). The discussions (interpretation/scientific implications-study limitations including animal model, implications for the 3Rs) were clearly inadequate in one article (1.8%), possibly adequate in 51 article (89.4%) and clearly adequate in 5 article (8.8%). Discussions (generalizability/translation-relevance to human biology) were inadequate in 2 articles (3.5%), possibly adequate in 46 article (80.7%) and clearly adequate in 9 article (15.8%). Discussions (funding-sources, role of the funders) were clearly

inadequate in 3 articles (5.3%) and clearly adequate in 54 articles (94.7%). **Table 5.5** represents the assessment of the quality of the published articles included in the review.

Table 5.5: Quality assessment of articles included using ARRIVE guidelines

Reference	Items																					Score
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	T(36)	
Abramson[438]	1	0	2	1	0	2	0	1	0	2	1	0	0	0	0	1	0	0	0	2	**14**	**0.38**
Alden[439]	1	1	2	1	0	1	1	1	0	2	1	1	0	0	0	2	0	2	2	2	**21**	**0.58**
Ashinoff[440]	1	1	2	1	2	2	2	2	1	2	1	2	2	1	2	2	2	1	1	2	**33**	**0.91**
Chang[441]	1	2	2	1	2	2	2	2	0	2	1	2	2	1	2	1	2	2	2	2	**34**	**0.94**
Chang[442]	1	2	2	1	2	2	2	1	0	2	1	2	2	1	1	1	0	1	1	2	**28**	**0.77**
Chang[485]	1	1	2	1	0	1	1	2	0	2	1	2	2	1	1	2	2	1	1	2	**27**	**0.75**
Chen[479]	1	1	2	1	2	1	1	1	0	2	1	2	2	1	2	1	2	1	1	2	**28**	**0.77**
Chen[443]	1	2	2	1	2	1	1	1	0	1	1	1	2	1	2	2	1	1	1	2	**26**	**0.72**
Chen[478]	1	2	1	1	2	2	2	2	1	1	1	1	0	1	1	2	1	1	1	2	**26**	**0.72**
Cirelli[444]	1	2	2	1	2	2	2	2	1	2	1	2	2	0	1	2	0	1	2	2	**31**	**0.86**
Cao[449]	1	2	1	1	2	2	2	2	0	2	1	1	2	0	2	2	1	1	1	2	**28**	**0.77**
Dai[450]	1	2	2	1	2	2	1	1	0	2	1	2	2	0	1	1	0	1	1	2	**26**	**0.72**
Dunn[445]	1	2	2	1	2	2	2	2	0	2	0	2	2	0	1	1	0	1	1	2	**27**	**0.75**
Hu[451]	1	1	2	1	2	2	2	2	0	2	1	2	2	1	1	2	2	1	1	2	**31**	**0.86**
Iglesias-Linares[481]	1	2	2	1	2	2	1	1	0	2	1	2	2	0	1	2	2	2	2	2	**31**	**0.86**
Jiang[486]	1	1	2	1	2	2	2	2	1	2	1	2	2	1	1	2	2	1	1	2	**32**	**0.88**
Jiang[454]	1	1	2	1	2	2	2	2	1	2	1	2	2	1	1	2	2	1	1	2	**32**	**0.88**
Jiang[452]	1	2	2	1	2	2	2	2	0	2	1	2	2	0	1	2	1	1	1	2	**30**	**0.83**
Jiang[453]	1	1	2	1	2	2	2	2	0	2	1	2	2	1	2	2	1	1	1	2	**31**	**0.86**
Jin[446]	1	2	2	1	2	2	2	1	0	2	1	2	2	0	1	2	1	1	1	2	**29**	**0.80**
Jin[447]	1	2	2	1	0	2	2	2	0	2	1	2	2	0	1	2	1	1	1	2	**28**	**0.77**
Kanzaki[480]	1	2	2	1	2	2	1	1	0	2	1	2	2	1	1	2	2	2	2	2	**32**	**0.88**
Kroczek[487]	1	2	2	1	2	1	2	2	0	2	0	2	2	1	1	2	1	1	1	2	**29**	**0.80**
kuboki[493]	1	1	2	1	2	2	2	1	0	2	1	2	0	0	1	1	2	1	1	2	**26**	**0.72**
Lai[455]	1	1	2	1	2	2	2	1	0	2	1	2	2	1	1	2	2	1	1	2	**30**	**0.83**
Lai[456]	1	1	2	1	2	2	2	2	0	2	1	2	2	1	1	2	2	1	1	2	**31**	**0.86**
Lattanzi[483]	1	1	2	1	2	2	2	1	0	2	1	2	0	1	2	2	1	1	1	2	**28**	**0.77**
Li[457]	1	1	2	1	2	2	1	1	0	2	1	2	2	0	1	2	1	1	1	2	**27**	**0.75**
Li[458]	1	1	2	1	2	2	1	2	0	2	1	2	2	0	1	2	1	1	1	2	**28**	**0.77**
Li[378]	1	2	2	1	1	2	1	1	0	1	1	1	2	0	0	1	1	1	1	2	**22**	**0.61**
Long[459]	1	1	2	1	2	2	2	2	0	2	1	2	2	0	1	2	2	1	1	2	**30**	**0.83**
Park J[482]	1	2	2	1	0	2	2	1	1	2	1	2	0	0	1	1	1	1	1	2	**25**	**0.69**
Park S[484]	1	2	2	1	2	2	1	1	0	2	1	2	2	0	1	2	1	2	1	2	**29**	**0.80**
Rabie[460]	1	2	2	1	2	1	1	1	0	2	1	2	2	0	1	2	1	1	1	2	**27**	**0.75**
Steinhardt[488]	1	2	2	1	2	1	2	1	0	0	0	2	2	0	1	2	1	1	2	2	**25**	**0.69**
Su[461]	1	1	2	1	2	2	2	2	1	2	1	2	2	0	1	1	1	1	1	0	**27**	**0.75**
Sun[489]	1	1	2	1	2	2	2	2	1	2	1	2	2	1	1	2	2	1	1	2	**32**	**0.88**
Sun[463]	1	1	2	1	2	2	2	1	1	2	1	2	2	1	1	1	1	1	2	0	**28**	**0.77**
Sun[462]	1	2	2	1	2	2	1	2	0	2	1	2	2	0	1	2	2	1	1	2	**30**	**0.83**
Sun[464]	1	2	0	0	0	1	1	2	0	2	0	1	2	0	0	2	1	1	0	2	**18**	**0.5**
Tan[465]	1	2	2	1	2	1	1	2	1	2	1	2	2	1	1	2	2	1	2	2	**32**	**0.88**
Tang[466]	1	1	2	1	2	2	2	2	1	2	1	2	2	0	1	1	2	1	1	2	**30**	**0.83**
Tang[467]	1	1	2	1	2	1	1	2	0	2	1	2	0	0	1	2	1	1	1	2	**25**	**0.69**
Wang[468]	1	2	2	1	2	2	2	2	0	2	1	2	2	1	1	1	1	1	1	2	**30**	**0.83**
Wei[490]	1	1	2	1	2	2	1	2	0	2	1	2	2	0	1	2	1	1	1	2	**28**	**0.77**
Wen[469]	1	1	2	1	2	2	2	1	1	2	1	2	2	0	1	2	1	1	1	2	**29**	**0.80**
Yang[491]	1	1	2	1	2	2	2	1	0	2	1	2	2	0	1	1	1	1	1	2	**27**	**0.75**
Ye[470]	1	1	2	1	2	2	2	2	1	2	1	2	2	0	1	2	1	1	1	2	**30**	**0.83**
Yu[448]	1	1	2	1	2	2	1	1	1	2	1	2	2	0	1	1	1	1	1	2	**27**	**0.75**
Zhang[473]	1	0	2	1	2	1	1	1	0	2	1	2	2	1	1	2	1	1	1	2	**26**	**0.72**
Zhang[472]	1	0	2	1	2	1	1	1	0	2	1	2	2	1	1	1	1	1	1	2	**25**	**0.69**
Zhang[471]	1	1	2	1	2	2	2	2	1	2	1	2	2	0	1	1	1	1	1	2	**28**	**0.77**
Zhao[474]	1	1	2	1	2	2	2	2	0	2	1	2	2	0	1	2	1	1	1	2	**29**	**0.80**
Zhao[492]	1	1	2	1	2	2	1	2	1	2	0	2	2	1	1	2	2	1	2	2	**31**	**0.86**
Zhou[476]	1	1	2	1	2	2	2	1	0	2	1	2	2	0	1	2	1	1	1	2	**28**	**0.77**
Zhou[475]	1	1	2	1	2	2	1	2	0	2	1	2	2	1	2	2	1	1	1	0	**27**	**0.75**
Zou[477]	1	2	2	1	2	2	2	2	0	2	1	2	2	0	1	2	1	1	1	2	**30**	**0.83**
Category score (quality obtained)	**57**	**76**	**108**	**56**	**100**	**100**	**89**	**88**	**16**	**110**	**52**	**105**	**100**	**25**	**61**	**96**	**68**	**60**	**63**	**110**		
Max category score (quality expected)	**57**	**114**	**114**	**57**	**114**	**114**	**114**	**114**	**114**	**114**	**57**	**114**	**114**	**57**	**114**	**114**	**114**	**114**	**114**	**114**		

T: Total score for all the 20 items (36 points), Score: score of the items/the total score.

Risk of bias assessment of the included articles

Overall, all the studies were having low risk of bias in publishing in peer-reviewed journals. Random allocation of the treatment or control were reported in 29 articles [378, 439, 446, 447, 449-456, 459-461, 465, 468, 469, 474, 475, 477, 479, 483, 486, 489-492] and three studies [454, 457, 462] had a low risk of bias in random allocation concealment. Blinding of outcome assessment was performed in 13 studies [440, 444-447, 449-451, 453, 455, 456, 468, 481] and only two studies [442, 450] were reporting sample size calculation The statement of compliance with animal welfare regulations were reported in 51 studies [378, 440-445, 447-463, 465-481, 483, 484, 486-493] while conflict of interest were in 11 studies [441-443, 448, 449, 461, 468, 469, 489, 490]. More details about possible risk of bias were presented in **Figure 5.2.**

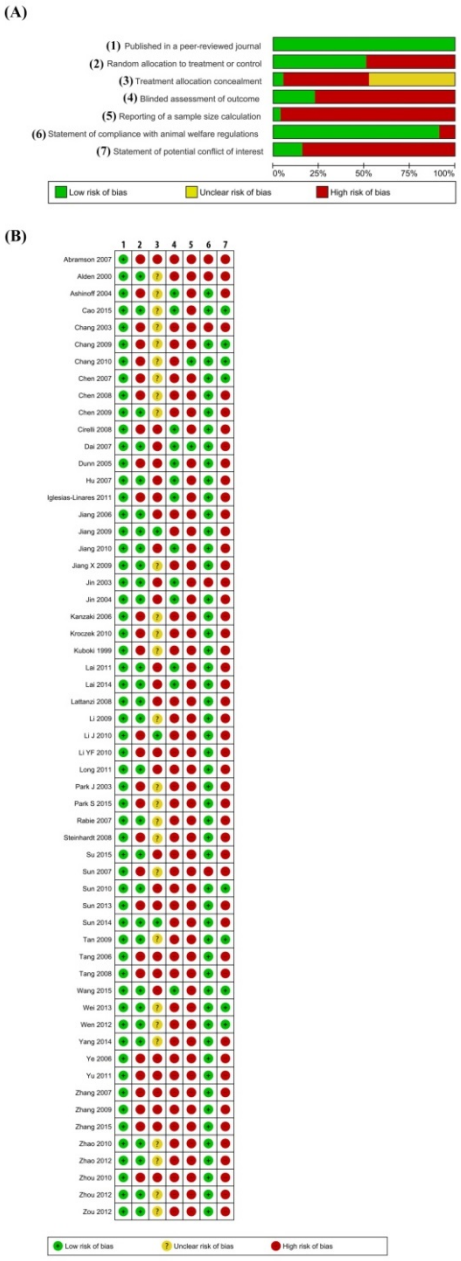


Figure 5.2: Risk of bias graph for the studies included in this systematic review.

Assessment of risk of bias using modified CAMARADES tool. Panel (A) Risk of bias of all included studies with the percentage of risk of bias for each item of assessment; Panel (B) Author name of each study and with their respective result in each item of assessment. Item (1) published in a peer-reviewed journal; (2) random allocation to treatment or control; (3) treatment allocation concealment; (4) blinded assessment of outcome; (5) reporting of a sample size calculation; (6) statement of compliance with animal welfare regulations and (7) statement of potential conflict of interest respectively.

Meta-analysis

Fourteen studies were included in the histological meta-analysis of percentage of area of newly formed bone by gene therapy whereas three studies were included in percentage of volume of newly formed bone. However, four studies were included in the radiographic meta-analysis of the bone formation by calculating the bone volume fraction. **Figure 5.3** summarizes the results of forest plot of gene therapy treatment versus control treatment.

Percentage of area of bone formation by histology:

Pooled data from *gene vs reporter* comprising of 9 inter-group comparisons generated from 7 original studies involving 204 animals (102 treated and 102 control groups) was (SMD=1.74, 95% CI, I^2=64%, P<0.00001) while data from *gene vs scaffold* comprising of 5 inter-group comparisons generated from 4 original studies involving 68 animals (34 treated and 34 control groups) was (SMD=1.17, 95% CI, I^2=87%, P=0.0004).

Pooled data from *gene/scaffold vs reporter/scaffold* comprising of 6 inter-group comparisons generated from 4 original studies involving 48 animals (24 treated and 24 control groups) was (SMD=1.31, 95% CI, I^2=45%, P=0.0006). However, data from *gene/scaffold vs scaffold* comprising of 4 inter-group comparisons generated from 4 original studies involving 48 animals (24 treated and 24 control groups) was (SMD=2.12, 95% CI, I^2=82%, P<0.00001).

Finally, pooled data from *gene/scaffold vs untransfected cells/scaffold* comprising of 3 inter-group comparisons generated from 3 original studies involving 46 animals (23 treated and 23 control groups) was (SMD=1.62, 95% CI, I^2=67%, P<0.00001).

Percentage of area of bone formation by histology is presented in (**Figure 5.3A**).

Percentage of volume of bone formation by histology:

Pooled data from *gene vs reporter* comprising of 2 inter-group comparisons generated from 2 original studies involving 52 animals (36 treated and 36 control groups) was (SMD=1.71, 95% CI, I^2=48%, P<0.00001) while data from *gene vs saline* comprising of 3 inter-group

comparisons generated from 3 original studies involving 108 animals (54 treated and 54 control groups) was (SMD=2.34, 95% CI, I^2=0%, P<0.00001).

Percentage of volume of bone formation by histology is presented in (**Figure 5.3B**).

Bone volume fraction for bone formation by radiograph:

Pooled data from *gene vs reporter* comprising of 4 inter-group comparisons generated from 4 original studies was performed involving 84 animals (42 treated and 42 control groups), (SMD=1.36, 95% CI, I^2=86%, P<0.00001). Bone volume fraction for bone formation by radiograph is presented in (**Figure 5.3C**)

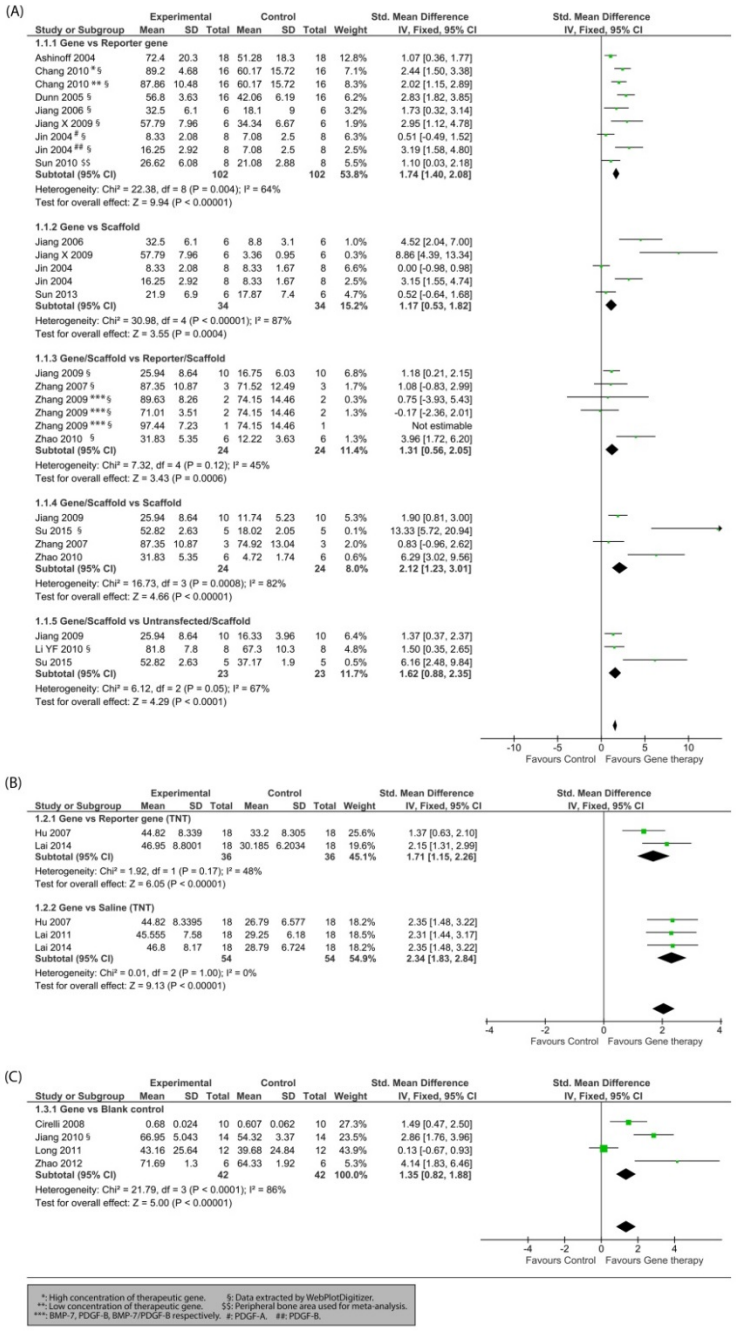


Figure 5.3: Forest plot of standard mean difference (SMD), with 95% Confidence Interval (CI) in bone formation by histology and radiograph comparing different subgroups.
Panel (A) represents forest plot of percentage area of bone formation by histology. Several subgroups were analysed as: Gene vs Reporter gene, Gene vs Scaffold, Gene/Scaffold vs Reporter/Scaffold, Gene/Scaffold vs Scaffold, Gene/Scaffold vs Untransfected cells/Scaffold. Panel (B) represents forest plot of percentage volume of bone formation by histology. Panel (C) represents forest plot of bone volume fraction detected by 3D µCT. the diamond represents the overall effect within each subgroup.

Publication bias

Funnel plots of the study results are shown in **Figure 5.4.** Symmetrical funnel plots were obtained in all the models. The funnel plot of the study standard error by effect size (SMD) was symmetric. The funnel plot of standard error versus effect size (standard mean difference) was symmetrical indicating the absence of potential publication bias among the meta-analysis of bone formation by histology (**Figure 5.4A&B**) or radiograph (**Figure 5.4C**).

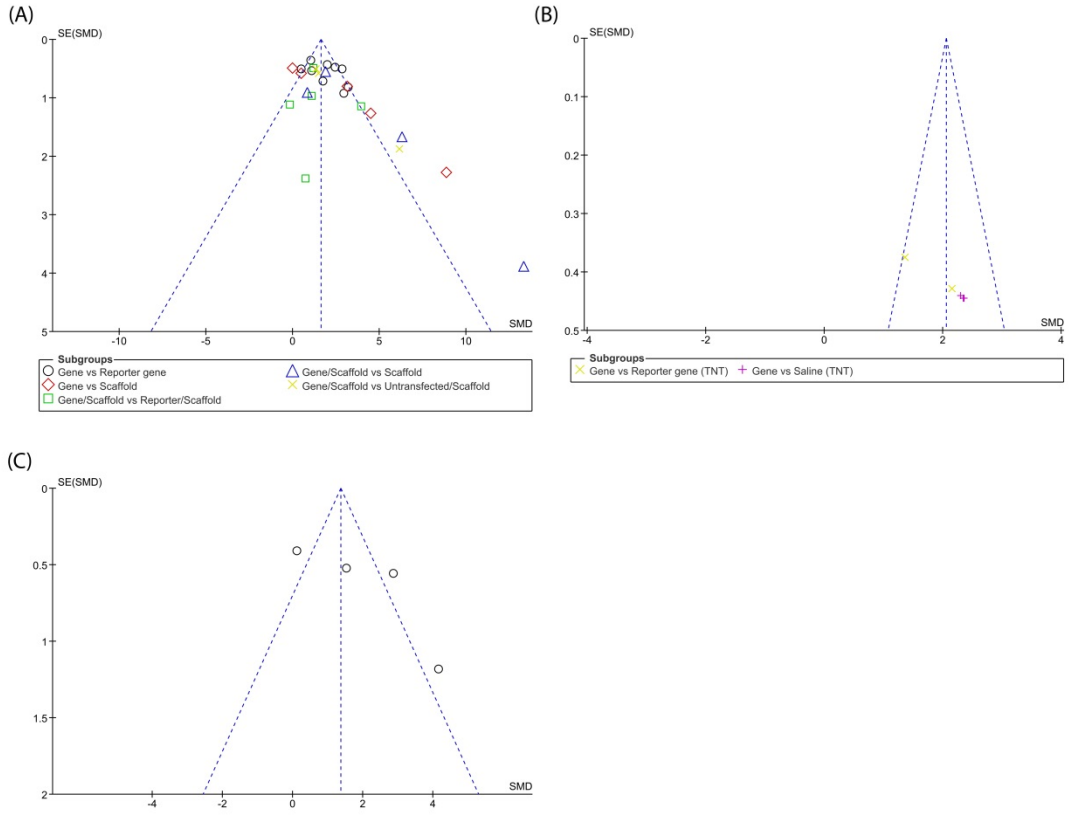


Figure 5.4: Funnel plot showing publication bias among the studies.
The symmetry of the funnel plot shows there was no evidence of publication bias among the studies. Each symbol on the funnel plot represents an individual study estimate included in the meta-analysis. The y-axis displays the standard error and the x-axis displays the standardized mean difference. SE: Standard Error; SMD: Standardized mean difference.

DISCUSSION

Several literature reviews have focused on gene therapy in bone tissue engineering, dentistry or oral and maxillofacial surgery [44, 428, 498, 499]. However, there has been no systematic review or meta-analysis with a specific focus on research covering gene therapy in the field of

Oral and Maxillofacial Surgery. Thus, we have conducted a comprehensive systematic review of the studies addressing efforts made in the field of gene therapy for healing of maxillofacial defects revealing the raised success rate during the recent years. Our meta-analysis results provided evidence that gene therapy was beneficial in treating maxillofacial defects in terms of improving bone formation based on histological and radiographic measures. However, it is important to keep in mind that several factors such as variability in research methods, characteristics of laboratory animals, interventions and outcome measures play role in meta-analysis of animal studies.

Although gene therapy was initially considered as a means of correcting hereditary disorders by changing the genes that cause the disease [500], more recent research is applying gene therapy to produce continuous amounts of biologically active molecules in the defects such as its potent ability for alveolar bone regeneration, periodontal healing and dental implants osseo-integration [471]. Clinical trials using gene therapy are now underway in salivary gland regeneration for dental application (https://clinicaltrials.gov/ct2/show/NCT00004178) and bone regeneration (https://clinicaltrials.gov/ct2/show/NCT02293031). However, future clinical trials for the use of gene therapy in periodontal regeneration remain hopeful for the near future.

From our results, multiple genes were used as osteogenic factors for gene therapy in the maxillofacial region because of their potent induction of de novo bone formation in vivo with varying results as soluble growth factors (PDGF, FGFs), morphogens (BMPs), angiogenetic factors (VEGF), intracellular regulators (LIM mineralization protein-1: LMP-1), transcription factors associated with bone/cartilage-related gene expression (Runx2) [501, 502]. All of these biological factors have been investigated for their potential use in bone tissue engineering and repair. However, BMPs were preferred candidates for local gene therapy for bone regeneration as they are the only group that can initiate and sustain the entire bone

formation cascade [503]. Some studies proved the feasibility of transferring BMP genes [504, 505]. On the other hand, previous investigations had reported the effect of PDGF on osseous wound healing showing that PDGF signalling plays role in chemotaxis and proliferation of osteoblasts and fibroblasts [506]. However, PDGF's ability to induce osteogenic differentiation is less clear. Recently, LMP-1 proved the initiation of membranous bone formation *in vitro* and *in vivo* [507]. Unlike BMPs acting extracellularly through cell surface receptors, LMP-1 is an intracellular signalling molecule involved in osteoblast differentiation [499].

Another critical element of gene therapy is the vector which is the vehicle that facilitates the transfer of genetic material into the target cell nucleus without degradation or causing toxicity. Two kinds of vectors have been employed as vehicles: viral and non-viral vectors. Gene transfer via viral vectors is called transduction while transfer via the non-viral vectors is transfection. Different viral vectors have been introduced as DNA-based like adenoviruses, adeno-associated viruses or RNA-based viral vectors as retroviruses and lentiviruses. Non-viral vectors can be plasmids, liposomes or polyplexes. Each vector has its own advantages and disadvantages. Viral vectors have the advantage of its ability to carry the gene efficiently and ensure long-term expression but they can only trigger short-term gene expression and are highly immunogenic. Another advantage of viral vectors is that they are non-virulent due to their modified genome in which the essential viral genes are replaced by the therapeutic gene being unable to replicate in the absence of these critical gene products. Non-viral vectors could be also used due to their safety profile and minimal immunogenicity. However, the main disadvantage in their use is the insufficient transfection efficiencies. [41, 501, 508-511].

For viral-based gene therapy, it is necessary to allow continuous high-titre virus production. The viruses are replicated in either human or non-human cell lines. A whole panel of different cell lines has been used all-over the years to generate viral vector to be used as therapeutic

product. HEK293 cells and their derivatives have been extensively used for production of different vectors because of their easy handling and the possibility to grow them as adherent as well as suspension cells [512, 513]. In line with our findings, several studies had proved the efficiency of viral vector in the transfer of the DNA [514-516] while other studies have used non-viral vectors [517, 518].

Reporter gene assays have emerged as a rapid and sensitive strategy for indirectly monitoring transgene expression by cloning the promoter region of the gene of interest correlated to the reporter gene and measure reporter gene expression as a reflection of the expression of the gene of interest [519]. It is important to use a reporter gene that is not naturally expressed in the cell or organism under study. Different strategies of making the fusion construct and their applications have been reported [520]. Commonly used reporter genes that induce visually identifiable characteristics usually involve fluorescent proteins as green fluorescent protein (GFP), which causes cells that express it to glow green under UV light and the enzyme luciferase [521], which catalyses a reaction with a luciferin to produce light. Another common reporter gene is the lacZ expressed in bacteria, which encodes the protein β-galactosidase. This enzyme causes bacteria expressing the gene to appear blue when grown on a medium that contains the substrate analogue X-gal. In our results, several reporter genes have been used which gives an add-on to the experiments being an internal control for the expression of the gene of interest.

Various biological delivery systems have been applied for directing therapeutic gene to target cells. In the in-vivo approach, cells can be genetically modified in situ or the vector is administered to the defect via systemic or local direct injection associated with a biomaterial. The latter combination of vector and biomaterial is called gene activated matrix (GAM). GAMs are three-dimensional biomaterials acting as a scaffold for vectors introduced to a localised area and useful for avoiding unintended spread of transfection to local tissues.

Regarding the ex vivo approach, cells are removed, genetically modified and re-implanted in the defect by direct injection or using a biomaterial as carrier [522-524].

Genetic modification of stem or progenitor cells serves as an important advancement in regenerative medicine to improve their in-vivo performance. By combining gene with cell therapy, stem cell function may be enhanced by improving proliferative capacity or differentiation of the stem cells. Another important function of stem cells is for drug delivery exerting paracrine or endocrine actions. The most common cell source is mesenchymal stem cells (MSC) which can be isolated from bone marrow, muscle tissue, peripheral blood, umbilical cord, adipose tissue, liver, multiple dental tissues or induced pluripotent stem cells (iPSC) [525, 526]. MSC are adult stem cells capable of self-renewal and differentiation into multiple lineages including cartilage, adipose, and bone which have been used for treating bone-related diseases [527]. The induced pluripotent stem cells (iPSCs) is a new source of stem cell generated from human somatic cells into a pluripotent stage [528]. Various cells such as gingival or dermal fibroblasts, periosteal cells, primary articulated joint chondroblasts, bone marrow stromal cells/ MSCs, muscle-derived stem cells, fat-derived stem cells, osteoblasts and myoblasts have been successfully transduced using in vivo or ex vivo techniques and the different vector systems [501]. From our results, the most commonly used stem cells in the maxillofacial region were genetically modified bone marrow, adipose, periodontal and dental pulp stem cells. Other studies used the same cells for regeneration of bone and other organs: BMMSCs [529, 530], ADSCs [531-533], PDLSCs [534], DPSCs [535].

Animal models are valuable tools in biomedical research in particular gene therapy to test the safety, efficacy, dosage and localization of transgene expression in models that closely resemble human diseases. Animal craniofacial models for gene therapy exist not only for bone [536] but also for periodontal ligaments [447], TMJ [460], cartilage [537] as well as

salivary glands [538]. Such models have critical-size defects with the absence of spontaneous complete osseous regeneration of the created defects during the lifetime of the animals [5, 39].

Considering limitation of our systematic review, meta-analysis was conducted for only few included studies due to the high level of heterogeneity in reporting the treatment outcomes. Moreover, the studies which were included in our meta-analysis generally used animal models for gene therapy. Therefore, randomized clinical studies in humans are needed to confirm our conclusions. However, meta-analysis was performed only to articles that had clearly reported bone formation (primary outcome) either by percentage of area or volume histologically as well as radiographically.

CONCLUSION

Challenging approaches had emerged for oral and maxillofacial reconstruction in the last decade due to the complex nature of craniofacial defects. Tissue engineering is attracting the spotlights as a new paradigm for bone regeneration which requires the collaboration of multidisciplinary teams of surgeons, biologists and biomedical engineers. Gene therapy is on the top list of innovative strategies in tissue engineering that developed in the last 10 years. While significant progress has been made towards preclinical studies of gene therapy in the maxillofacial region building the scientific basis of this technique, gene therapy is still in the clinical trials phase in salivary glands and craniofacial defects.

6. PUBLICATION VI

PATHOGENESIS OF ANTIRESORPTIVE DRUG-RELATED OSTEONECROSIS OF THE JAW

Riham Fliefel and Sven Otto. Pathogenesis of antiresorptive drug-related osteonecrosis of the jaw. In: Kenneth E Fleisher, Risto Kontio, Sven Otto. Antiresorptive Drug-related Osteonecrosis of the Jaw (ARONJ)—a Guide to Research. Switzerland: AOCMF; 2016. p64. ISBN: 978-3-905363-10-4.

ABSTRACT

In this chapter, three questions are raised and discussed:

- Which theories exist for the pathogenesis of antiresorptive drug-related osteonecrosis of the jaw (ARONJ)?
- Why jaw bones are predominantly affected?
- Why can nitrogen-containing bisphosphonates and denosumab cause ARONJ?

Bones are constantly remodelled through osteoblastic (bone formation) and osteoclastic (bone resorption) activity to maintain skeletal strength and integrity. However, imbalance between these phenomena affects bone mineral density leading to bone disorders as osteoporosis, Paget's disease, myeloma, bone metastases secondary to cancer as well as osteogenesis imperfecta and inflammatory bone loss. One of the recent treatment of bone disorders is the use of antiresorptive drugs including hormone replacement therapy, selective estrogen receptor modulators, bisphosphonates and denosumab which reduce the occurrence of bone pain, pathological fracture and spinal cord compression [539-542].

Among the antiresorptive drugs, bisphosphonates (BPs) are stable analogues of natural inorganic pyrophosphates [217, 543, 544]. They can be classified into non-nitrogen- BPs that metabolically interfere with adenosine triphosphate-dependent (ATP) intracellular pathways and nitrogen BPs which inhibit farnesyl pyrophosphate synthase [545, 546]. Denosumab is a new antiresorptive drug with a novel mechanism of action [547]. Both denosumab and bisphosphonates target osteoclasts. However, their effects on osteoblasts are largely indirect [548].

The mechanisms of action of BPs in bone metabolism are complex and multifactorial altering the osteoclast cytoskeleton stimulating apoptosis and reducing proton-pump expression [549-551]. They interfere with chemotaxis and attachment of osteoclast to bone together with suppressing mature osteoclast function by defective intracellular vesicle transport which in turn prevents osteoclast from forming a tight scaling zone or ruffled border, required for bone resorption [55, 552, 553]. In addition, they inhibit recruitment, activation and differentiation of osteoclast precursors [554]. The clinical efficacy of bisphosphonates rises from their ability to bind strongly to bone mineral [544]. The initial clearance of BPs occurs through renal excretion or adsorption to bone mineral extending over a period of weeks to years [555]. During bone resorption, the acidic pH in the resorption lacuna increase the dissociation of BP

from bone [556]. This is followed by the uptake of the BP most likely by fluid-phase endocytosis [557].

Bone resorption is regulated through RANK/RANKL/OPG pathway [548, 558]. Receptor activator of nuclear factor kappa-B ligand (RANKL) is a transmembrane and soluble protein highly expressed by osteoblasts [559, 560]; its receptor, Receptor activator of nuclear factor kappa-B (RANK), is located on the cell membrane of osteoclasts and preosteoclasts [560, 561]. RANK/RANKL binding stimulates the formation, activity, and survival of osteoclasts, resulting in increased bone resorption [562]. Osteoprotegerin (OPG) is a naturally occurring soluble, non-signalling “decoy receptor” for RANKL. OPG inhibits osteoclast activity by binding to RANKL preventing its interaction with RANK [562-564]. Both RANKL and OPG are produced by osteoblasts [565].

Denosumab is a fully human monoclonal antibody that was developed specifically to interact with RANK/RANKL/OPG pathway [544]. By binding to RANKL, it prevents the maturation and differentiation of preosteoclasts in the extracellular environment and promotes apoptosis of osteoclasts [566]. It has several advantages over bisphosphonates, including better tolerability, ease of subcutaneous injection, shorter half-life and reduced incidence of nephrotoxicity rendering it the drug of choice for patients with renal diseases or prostate cancer [567]. In contrast to the bisphosphonates, denosumab does not become embedded within bone tissue [547, 548]. Denosumab is cleared from the bloodstream through the reticuloendothelial system, with a half-life of approximately 26 days without inducing the formation of neutralising antibodies [568].

Antiresorptive drugs have numerous side effects including the upper gastrointestinal where nausea, vomiting, epigastric pain and dyspepsia occurs after oral administration of the drugs for the treatment of osteoporosis. Subsequently, several cases of renal failure were reported with the use of intravenous bisphosphonates. A possible mechanism of the renal toxicity was

the strong affinity of the bisphosphonates for metal ions and their tendency to form complexes and aggregates with metal ions. Non-specific conjunctivitis is the most common ocular side effect of bisphosphonates which usually improves without therapy and despite continuing treatment with bisphosphonates. Transient hypocalcaemia with secondary hyperparathyroidism is also a side effect of bisphosphonate administration. There is a possibility of severe and sometimes incapacitating bone, joint, and/or muscle (musculoskeletal) pain in patients taking bisphosphonates [569, 570].

No potential adverse effect of antiresorptive drugs has been more widely reported than medication-related osteonecrosis of the jaw (MRONJ) that ranges in severity from painless, small areas of exposed bone to significant bone exposure associated with severe pain, sequestration, infection, fistula or jaw fracture [29, 140, 354, 571]. The pathogenesis of the disease is certain with many questions regarding the potential mechanisms underlying the pathophysiology [186, 558, 572]. Five main mechanisms had also been proposed: i) impaired healing; ii) angiogenesis; iii) local toxicity; iv) immunomodulation; and v) infections. Most likely a combination of these facilitate development of MRONJ [573]. However, the leading theory to explain the mechanism suggests that it is caused by cessation of bone remodelling and bone turnover by the inhibition of osteoclasts [134].

MRONJ most commonly occurs in the oral cavity as the jaws are covered by a thin layer of periosteum and epithelium. The alveolar bone of the jaws is daily remodelled with a high rate of bone turnover. and the presence of teeth and gum providing an easy entrance for bacterial infection [572, 574].The oral structures are subjected to a wide variety of stresses, which may be physiologic, iatrogenic or inflammatory. The constant stress leads to trauma to the mucosa with exposure of bone [572]. Prolonged use of bisphosphonates may suppress bone turnover with accumulation of microcracks resulting in decreased biomechanical competence [140, 344]. BPs cause excessive reduction of bone turnover resulting in an increased risk of bone

necrosis in osseous repair [575, 576]. However, this theory failed to explain why exposed necrotic lesions are rarely seen in bones other than the jaws. MRONJ does not appear to occur in other conditions associated with reduced bone turnover, such as hypoparathyroidism and in patients with reported MRONJ, the bone turnover markers were not suppressed [577, 578]. In patients with breast cancer and bone metastases treated with zoledronate or denosumab, bone scintigraphy images suggest that the bone turnover of the mandible and the maxilla is not overtly changed when compared to other bones [579].

Blood supply may play role in MRONJ as its reduction might lead to delayed wound healing due to the antiangiogenic effect [230]. Antiresorptive medications may inhibit angiogenesis by inhibiting the formation of blood vessels, endothelial cells, fibroblast growth factor, and endothelial growth factor impairing endothelial cell (EC) functions leading to altered adhesion and migration. Furthermore, there is reduced proliferation, increased apoptosis, and decreased capillary-like tube formation in ECs that might cause bone necrosis [386, 580, 581]. In a study by Wehrhan et al [582], mucoperiosteal tissue samples from BRONJ patients and controls were assessed for vascularization with CD31 staining and neo-angiogenesis by CD105. Although there was no difference in vascularization between sample groups, there were significantly fewer CD105-positive vessels in BRONJ samples suggesting that neo-angiogenesis was suppressed in BRONJ patients. Histological evaluation of BRONJ tissue revealed decreased p63 gene expression, indicating a reduction in basal cell progenitors and might lead to impaired healing of the oral mucosa [583]. Although bisphosphonates, bevacizumab and sunitinib all have antiangiogenic effects, the effects of denosumab on angiogenesis is largely unknown. [584-586]. As such, impaired vascularization may play only a minor role in development of MRONJ [587].

Soft tissue cytotoxicity might also play a role explaining why bone is directly exposed to the oral environment through teeth and periodontal ligaments [588]. Local infection, tooth

extraction in particular, could result in the release of bisphosphonates into the local tissues. Provided that the local concentration of drug is high enough, the proliferation of adjacent epithelial cells could be inhibited and thus slow down the healing of the breached mucosal barrier [589]. However, soft tissue toxicity has not been reported with denosumab. BPs was explored on a variety of cells, including gastrointestinal cells, cervical epithelial cells, renal cells, prostate epithelial cells, and oral mucosal cells [572]. Antiresorptive drugs also acts on immunity including the impairment of myeloid cells function [590, 591], dendritic cell [592] and T-cell upregulation [593]. They increase the antigenicity of cancer cells as targets and increase adaptive immunity. This impairment of local immunity with an infectious tendency may be a key element in MRONJ [573].

Infection and periodontal disease are critical factors associated with MRONJ. However, controversy exists as to whether (1) BP inhibition of bone remodelling results in necrosis with subsequent infection or (2) the direct toxic effects of BPs on the oral mucosa allow for invasion of oral pathogens causing infection with subsequent necrosis [293, 594]. Among all the bones, jaw seems to be the most liable to bacterial infection since mucosa covering the alveolar bone is very thin and vulnerable and teeth easily become a pathway for bacteria from the outside into the bone. After administration, BPs accumulate in the bone and during physiological remodelling, osteocytes are exposed to BPs in bone [595]. BPs bind to bone at neutral pH and released from bone in an acidic milieu; thus, pH and infections might play an important role in the pathogenesis of MRONJ. This physiologic mechanism takes place in the resorption lacunas during bone resorption, where acid pH increases the dissociation between BP and hydroxyapatite. To date, this well-known feature has not been linked to the pathogenesis of BRONJ, but may prove to be the missing part in the multifactorial puzzle [18, 173].

Aghaloo et al. [596] found that necrosis of the alveolar bones developed after placement of a wire ligature around the crown of maxillary molar in a rat periodontal disease model. The results showed that periodontitis, which is presumably infection-related can trigger osteonecrosis. When periodontitis occurs, inflammatory cells are recruited to the sites to eliminate the causative pathogens. However, the blockade of bone resorption with BP may render it difficult for these cells to access to the pathogens, allowing the infection to persist. The resulting accumulation of bacterial toxins and inflammation generated superoxides will promote bone necrosis. [595]. Mechanism of MRONJ is so much related to immunity and infections rather than being aseptic or avascular in origin [584]. It is mostly following invasive dental procedures suggesting that MRONJ likely involves a drug-induced compromise in the bone response to invasive trauma. Even though the underlying indication for dental extraction in these patients may have been infection, MRONJ did not manifest until after extraction in most cases. For a direct in-vivo mechanism to be identified, it is yet unclear whether invasive trauma by itself is sufficient to precipitate MRONJ in bisphosphonate-treated individuals [29, 578]. Polymicrobial infection and periodontal disease may contribute to development of MRONJ as a biofilm-associated infection. Filleul et al. [359] found out that actinomyces were present in 70% of all cases. Thumbigere-Math et al. [198] found Actinomyces-like microorganisms in all bone specimens of patients during microbiological examination. In an animal models treated with BPs, bacterial infection was sufficient enough to cause MRONJ [597]. Sterile inflammation alone in the soft tissues surrounding the jaw is not enough to induce MRONJ [598]. Treatment with antibiotics in animal models [599] and mucoperiosteal coverage on the day of tooth extraction in a rat model prevented the development of MRONJ [600].

The presence of the infectious component in MRONJ is the most dangerous aspect. Oral pathogens should be prevented from reaching the bone surface, and optimum oral hygiene is

essential. The current regimens which consist of oral antiseptics and antibiotics are not always successful. Ideally, treatment aims to eradicate the underlying infection, prevent secondary infection, stop the disease process and control symptoms [601]. Traumatic intervention should be avoided, but where it must be undertaken, strict adherence is necessary. The proposed sequence of events in the development of MRONJ with infection could justify temporary discontinuation of the drug to allow recovery of macrophage production and function [31].

7. PUBLICATION VII

NEW AND INNOVATIVE TREATMENT STRATEGIES FOR MEDICATION-RELATED OSTEONECROSIS OF THE JAW

Riham Fliefel and Pit Voss. New and Innovative Treatment Strategies for Medication-Related Osteonecrosis of the Jaw. In: Sven Otto. Medication-Related Osteonecrosis of the Jaws: Bisphosphonates, Denosumab, and New Agents. Heidelberg: Springer; 2015. p220. ISBN: 978-3-662-43732-2.

ABSTRACT

A large variety of treatment options have been proposed for the management of medication-related osteonecrosis of the jaw in particular for osteonecrosis of the jaw due to bisphosphonate intake. More recently, regenerative concepts using stem cells from different sources and growth factors have been introduced for the treatment of medication-related osteonecrosis of the jaws. These new and innovative concepts seem to be promising future options in the management of osteonecrosis of the jaws.

In the current literature, treatment options for patients with established medication-related osteonecrosis of the jaw differ. While the first guidelines focused on preserving the patient's quality of life by controlling pain and secondary infection, nowadays there is a trend to a more surgical approach with the aim of complete mucosal healing of the lesions [419, 602]. As described in the previous chapters, a large variety of treatment modalities have been reported including conservative medical management, various types of surgery, hyperbaric oxygen, and ozone and laser therapy[256, 603, 604]. In large lesions with pathological fractures, reconstruction with vascularized or non-vascularized bone has been described, but remains problematic due to poor bone healing and an obligatory graft resorption phase, donor site morbidity, and infection of foreign material. Because bisphosphonates are often administered in patients with generalized bone pathologies and the molecules not only bind to the jaws, it is not unlikely that the transferred bone will either be affected by bony metastases or also develop osteonecrosis of the jaws [605, 606]. In osteonecrotic lesions, among others, the lack of osteogenic precursors and a shortage of endothelial progenitor cells (EPCs) cause an insufficient vascular support, so that safe alternative therapies are needed to enhance the osteogenesis and vasculogenesis [581, 607]. While tissue engineering is the branch that brings biology, bioengineering, clinical sciences, and biotechnology together for the purpose of generating new tissues and organs and the development of biologic substitutes that can restore and maintain normal function, a variety of approaches are utilized that combine the use of morphogens, growth factors, and cytokines, with scaffolds and carriers and cells [608-610]. During the last years, the increased interest on stem cells allowed the evolution of new horizons in treatment perspectives. Stem cells are immature, undifferentiated cells that can divide and multiply for an extended period of time, differentiating into specific types of cells and tissues. They are defined as cells that self-replicate and are able to differentiate into at least two different cell types, and both criteria must be present for a cell to be called a "stem

cell" [611, 612]. Embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs) represent the three different major types of stem cells [613]. During embryonic development, embryonic stem cells are derived from cells of the inner cell mass of the blastocysts. They are pluripotent and give rise to all derivatives of the three primary germ layers. The most important and potential use of ESCs is clinically in transplantation medicine, where they can be used to develop cell replacement therapies [611, 612, 614, 615]. In contrast, iPSCs refer to adult or somatic stem cells that have been genetically reprogrammed to behave like ESC [616].

ASCs are multipotent because their potential is normally limited to one or more lineages of specialized cells [614]. In addition to bone marrow, various tissues have been found to harbour mesenchymal stem cell (MSC)-like populations including adipose tissues, muscles, tendons, dental pulps, periodontal ligaments, umbilical cord blood, placenta, periosteum, liver, cartilage, synovium, synovial fluid, spleen, and thymus [617-623]. In vitro expanded bone marrow stem cells (BMMSCs) may be a rich source of osteogenic progenitor cells that are capable of promoting the repair or regeneration of skeletal defects when cultured in the presence of dexamethasone, inorganic phosphate, and vitamin C. BMMS can be induced to become osteoblast-like eel in vitro and form calcified nodules [624, 625].

Cell-Based Therapy in Craniofacial Tissue Engineering

The bone is the second most frequently transplanted tissue with increasing frequency. Reconstruction of craniofacial components is of the most important and intricate objectives stem cell-mediated regenerative medicine [626-628]. The craniofacial bone has an essential to in supporting the adjacent soft tissue, providing anchoring for dental structures and providing stable although flexible framework for craniofacial cartilage structures. Embryologically, most craniofacial bones are derived from mesenchymal tissue through membranous ossification [629].

Facial development, including that of the teeth and oral cavity, is a classic act of interactions by stem cells of the epithelium, craniofacial mesoderm and neural crest-derived mesenchyme [630, 631]. Cranial neural crest cells (CNC) play an important role in development of the teeth, alveolar crest, and jaw bone [632]. Thus, the biologically unique features of cranial neural crest cell-derived bone should be considered in the etiopathology of antiresorptive drug-induced osteonecrosis of the jaw.

Stem cell-based strategies are currently a promising approach in craniofacial bone tissue engineering as they supply sufficient numbers of cells that can not only form bone and associated tissue but also maintain bone as it undergoes turnover throughout life [610, 633]. Regenerative medicine for bone healing has reached the patient in the form of cell therapy approaches to treat localized bone defects or systemic diseases of the skeleton [634]. Mesenchymal stem cells (MSCs) have been isolated from a variety of mesenchymal tissues and they can differentiate into a wide array of cell types, including osteoblasts, chondrocytes and adipocytes. They participate in regeneration injured tissues in different ways. On one hand, they directly differentiate into tissue-specific cells a1 thus substitute damaged or lost cells. On the other hand, they indirectly influence tissue regeneration by secretion of soluble factors. Thirdly, they are able to modulate the inflammatory response. Thus, they can promote vascularization, cell proliferation, and differentiation and modulate inflammatory processes [635].

As a result of their slower growth rate and the absence of telomerase activity in vitro, mesenchymal stem cells (MSCs) are presumed to have a lower risk for tumour formation compared with embryonic stem cells (ESCs) [636]. This suggests that mesenchymal stem cells may have broader therapeutic applications compared to other adult stem cells. Bone marrow-derived mesenchymal stem cells (BMMSCs) can be concentrated from bone marrow aspirate with different techniques. The FICOLL method (synthetic polysaccharide) and the

BMAC method (bone marrow aspirate concentrate) are established methods for mononuclear cell concentration from iliac crest aspirate [626]. Percutaneous or intraoperative local administration of cell suspensions delivers progenitor or lineage-committed cells directly to the wound site. Mesenchymal stem cells functional properties have been proved by several experimental and clinical studies using autologous BMMSC implants for healing, cell architecture repair, and recovery of local blood flow on injured and ischemic tissues for alveolar ridge augmentation and long bone defects [637-639]. Autologous bone marrow or autologous mesenchymal stem cells were successfully implanted in a number of patients to enhance fracture and osteotomy healing; fill bone defects; treat pseudoarthrosis, bone cysts, and osteonecrosis or enhance spinal fusion [635]. In a randomized controlled trial, it has been shown that the new bone formation in sinus lift procedures using autologous mesenchymal stem cells in combination with bovine bone mineral is equivalent to autologous bone and bovine bone mineral [640].

Experimental and Clinical Cell-Based Therapy in Medication-Related Osteonecrosis of the Jaw

Several authors have focused on the treatment of osteonecrosis of the jaw with mesenchymal stem cells. With the ability to induce ectopic bone formation and angiogenesis, MSCs might become a promising treatment option for antiresorptive drug-induced osteonecrosis of the jaws [641]. In a mouse model, a mesenchymal stem cell-based approach to treat osteonecrosis of the jaw was tested. At 2 weeks after tooth extraction, ONJ-like wild-type mice receiving intravenous infusions with mesenchymal stem cells healed with complete soft tissue and bone regeneration at the extracted alveolar socket suggesting that cell-based immunotherapy using T regulatory cells (Tregs) or mesenchymal stem cells are promising therapeutic strategies to prevent and treat ONJ-like lesions in wild-type mice. It is discussed that cell-based therapy using systemic mesenchymal stem cell infusions can prevent or cure antiresorptive drug-

induced osteonecrosis of the jaws via re-establishment of the immune balance between inhibition of T-helper-producing interleukin 17 cells (th17) and increase in Tregs [642].

In a swine model, Li et al. reported the treatment of ONJ lesions with allogenic mesenchymal stem cells and concluded to have discovered that allogenic mesenchymal stem cell-based infusions provide a safe and effective therapeutic modality for treating ONJ lesions, which sheds light on potential clinical applications for treating patients suffering from medication-related osteonecrosis of the jaws [643].

In a case report, Cella et al. published to have cured a patient with refractory osteonecrosis of the jaw, with autologous mesenchymal stem cells that were aspirated from the iliac crest and transplanted intra-lesionally on a gelatine sponge carrier after concentration with the FICOLL method. This procedure allowed a clinical improvement of symptoms and induced novel ossification with complete remission from a stage 3 bisphosphonate-induced osteonecrosis of the jaw [260]. In another case report, Elad et al. presented a patient with bisphosphonate-induced osteonecrosis of the jaw, where bone marrow cells were re-suspended in saline and injected along the mucosal margins of two areas of exposed bone. No complications were observed with considerable reduction in the size of the alveolar bone exposures following the local infiltration of the hematopoietic stem cells. Complete healing of the lesion was achieved within a few months of the procedure showing great potential of hematopoietic stem cells to treat osteonecrosis of the jaws [607, 644].

In our own experience, a case series of 8 patients with refractory bisphosphonate-induced osteonecrosis of the jaws, the lesions was managed with surgical resection of necrotic bone followed by mesenchymal stem cell grafting. Marrow derived cells were aspirated from the iliac crest and concentrated using a chair-side bone marrow mesenchymal stem cells. These MSCs were then grafted into the defect with autologous thrombin and a BioGide membrane. In all cases bony edges were rounded and the wound closed using a three-layer technique. At

12-15 months follow-up, all patients showed satisfactory healing with no signs of wound infection, dehiscence, or recurrence of osteonecrosis of the jaw. Only one patient developed significant complications, that of sepsis of unknown origin, 2 months postoperatively (unpublished own data).

Growth Factors in Treatment of Medication-Related Osteonecrosis of the Jaw

Growth factors are soluble-secreted signalling polypeptides capable of instructing specific cellular responses in a biological environment [645]. The specific cellular response triggered by growth factor signalling can result in a very wide range of cell actions, including cell survival, control over migration, differentiation, or proliferation of a specific subset of cells [646]. A variety of growth factors produced by osteogenic cells, platelets, and inflammatory cells-including bone morphogenetic proteins (BMPs), insulin-like growth factors 1 and 2, transforming growth factor-β1 (TGF-β1), platelet-derived growth factor, and fibroblast growth factor 2-are functionally involved in bone healing. The bone matrix serves as a reservoir for these growth factors [647-649]. Growth factor application to patients suffering osteonecrosis of the jaws can be considered a challenge because of improving the soft and hard tissues healing. Acting like chemotactic agents, they stimulate angiogenesis, migration, proliferation, and differentiation of stem cells from the surrounding mesenchymal tissues into bone forming cells in an area of injury [330, 395]. The discovery of bone morphogenetic proteins (BMPs) as osteoinductive factors and the subsequent development of commercially available recombinant forms of BMPs have offered the potential to replace traditional grafting techniques with de novo bone formation [650, 651]. Bone morphogenetic protein type 2 (BMP-2) application substituting the necrotic bone removal could be considered a therapeutic option for reconstruction of localized bone defects of medication-related osteonecrosis of the jaws. rhBMP-2 was applied using an absorbable collagen sponge carrier to 20 patients who underwent surgical removal of necrotic bone related to bisphosphonate therapy. The collagen

was fixed to the soft tissue by an absorbable suture. The postoperative controls showed an increase in the soft tissue healing and new bone formation of the treated sites [282].

Some researchers have proposed also the use of platelet-rich plasma (PRP) in ONJ surgery based on surgical debridement and reconstruction combined with the use of platelet-rich plasma produced from the patient's autologous blood [258, 259, 291, 305, 328, 396, 652-656]. The rationale for the employment of PRP in patients affected by osteonecrosis of the jaws is based on the thesis that the presence of growth factors constitutes stimulations for bone healing, which is similar to physiological healing. The growth factors in platelet-rich plasma might accelerate epithelial wound healing, decrease tissue inflammation after surgery, improve the regeneration of bone and soft tissues, and promote tissue vascularization. The additional advantages related to the use of this product are its biocompatibility and safety as an autologous product [657, 658].

In a prospective study, Scoletta et al. reported of only one wound dehiscence after extraction of 202 teeth in 63 patients under intravenous bisphosphonate treatment. After extraction, the sockets were filled with scaffold-like autologous PRP [659]. In a case series of 25 patients with osteonecrotic lesions due to bisphosphonate intake, treatment of ONJ with a combination of bone resection and platelet-rich plasma was found to be an effective therapy that should be considered an alternative treatment modality for the management of advanced ONJ cases [660].

Lee et al. also described the successful management of complications of dental implant surgery of 2 patients taking the oral form of bisphosphonates, including platelet-rich plasma and hyperbaric oxygen [396]. Several other studies reported of enhanced mucosal healing of patients with ONJ due to bisphosphonate intake treated with surgical removal of the exposed bone, platelet-rich plasma, and primary closure under antibiotic coverage [259, 305, 328, 653].

Nitrogen-containing bisphosphonates are able to inhibit pyrophosphate synthase in the mevalonate pathway. The consequently decreased synthesis of the metabolite geranylgeraniol is believed to largely account for the development of bisphosphonate-induced osteonecrosis of the jaws. In an in vitro study, Ziebart et al. demonstrated that geranylgeraniol can rescue the negative effect of bisphosphonates in human umbilical cord vein endothelial cells, fibroblasts, and osteogenic cells [661]. Geranylgeraniol could lead to new treatment strategies for bisphosphonate-induced osteonecrosis of the jaws that have to be proven in animal studies.

Conclusion

The implementation of stem cell-based concepts and the use of growth factors are promising future treatment modalities for patients suffering from medication-related osteonecrosis of the jaw.

OTHER PROJECTS DURING PHD

- Regeneration of Critical-sized defects in oral and maxillofacial surgery in minipigs. Dr. Florian Probst. AO Grant Jan 2013.
- Large animal model for antiresorptive drug induced osteonecrosis of the jaw. PD Dr. Dr. Sven Otto. AO Grant May 2014.

REFERENCES

[1] Malik NA. Textbook of Oral and Maxillofacial Surgery: Jaypee Brothers,Medical Publishers Pvt. Limited; 2012.
[2] Nussenbaum B, Krebsbach PH. The role of gene therapy for craniofacial and dental tissue engineering. Advanced Drug Delivery Reviews. 2006;58:577-91.
[3] Scolozzi P, Martinez A, Jaques B. Complex orbito-fronto-temporal reconstruction using computer-designed PEEK implant. J Craniofac Surg. 2007;18:224-8.
[4] Elsalanty ME, Genecov DG. Bone grafts in craniofacial surgery. Craniomaxillofac Trauma Reconstr. 2009;2:125-34.
[5] Li Y, Chen S-K, Li L, Qin L, Wang X-L, Lai Y-X. Bone defect animal models for testing efficacy of bone substitute biomaterials. Journal of Orthopaedic Translation. 2015;3:95-104.
[6] Christensen LV, McKay DC. Rotational and translational loading of the temporomandibular joint. Cranio : the journal of craniomandibular practice. 2000;18:47-57.
[7] van Eijden TM. Biomechanics of the mandible. Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists. 2000;11:123-36.
[8] Dimitriou R, Jones E, McGonagle D, Giannoudis PV. Bone regeneration: current concepts and future directions. BMC Medicine. 2011;9:1-10.
[9] Rosetti EP, Marcantonio RA, Cirelli JA, Zuza EP, Marcantonio E, Jr. Treatment of gingival recession with collagen membrane and DFDBA: a histometric study in dogs. Brazilian oral research. 2009;23:307-12.
[10] Guimaraes EP, Pedreira FR, Jham BC, de Carli ML, Pereira AA, Hanemann JA. Clinical management of suppurative osteomyelitis, bisphosphonate-related osteonecrosis, and osteoradionecrosis: report of three cases and review of the literature. Case Rep Dent. 2013;2013:402096.
[11] Sanchez CJ, Jr., Ward CL, Romano DR, Hurtgen BJ, Hardy SK, Woodbury RL, et al. Staphylococcus aureus biofilms decrease osteoblast viability, inhibits osteogenic differentiation, and increases bone resorption in vitro. BMC musculoskeletal disorders. 2013;14:187.
[12] Sax H, Lew D. Osteomyelitis. Current infectious disease reports. 1999;1:261-6.
[13] Teitelbaum SL, Tondravi MM, Ross FP. Osteoclasts, macrophages, and the molecular mechanisms of bone resorption. Journal of leukocyte biology. 1997;61:381-8.
[14] Singh M, Singh S, Jain J, Singh KT. Chronic suppurative osteomyelitis of maxilla mimicking actinimycotic osteomyelitis: A rare case report. National journal of maxillofacial surgery. 2010;1:153-6.
[15] Chihara S, Segreti J. Osteomyelitis. Disease-a-month : DM. 2010;56:5-31.
[16] Henderson B, Nair SP. Hard labour: bacterial infection of the skeleton. Trends in microbiology. 2003;11:570-7.
[17] Wagner C, Kondella K, Bernschneider T, Heppert V, Wentzensen A, Hansch GM. Post-traumatic osteomyelitis: analysis of inflammatory cells recruited into the site of infection. Shock (Augusta, Ga). 2003;20:503-10.
[18] Otto S, Hafner S, Mast G, Tischer T, Volkmer E, Schieker M, et al. Bisphosphonate-related osteonecrosis of the jaw: is pH the missing part in the pathogenesis puzzle? Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2010;68:1158-61.
[19] Humber CC, Albilia JB, Rittenberg B. Chronic osteomyelitis following an uncomplicated dental extraction. Journal. 2011;77:b98.
[20] Mori G, Brunetti G, Colucci S, Ciccolella F, Coricciati M, Pignataro P, et al. Alteration of activity and survival of osteoblasts obtained from human periodontitis patients: role of TRAIL. Journal of biological regulators and homeostatic agents. 2007;21:105-14.

[21] Pavlukhina S, Lu Y, Patimetha A, Libera M, Sukhishvili S. Polymer multilayers with pH-triggered release of antibacterial agents. Biomacromolecules. 2010;11:3448-56.
[22] Bistrian B. Systemic response to inflammation. Nutrition reviews. 2007;65:S170-2.
[23] Issekutz AC, Bhimji S. Role for endotoxin in the leukocyte infiltration accompanying Escherichia coli inflammation. Infection and immunity. 1982;36:558-66.
[24] Ma L, Liu M, Liu H, Chen J, Cui D. In vitro cytotoxicity and drug release properties of pH- and temperature-sensitive core-shell hydrogel microspheres. International journal of pharmaceutics. 2010;385:86-91.
[25] Romas E, Gillespie MT. Inflammation-induced bone loss: can it be prevented? Rheumatic diseases clinics of North America. 2006;32:759-73.
[26] Thomas MV, Puleo DA. Infection, Inflammation, and Bone Regeneration: a Paradoxical Relationship. Journal of Dental Research. 2011;90:1052-61.
[27] Licata AA. Discovery, clinical development, and therapeutic uses of bisphosphonates. The Annals of pharmacotherapy. 2005;39:668-77.
[28] Peer A, Khamaisi M. Diabetes as a Risk Factor for Medication-Related Osteonecrosis of the Jaw. Journal of Dental Research. 2015;94:252-60.
[29] Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons Position Paper on Medication-Related Osteonecrosis of the Jaw—2014 Update. Journal of Oral and Maxillofacial Surgery. 2014;72:1938-56.
[30] Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons position paper on medication-related osteonecrosis of the jaw--2014 update. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2014;72:1938-56.
[31] Katsarelis H, Shah NP, Dhariwal DK, Pazianas M. Infection and medication-related osteonecrosis of the jaw. Journal of dental research. 2015;94:534-9.
[32] Pushalkar S, Li X, Kurago Z, Ramanathapuram LV, Matsumura S, Fleisher KE, et al. Oral microbiota and host innate immune response in bisphosphonate-related osteonecrosis of the jaw. International journal of oral science. 2014;6:219-26.
[33] Li CL, Seneviratne CJ, Huo L, Lu WW, Zheng LW. Impact of Actinomyces naeslundii on bisphosphonate-related osteonecrosis of the jaws in ovariectomized rats with periodontitis. Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery. 2015;43:1662-9.
[34] Sedghizadeh PP, Yooseph S, Fadrosh DW, Zeigler-Allen L, Thiagarajan M, Salek H, et al. Metagenomic investigation of microbes and viruses in patients with jaw osteonecrosis associated with bisphosphonate therapy. Oral surgery, oral medicine, oral pathology and oral radiology. 2012;114:764-70.
[35] Fliefel R, Tröltzsch M, Kühnisch J, Ehrenfeld M, Otto S. Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. International Journal of Oral and Maxillofacial Surgery. 2015;44:568-85.
[36] Perry CR. Bone and Joint Infections: Taylor & Francis; 1996.
[37] Aimola P, Desiderio V, Graziano A, Claudio PP. Stem cells in cancer therapy: From their role in pathogenesis to their use as therapeutic agents. Drug news & perspectives. 2010;23:175-83.
[38] Takato T, Mori Y, Fujihara Y, Asawa Y, Nishizawa S, Kanazawa S, et al. Preclinical and clinical research on bone and cartilage regenerative medicine in oral and maxillofacial region. Oral Science International. 2014;11:45-51.
[39] Ward BB, Brown SE, Krebsbach PH. Bioengineering strategies for regeneration of craniofacial bone: a review of emerging technologies. Oral Dis. 2010;16:709-16.

[40] Schulthess B, Bloemberg GV, Zbinden R, Bottger EC, Hombach M. Evaluation of the Bruker MALDI Biotyper for identification of Gram-positive rods: development of a diagnostic algorithm for the clinical laboratory. Journal of clinical microbiology. 2014;52:1089-97.
[41] Ibraheem D, Elaissari A, Fessi H. Gene therapy and DNA delivery systems. International journal of pharmaceutics. 2014;459:70-83.
[42] Jo J, Tabata Y. Non-viral gene transfection technologies for genetic engineering of stem cells. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2008;68:90-104.
[43] Waerzeggers Y, Monfared P, Viel T, Winkeler A, Voges J, Jacobs AH. Methods to monitor gene therapy with molecular imaging. Methods (San Diego, Calif). 2009;48:146-60.
[44] Scheller EL, Villa-Diaz LG, Krebsbach PH. Gene therapy: implications for craniofacial regeneration. J Craniofac Surg. 2012;23:333-7.
[45] Petrovic V, Zivkovic P, Petrovic D, Stefanovic V. Craniofacial bone tissue engineering. Oral surgery, oral medicine, oral pathology and oral radiology. 2012;114:e1-9.
[46] Scheller EL, Krebsbach PH. Gene therapy: design and prospects for craniofacial regeneration. Journal of dental research. 2009;88:585-96.
[47] Spector JA, Mehrara BJ, Greenwald JA, Saadeh PB, Steinbrech DS, Bouletreau PJ, et al. Osteoblast expression of vascular endothelial growth factor is modulated by the extracellular microenvironment. American journal of physiology Cell physiology. 2001;280:C72-80.
[48] Hatzenbuehler J, Pulling TJ. Diagnosis and management of osteomyelitis. American family physician. 2011;84:1027-33.
[49] Redlich K, Smolen JS. Inflammatory bone loss: pathogenesis and therapeutic intervention. Nature reviews Drug discovery. 2012;11:234-50.
[50] Marriott I, Gray DL, Tranguch SL, Fowler VG, Jr., Stryjewski M, Scott Levin L, et al. Osteoblasts express the inflammatory cytokine interleukin-6 in a murine model of Staphylococcus aureus osteomyelitis and infected human bone tissue. The American journal of pathology. 2004;164:1399-406.
[51] Eriksen EF. Cellular mechanisms of bone remodeling. Reviews in endocrine & metabolic disorders. 2010;11:219-27.
[52] Chakkalakal DA, Mashoof AA, Novak J, Strates BS, McGuire MH. Mineralization and pH relationships in healing skeletal defects grafted with demineralized bone matrix. Journal of biomedical materials research. 1994;28:1439-43.
[53] Iyemere VP, Proudfoot D, Weissberg PL, Shanahan CM. Vascular smooth muscle cell phenotypic plasticity and the regulation of vascular calcification. Journal of internal medicine. 2006;260:192-210.
[54] Kohn DH, Sarmadi M, Helman JI, Krebsbach PH. Effects of pH on human bone marrow stromal cells in vitro: implications for tissue engineering of bone. Journal of biomedical materials research. 2002;60:292-9.
[55] Green J. Cytosolic pH regulation in osteoblasts. Mineral and electrolyte metabolism. 1994;20:16-30.
[56] Arnett TR, Dempster DW. Protons and osteoclasts. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 1990;5:1099-103.
[57] Wu LN, Wuthier MG, Genge BR, Wuthier RE. In situ levels of intracellular Ca2+ and pH in avian growth plate cartilage. Clinical orthopaedics and related research. 1997:310-24.
[58] Ramp WK, Lenz LG, Kaysinger KK. Medium pH modulates matrix, mineral, and energy metabolism in cultured chick bones and osteoblast-like cells. Bone and mineral. 1994;24:59-73.
[59] Kaysinger KK, Ramp WK. Extracellular pH modulates the activity of cultured human osteoblasts. Journal of cellular biochemistry. 1998;68:83-9.

[60] Shen Y, Liu W, Wen C, Pan H, Wang T, Darvell BW, et al. Bone regeneration: importance of local pH-strontium-doped borosilicate scaffold. Journal of Materials Chemistry. 2012;22:8662-70.
[61] Muzylak M, Arnett TR, Price JS, Horton MA. The in vitro effect of pH on osteoclasts and bone resorption in the cat: Implications for the pathogenesis of FORL. Journal of cellular physiology. 2007;213:144-50.
[62] Han S-H, Chae S-W, Choi J-Y, Kim E-C, Chae H-J, Kim H-R. Acidic pH environments increase the expression of cathepsin B in osteoblasts: The significance of ER stress in bone physiology. Immunopharmacology and Immunotoxicology. 2009;31:428-31.
[63] Wei X, Yang X, Han Z-p, Qu F-f, Shao L, Shi Y-f. Mesenchymal stem cells: a new trend for cell therapy. Acta Pharmacol Sin. 2013;34:747-54.
[64] Larsen MJ, Jensen SJ. The hydroxyapatite solubility product of human dental enamel as a function of pH in the range 4.6-7.6 at 20 degrees C. Archives of oral biology. 1989;34:957-61.
[65] Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. Science (New York, NY). 1999;284:143-7.
[66] Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem cells (Dayton, Ohio). 2001;19:180-92.
[67] Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, et al. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proceedings of the National Academy of Sciences of the United States of America. 1995;92:4857-61.
[68] Kim DH, Yoo KH, Choi KS, Choi J, Choi SY, Yang SE, et al. Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell. Cytokine. 2005;31:119-26.
[69] Nuschke A, Rodrigues M, Stolz DB, Chu CT, Griffith L, Wells A. Human mesenchymal stem cells/multipotent stromal cells consume accumulated autophagosomes early in differentiation. Stem cell research & therapy. 2014;5:140.
[70] Qin Y, Guan J, Zhang C. Mesenchymal stem cells: mechanisms and role in bone regeneration. Postgraduate medical journal. 2014;90:643-7.
[71] Knight MN, Hankenson KD. Mesenchymal Stem Cells in Bone Regeneration. Advances in wound care. 2013;2:306-16.
[72] Wuertz K, Godburn K, Neidlinger-Wilke C, Urban J, Iatridis JC. Behavior of mesenchymal stem cells in the chemical microenvironment of the intervertebral disc. Spine. 2008;33:1843-9.
[73] Moore KA, Lemischka IR. Stem cells and their niches. Science (New York, NY). 2006;311:1880-5.
[74] Swenson O, Claff CL. Changes in the hydrogen ion concentration of healing fractures. Proceedings of the Society for Experimental Biology and Medicine Society for Experimental Biology and Medicine (New York, NY). 1946;61:151-4.
[75] Arnett TR, Dempster DW. Effect of pH on bone resorption by rat osteoclasts in vitro. Endocrinology. 1986;119:119-24.
[76] Newman RJ, Francis MJ, Duthie RB. Nuclear magnetic resonance studies of experimentally induced delayed fracture union. Clinical orthopaedics and related research. 1987:253-61.
[77] Alberton P, Popov C, Pragert M, Kohler J, Shukunami C, Schieker M, et al. Conversion of human bone marrow-derived mesenchymal stem cells into tendon progenitor cells by ectopic expression of scleraxis. Stem cells and development. 2012;21:846-58.

[78] Kohler J, Popov C, Klotz B, Alberton P, Prall WC, Haasters F, et al. Uncovering the cellular and molecular changes in tendon stem/progenitor cells attributed to tendon aging and degeneration. Aging cell. 2013;12:988-99.
[79] Popov C, Radic T, Haasters F, Prall WC, Aszodi A, Gullberg D, et al. Integrins alpha2beta1 and alpha11beta1 regulate the survival of mesenchymal stem cells on collagen I. Cell death & disease. 2011;2:e186.
[80] Naciri M, Kuystermans D, Al-Rubeai M. Monitoring pH and dissolved oxygen in mammalian cell culture using optical sensors. Cytotechnology. 2008;57:245-50.
[81] Mackenzie CG, Mackenzie JB, Beck P. The effect of pH on growth, protein synthesis, and lipid-rich particles of cultured mammalian cells. The Journal of biophysical and biochemical cytology. 1961;9:141-56.
[82] Arnett TR. Extracellular pH regulates bone cell function. The Journal of nutrition. 2008;138:415S-8S.
[83] Moghadam FH, Tayebi T, Dehghan M, Eslami G, Nadri H, Moradi A, et al. Differentiation of bone marrow mesenchymal stem cells into chondrocytes after short term culture in alkaline medium. International journal of hematology-oncology and stem cell research. 2014;8:12-9.
[84] Uskokovic V, Hoover C, Vukomanovic M, Uskokovic DP, Desai TA. Osteogenic and antimicrobial nanoparticulate calcium phosphate and poly-(D,L-lactide-co-glycolide) powders for the treatment of osteomyelitis. Materials science & engineering C, Materials for biological applications. 2013;33:3362-73.
[85] Kinnari TJ, Esteban J, Martin-de-Hijas NZ, Sanchez-Munoz O, Sanchez-Salcedo S, Colilla M, et al. Influence of surface porosity and pH on bacterial adherence to hydroxyapatite and biphasic calcium phosphate bioceramics. Journal of medical microbiology. 2009;58:132-7.
[86] Musgrove E, Seaman M, Hedley D. Relationship between cytoplasmic pH and proliferation during exponential growth and cellular quiescence. Experimental cell research. 1987;172:65-75.
[87] Taylor IW, Hodson PJ. Cell cycle regulation by environmental pH. Journal of cellular physiology. 1984;121:517-25.
[88] Wuertz K, Godburn K, Iatridis JC. MSC response to pH levels found in degenerating intervertebral discs. Biochemical and biophysical research communications. 2009;379:824-9.
[89] Teo A, Mantalaris A, Lim M. Influence of culture pH on proliferation and cardiac differentiation of murine embryonic stem cells. Biochemical Engineering Journal. 2014;90:8-15.
[90] Laffey JG, Engelberts D, Kavanagh BP. Injurious effects of hypocapnic alkalosis in the isolated lung. American journal of respiratory and critical care medicine. 2000;162:399-405.
[91] Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. Nature reviews Molecular cell biology. 2007;8:729-40.
[92] Busa WB, Nuccitelli R. Metabolic regulation via intracellular pH. The American journal of physiology. 1984;246:R409-38.
[93] Brunelle JK, Letai A. Control of mitochondrial apoptosis by the Bcl-2 family. Journal of cell science. 2009;122:437-41.
[94] D'Arcangelo D, Facchiano F, Barlucchi LM, Melillo G, Illi B, Testolin L, et al. Acidosis inhibits endothelial cell apoptosis and function and induces basic fibroblast growth factor and vascular endothelial growth factor expression. Circulation research. 2000;86:312-8.
[95] Cutaia M, Black AD, Cohen I, Cassai ND, Sidhu GS. Alkaline stress-induced apoptosis in human pulmonary artery endothelial cells. Apoptosis : an international journal on programmed cell death. 2005;10:1457-67.

[96] Webster KA, Discher DJ, Kaiser S, Hernandez O, Sato B, Bishopric NH. Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53. The Journal of clinical investigation. 1999;104:239-52.
[97] Aoyama K, Burns DM, Suh SW, Garnier P, Matsumori Y, Shiina H, et al. Acidosis causes endoplasmic reticulum stress and caspase-12-mediated astrocyte death. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism. 2005;25:358-70.
[98] Brandao-Burch A, Utting JC, Orriss IR, Arnett TR. Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. Calcified tissue international. 2005;77:167-74.
[99] Kuilman T, Michaloglou C, Mooi WJ, Peeper DS. The essence of senescence. Genes & development. 2010;24:2463-79.
[100] Di Benedetto A, Brunetti G, Posa F, Ballini A, Grassi FR, Colaianni G, et al. Osteogenic differentiation of mesenchymal stem cells from dental bud: Role of integrins and cadherins. Stem Cell Research. 2015;15:618-28.
[101] Simao AM, Bolean M, Hoylaerts MF, Millan JL, Ciancaglini P. Effects of pH on the production of phosphate and pyrophosphate by matrix vesicles' biomimetics. Calcified tissue international. 2013;93:222-32.
[102] Richards P, Chamberlain MJ, Wrong OM. Treatment of osteomalacia of renal tubular acidosis by sodium bicarbonate alone. Lancet. 1972;2:994-7.
[103] Disthabanchong S, Domrongkitchaiporn S, Sirikulchayanonta V, Stitchantrakul W, Karnsombut P, Rajatanavin R. Alteration of noncollagenous bone matrix proteins in distal renal tubular acidosis. Bone. 2004;35:604-13.
[104] Wu LN, Yoshimori T, Genge BR, Sauer GR, Kirsch T, Ishikawa Y, et al. Characterization of the nucleational core complex responsible for mineral induction by growth plate cartilage matrix vesicles. The Journal of biological chemistry. 1993;268:25084-94.
[105] Valhmu WB, Wu LN, Wuthier RE. Effects of Ca/Pi ratio, Ca2+ x Pi ion product, and pH of incubation fluid on accumulation of 45Ca2+ by matrix vesicles in vitro. Bone and mineral. 1990;8:195-209.
[106] Wu LN, Genge BR, Wuthier RE. Analysis and molecular modeling of the formation, structure, and activity of the phosphatidylserine-calcium-phosphate complex associated with biomineralization. The Journal of biological chemistry. 2008;283:3827-38.
[107] Marie PJ. Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys. 2008;473:98-105.
[108] Masrour Roudsari J, Mahjoub S. Quantification and comparison of bone-specific alkaline phosphatase with two methods in normal and paget's specimens. Caspian journal of internal medicine. 2012;3:478-83.
[109] Kaunitz JD, Yamaguchi DT. TNAP, TrAP, ecto-purinergic signaling, and bone remodeling. Journal of cellular biochemistry. 2008;105:655-62.
[110] Harada M, Udagawa N, Fukasawa K, Hiraoka BY, Mogi M. Inorganic pyrophosphatase activity of purified bovine pulp alkaline phosphatase at physiological pH. Journal of dental research. 1986;65:125-7.
[111] Leem YH, Nam TS, Kim JH, Lee KS, Lee DH, Yun J, et al. The Effects of Extracellular pH on Proliferation and Differentiation of human Bone Marrow Stem Cells. Korean J Bone Metab. 2012;19:35-46.
[112] Cheng S, Wang W, Lin Z, Zhou P, Zhang X, Zhang W, et al. Effects of extracellular calcium on viability and osteogenic differentiation of bone marrow stromal cells in vitro. Human cell. 2013;26:114-20.

[113] McLean FM, Keller PJ, Genge BR, Walters SA, Wuthier RE. Disposition of preformed mineral in matrix vesicles. Internal localization and association with alkaline phosphatase. The Journal of biological chemistry. 1987;262:10481-8.
[114] Genge BR, Sauer GR, Wu LN, McLean FM, Wuthier RE. Correlation between loss of alkaline phosphatase activity and accumulation of calcium during matrix vesicle-mediated mineralization. The Journal of biological chemistry. 1988;263:18513-9.
[115] Kohri K, Nomura S, Kitamura Y, Nagata T, Yoshioka K, Iguchi M, et al. Structure and expression of the mRNA encoding urinary stone protein (osteopontin). The Journal of biological chemistry. 1993;268:15180-4.
[116] Frick KK, Bushinsky DA. Chronic metabolic acidosis reversibly inhibits extracellular matrix gene expression in mouse osteoblasts. The American journal of physiology. 1998;275:F840-7.
[117] Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, et al. Increased bone formation in osteocalcin-deficient mice. Nature. 1996;382:448-52.
[118] Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell. 1997;89:755-64.
[119] Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, et al. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell. 1997;89:765-71.
[120] Jonason JH, Xiao G, Zhang M, Xing L, Chen D. Post-translational Regulation of Runx2 in Bone and Cartilage. Journal of dental research. 2009;88:693-703.
[121] Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell. 1997;89:747-54.
[122] Sprague SM, Krieger NS, Bushinsky DA. Greater inhibition of in vitro bone mineralization with metabolic than respiratory acidosis. Kidney Int. 1994;46:1199-206.
[123] Disthabanchong S, Radinahamed P, Stitchantrakul W, Hongeng S, Rajatanavin R. Chronic metabolic acidosis alters osteoblast differentiation from human mesenchymal stem cells. Kidney Int. 2006;71:201-9.
[124] Ali SY, Sajdera SW, Anderson HC. Isolation and characterization of calcifying matrix vesicles from epiphyseal cartilage. Proceedings of the National Academy of Sciences of the United States of America. 1970;67:1513-20.
[125] Anderson HC. Molecular biology of matrix vesicles. Clinical orthopaedics and related research. 1995:266-80.
[126] Golub EE. Role of matrix vesicles in biomineralization. Biochimica et biophysica acta. 2009;1790:1592-8.
[127] Anderson HC, Garimella R, Tague SE. The role of matrix vesicles in growth plate development and biomineralization. Frontiers in bioscience : a journal and virtual library. 2005;10:822-37.
[128] Millan JL. The role of phosphatases in the initiation of skeletal mineralization. Calcified tissue international. 2013;93:299-306.
[129] Thylstrup A, Fejerskov O. Textbook of Cariology: Wiley-Blackwell; 1986.
[130] Pautke C, Bauer F, Otto S, Tischer T, Steiner T, Weitz J, et al. Fluorescence-Guided Bone Resection in Bisphosphonate-Related Osteonecrosis of the Jaws: First Clinical Results of a Prospective Pilot Study. Journal of Oral and Maxillofacial Surgery. 2011;69:84-91.
[131] Stockmann P, Vairaktaris E, Wehrhan F, Seiss M, Schwarz S, Spriewald B, et al. Osteotomy and primary wound closure in bisphosphonate-associated osteonecrosis of the jaw: a prospective clinical study with 12 months follow-up. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer. 2010;18:449-60.

[132] Voss PJ, Oshero JJ, Kovalova-Muller A, Merino EAV, Sauerbier S, Al-Jamali J, et al. Surgical treatment of bisphosphonate-associated osteonecrosis of the jaw: Technical report and follow up of 21 patients. Journal of Cranio-Maxillofacial Surgery. 2012;40:719-25.
[133] Carlson ER, Basile JD. The role of surgical resection in the management of bisphosphonate-related osteonecrosis of the jaws. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2009;67:85-95.
[134] Marx RE, Sawatari Y, Fortin M, Broumand V. Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2005;63:1567-75.
[135] Hoff AO, Toth BB, Altundag K, Johnson MM, Warneke CL, Hu M, et al. Frequency and risk factors associated with osteonecrosis of the jaw in cancer patients treated with intravenous bisphosphonates. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2008;23:826-36.
[136] Montebugnoli L, Felicetti L, Gissi DB, Pizzigallo A, Pelliccioni GA, Marchetti C. Biphosphonate-associated osteonecrosis can be controlled by nonsurgical management. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2007;104:473-7.
[137] O'Ryan FS, Khoury S, Liao W, Han MM, Hui RL, Baer D, et al. Intravenous bisphosphonate-related osteonecrosis of the jaw: bone scintigraphy as an early indicator. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2009;67:1363-72.
[138] Watters AL, Hansen HJ, Williams T, Chou JF, Riedel E, Halpern J, et al. Intravenous bisphosphonate-related osteonecrosis of the jaw: long-term follow-up of 109 patients. Oral surgery, oral medicine, oral pathology and oral radiology. 2013;115:192-200.
[139] Fliefel R, Troltzsch M, Kuhnisch J, Ehrenfeld M, Otto S. Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. Int J Oral Maxillofac Surg. 2015;44:568-85.
[140] Khosla S, Burr D, Cauley J, Dempster DW, Ebeling PR, Felsenberg D, et al. Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2007;22:1479-91.
[141] Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B. American Association of Oral and Maxillofacial Surgeons Position Paper on Bisphosphonate-Related Osteonecrosis of the Jaws-2009 Update. Journal of Oral and Maxillofacial Surgery. 2009;67:2-12.
[142] Ristow O, Otto S, Troeltzsch M, Hohlweg-Majert B, Pautke C. Treatment perspectives for medication-related osteonecrosis of the jaw (MRONJ). Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery. 2015;43:290-3.
[143] Pautke C, Bauer F, Tischer T, Kreutzer K, Weitz J, Kesting M, et al. Fluorescence-guided bone resection in bisphosphonate-associated osteonecrosis of the jaws. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2009;67:471-6.
[144] Mucke T, Koschinski J, Deppe H, Wagenpfeil S, Pautke C, Mitchell DA, et al. Outcome of treatment and parameters influencing recurrence in patients with bisphosphonate-related osteonecrosis of the jaws. Journal of cancer research and clinical oncology. 2011;137:907-13.

[145] Hutchinson M, O'Ryan F, Chavez V, Lathon PV, Sanchez G, Hatcher DC, et al. Radiographic Findings in Bisphosphonate-Treated Patients With Stage 0 Disease in the Absence of Bone Exposure. Journal of Oral and Maxillofacial Surgery. 2010;68:2232-40.
[146] Fabbricini R, Catalano L, Pace L, Del Vecchio S, Fonti R, Salvatore M, et al. Bone scintigraphy and SPECT/CT in bisphosphonate-induced osteonecrosis of the jaw. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2009;50:1385; author reply
[147] Dore F, Filippi L, Biasotto M, Chiandussi S, Cavalli F, Di Lenarda R. Bone scintigraphy and SPECT/CT of bisphosphonate-induced osteonecrosis of the jaw. Journal of nuclear medicine : official publication, Society of Nuclear Medicine. 2009;50:30-5.
[148] Guggenberger R, Fischer DR, Metzler P, Andreisek G, Nanz D, Jacobsen C, et al. Bisphosphonate-induced osteonecrosis of the jaw: comparison of disease extent on contrast-enhanced MR imaging, [18F] fluoride PET/CT, and conebeam CT imaging. AJNR American journal of neuroradiology. 2013;34:1242-7.
[149] Assaf AT, Smeets R, Riecke B, Weise E, Grobe A, Blessmann M, et al. Incidence of bisphosphonate-related osteonecrosis of the jaw in consideration of primary diseases and concomitant therapies. Anticancer Res. 2013;33:3917-24.
[150] Pautke C, Tischer T, Neff A, Horch HH, Kolk A. In vivo tetracycline labeling of bone: an intraoperative aid in the surgical therapy of osteoradionecrosis of the mandible. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2006;102:e10-3.
[151] Otto S, Baumann S, Ehrenfeld M, Pautke C. Successful surgical management of osteonecrosis of the jaw due to RANK-ligand inhibitor treatment using fluorescence guided bone resection. Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery. 2013;41:694-8.
[152] Pautke C, Kreutzer K, Weitz J, Knodler M, Munzel D, Wexel G, et al. Bisphosphonate related osteonecrosis of the jaw: A minipig large animal model. Bone. 2012;51:592-9.
[153] Fleisher KE, Doty S, Kottal S, Phelan J, Norman RG, Glickman RS. Tetracycline-guided debridement and cone beam computed tomography for the treatment of bisphosphonate-related osteonecrosis of the jaw: a technical note. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2008;66:2646-53.
[154] Pautke C, Bauer F, Bissinger O, Tischer T, Kreutzer K, Steiner T, et al. Tetracycline bone fluorescence: a valuable marker for osteonecrosis characterization and therapy. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2010;68:125-9.
[155] Troeltzsch M, Probst F, Troeltzsch M, Ehrenfeld M, Otto S. Conservative management of medication-related osteonecrosis of the maxilla with an obturator prosthesis. The Journal of prosthetic dentistry. 2015;113:236-41.
[156] Vescovi P, Manfredi M, Merigo E, Meleti M. Early surgical approach preferable to medical therapy for bisphosphonate-related osteonecrosis of the jaws. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2008;66:831-2.
[157] Otto S, Marx RE, Tröltzsch M, Ristow O, Ziebart T, Al-Nawas B, et al. Comments on "Diagnosis and Management of Osteonecrosis of the Jaw: A Systematic Review and International Consensus". Journal of Bone and Mineral Research. 2015;30:1113-5.
[158] Nicolatou-Galitis O, Papadopoulou E, Sarri T, Boziari P, Karayianni A, Kyrtsonis M-C, et al. Osteonecrosis of the jaw in oncology patients treated with bisphosphonates: prospective experience of a dental oncology referral center. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontology. 2011;112:195-202.
[159] Kyrgidis A, Triaridis S, Kontos K, Patrikidou A, Andreadis C, Constantinidis J, et al. Quality of life in breast cancer patients with bisphosphonate-related osteonecrosis of the jaws

and patients with head and neck cancer: a comparative study using the EORTC QLQ-C30 and QLQ-HN35 questionnaires. Anticancer Res. 2012;32:3527-34.
[160] Then C, Horauf N, Otto S, Pautke C, von Tresckow E, Rohnisch T, et al. Incidence and risk factors of bisphosphonate-related osteonecrosis of the jaw in multiple myeloma patients having undergone autologous stem cell transplantation. Onkologie. 2012;35:658-64.
[161] Otto S, Schreyer C, Hafner S, Mast G, Ehrenfeld M, Sturzenbaum S, et al. Bisphosphonate-related osteonecrosis of the jaws - characteristics, risk factors, clinical features, localization and impact on oncological treatment. Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery. 2012;40:303-9.
[162] Bedogni A, Saia G, Bettini G, Tronchet A, Totola A, Bedogni G, et al. Long-term outcomes of surgical resection of the jaws in cancer patients with bisphosphonate-related osteonecrosis. Oral Oncol. 2011;47:420-4.
[163] Schubert M, Klatte I, Linek W, Mueller B, Doering K, Eckelt U, et al. The Saxon Bisphosphonate Register - Therapy and prevention of bisphosphonate-related osteonecrosis of the jaws. Oral Oncology. 2012;48:349-54.
[164] Jacobsen C, Metzler P, Obwegeser JA, Zemann W, Graetz KW. Osteopathology of the jaw associated with bone resorption inhibitors: what have we learned in the last 8 years? Swiss medical weekly. 2012;142:w13605.
[165] Graziani F, Vescovi P, Campisi G, Favia G, Gabriele M, Gaeta GM, et al. Resective surgical approach shows a high performance in the management of advanced cases of bisphosphonate-related osteonecrosis of the jaws: a retrospective survey of 347 cases. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2012;70:2501-7.
[166] Rupel K, Ottaviani G, Gobbo M, Contardo L, Tirelli G, Vescovi P, et al. A systematic review of therapeutical approaches in bisphosphonates-related osteonecrosis of the jaw (BRONJ). Oral Oncol. 2014;50:1049-57.
[167] Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gotzsche PC, Ioannidis JP, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. PLoS medicine. 2009;6:e1000100.
[168] Khan AA, Morrison A, Hanley DA, Felsenberg D, McCauley LK, O'Ryan F, et al. Diagnosis and management of osteonecrosis of the jaw: a systematic review and international consensus. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2015;30:3-23.
[169] Williams WB, O'Ryan F. Management of Medication-Related Osteonecrosis of the Jaw. Oral Maxillofac Surg Clin North Am. 2015;27:517-25.
[170] Vescovi P, Giovannacci I, Otto S, Manfredi M, Merigo E, Fornaini C, et al. Medication-Related Osteonecrosis of the Jaw: An Autofluorescence-Guided Surgical Approach Performed with Er:YAG Laser. Photomed Laser Surg. 2015;33:437-42.
[171] Ristow O, Pautke C. Auto-fluorescence of the bone and its use for delineation of bone necrosis. Int J Oral Maxillofac Surg. 2014;43:1391-3.
[172] Mitsimponas KT, Moebius P, Amann K, Stockmann P, Schlegel KA, Neukam FW, et al. Osteo-radio-necrosis (ORN) and bisphosphonate-related osteonecrosis of the jaws (BRONJ): the histopathological differences under the clinical similarities. International journal of clinical and experimental pathology. 2014;7:496-508.
[173] Otto S, Pautke C, Opelz C, Westphal I, Drosse I, Schwager J, et al. Osteonecrosis of the Jaw: Effect of Bisphosphonate Type, Local Concentration, and Acidic Milieu on the Pathomechanism. Journal of Oral and Maxillofacial Surgery. 2010;68:2837-45.
[174] Hansen T, Kunkel M, Springer E, Walter C, Weber A, Siegel E, et al. Actinomycosis of the jaws--histopathological study of 45 patients shows significant involvement in

bisphosphonate-associated osteonecrosis and infected osteoradionecrosis. Virchows Archiv : an international journal of pathology. 2007;451:1009-17.
[175] Hansen T, Kunkel M, Weber A, James Kirkpatrick C. Osteonecrosis of the jaws in patients treated with bisphosphonates - histomorphologic analysis in comparison with infected osteoradionecrosis. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2006;35:155-60.
[176] Lazarovici TS, Yahalom R, Taicher S, Elad S, Hardan I, Yarom N. Bisphosphonate-related osteonecrosis of the jaws: a single-center study of 101 patients. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2009;67:850-5.
[177] Hall V. Actinomyces--gathering evidence of human colonization and infection. Anaerobe. 2008;14:1-7.
[178] Kaplan I, Anavi K, Anavi Y, Calderon S, Schwartz-Arad D, Teicher S, et al. The clinical spectrum of Actinomyces-associated lesions of the oral mucosa and jawbones: correlations with histomorphometric analysis. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2009;108:738-46.
[179] Norouzi F, Aminshahidi M, Heidari B, Farshad S. Bacteremia Due to Actinomyces naeslundii in a T cell Lymphoma Child; a Case Report. Jundishapur J Microbiol. 2013;6:306-8.
[180] Ji X, Pushalkar S, Li Y, Glickman R, Fleisher K, Saxena D. Antibiotic effects on bacterial profile in osteonecrosis of the jaw. Oral Diseases. 2012;18:85-95.
[181] Hinson AM, Smith CW, Siegel ER, Stack BC, Jr. Is bisphosphonate-related osteonecrosis of the jaw an infection? A histological and microbiological ten-year summary. Int J Dent. 2014;2014:452737.
[182] Wragg P, Randall L, Whatmore AM. Comparison of Biolog GEN III MicroStation semi-automated bacterial identification system with matrix-assisted laser desorption ionization-time of flight mass spectrometry and 16S ribosomal RNA gene sequencing for the identification of bacteria of veterinary interest. Journal of microbiological methods. 2014;105:16-21.
[183] Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP, Raoult D. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. Journal of clinical microbiology. 2000;38:3623-30.
[184] Maurer P, Sandulescu T, Kriwalsky MS, Rashad A, Hollstein S, Stricker I, et al. Bisphosphonate-related osteonecrosis of the maxilla and sinusitis maxillaris. International Journal of Oral and Maxillofacial Surgery. 2011;40:285-91.
[185] Favia G, Pilolli GP, Maiorano E. Histologic and histomorphometric features of bisphosphonate-related osteonecrosis of the jaws: an analysis of 31 cases with confocal laser scanning microscopy. Bone. 2009;45:406-13.
[186] Allen MR, Burr DB. The pathogenesis of bisphosphonate-related osteonecrosis of the jaw: so many hypotheses, so few data. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2009;67:61-70.
[187] Kim KM, Rhee Y, Kwon YD, Kwon TG, Lee JK, Kim DY. Medication Related Osteonecrosis of the Jaw: 2015 Position Statement of the Korean Society for Bone and Mineral Research and the Korean Association of Oral and Maxillofacial Surgeons. Journal of bone metabolism. 2015;22:151-65.
[188] Hoefert S. Microbiology and Antibiotics in the Context of Medication-Related Osteonecrosis of the Jaw. In: Otto S, editor. Medication-related Osteonecrosis of the Jaws: Bisphosphonates, Denosumab, and New Agents Heidelberg Springer 2015. p. 121-29.
[189] Aspenberg P, Genant HK, Johansson T, Nino AJ, See K, Krohn K, et al. Teriparatide for acceleration of fracture repair in humans: a prospective, randomized, double-blind study

of 102 postmenopausal women with distal radial fractures. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2010;25:404-14.
[190] Sedghizadeh PP, Kumar SK, Gorur A, Schaudinn C, Shuler CF, Costerton JW. Microbial biofilms in osteomyelitis of the jaw and osteonecrosis of the jaw secondary to bisphosphonate therapy. Journal of the American Dental Association (1939). 2009;140:1259-65.
[191] Boff RC, Salum FG, Figueiredo MA, Cherubini K. Important aspects regarding the role of microorganisms in bisphosphonate-related osteonecrosis of the jaws. Archives of oral biology. 2014;59:790-9.
[192] Lau SK, Woo PC, Fung AM, Chan KM, Woo GK, Yuen KY. Anaerobic, non-sporulating, Gram-positive bacilli bacteraemia characterized by 16S rRNA gene sequencing. Journal of medical microbiology. 2004;53:1247-53.
[193] Elsayed S, George A, Zhang K. Intrauterine contraceptive device-associated pelvic actinomycosis caused by Actinomyces urogenitalis. Anaerobe. 2006;12:67-70.
[194] Kaya D, Demirezen S, Hascelik G, Gulmez Kivanc D, Beksac MS. Comparison of PCR, culturing and Pap smear microscopy for accurate diagnosis of genital Actinomyces. Journal of medical microbiology. 2013;62:727-33.
[195] Sato T, Matsuyama J, Takahashi N, Sato M, Johnson J, Schachtele C, et al. Differentiation of oral Actinomyces species by 16S ribosomal DNA polymerase chain reaction-restriction fragment length polymorphism. Archives of oral biology. 1998;43:247-52.
[196] Ruby JD, Li Y, Luo Y, Caufield PW. Genetic characterization of the oral Actinomyces. Archives of oral biology. 2002;47:457-63.
[197] Tang G, Samaranayake LP, Yip HK. Genotypic diversity of oral Actinomyces naeslundii genospecies 1 and 2 in caries-active preschool children. Oral microbiology and immunology. 2004;19:371-8.
[198] Thumbigere-Math V, Sabino MC, Gopalakrishnan R, Huckabay S, Dudek AZ, Basu S, et al. Bisphosphonate-related osteonecrosis of the jaw: clinical features, risk factors, management, and treatment outcomes of 26 patients. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2009;67:1904-13.
[199] Boonyapakorn T, Schirmer I, Reichart PA, Sturm I, Massenkeil G. Bisphosphonate-induced osteonecrosis of the jaws: prospective study of 80 patients with multiple myeloma and other malignancies. Oral Oncol. 2008;44:857-69.
[200] Vahtsevanos K, Kyrgidis A, Verrou E, Katodritou E, Triaridis S, Andreadis CG, et al. Longitudinal cohort study of risk factors in cancer patients of bisphosphonate-related osteonecrosis of the jaw. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2009;27:5356-62.
[201] Bamias A, Kastritis E, Bamia C, Moulopoulos LA, Melakopoulos I, Bozas G, et al. Osteonecrosis of the jaw in cancer after treatment with bisphosphonates: incidence and risk factors. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2005;23:8580-7.
[202] O'Ryan FS, Lo JC. Bisphosphonate-related osteonecrosis of the jaw in patients with oral bisphosphonate exposure: clinical course and outcomes. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2012;70:1844-53.
[203] Lopes RN, Rabelo GD, Rocha AC, Carvalho PAG, Alves FA. Surgical Therapy for Bisphosphonate-Related Osteonecrosis of the Jaw: Six-Year Experience of a Single Institution. Journal of Oral and Maxillofacial Surgery. 2015;73:1288-95.
[204] Lopes RN, Rabelo GD, Rocha AC, Carvalho PA, Alves FA. Surgical Therapy for Bisphosphonate-Related Osteonecrosis of the Jaw: Six-Year Experience of a Single

Institution. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2015;73:1288-95.
[205] Mozzati M, Arata V, Gallesio G. Tooth extraction in osteoporotic patients taking oral bisphosphonates. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2013;24:1707-12.
[206] Otto S, Troltzsch M, Jambrovic V, Panya S, Probst F, Ristow O, et al. Tooth extraction in patients receiving oral or intravenous bisphosphonate administration: A trigger for BRONJ development? Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery. 2015;43:847-54.
[207] Saia G, Blandamura S, Bettini G, Tronchet A, Totola A, Bedogni G, et al. Occurrence of bisphosphonate-related osteonecrosis of the jaw after surgical tooth extraction. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2010;68:797-804.
[208] Conte-Neto N, Bastos AS, Spolidorio LC, Marcantonio RAC, Marcantonio E. Oral bisphosphonate-related osteonecrosis of the jaws in rheumatoid arthritis patients: a critical discussion and two case reports. Head & Face Medicine. 2011;7.
[209] Patel S, Choyee S, Uyanne J, Nguyen AL, Lee P, Sedghizadeh PP, et al. Non-exposed bisphosphonate-related osteonecrosis of the jaw: a critical assessment of current definition, staging, and treatment guidelines. Oral Dis. 2012;18:625-32.
[210] Arantes HP, Silva AG, Lazaretti-Castro M. Bisphosphonates in the treatment of metabolic bone diseases. Arquivos brasileiros de endocrinologia e metabologia. 2010;54:206-12.
[211] Griz L, Caldas G, Bandeira C, Assuncao V, Bandeira F. Paget's disease of bone. Arquivos brasileiros de endocrinologia e metabologia. 2006;50:814-22.
[212] Lipton A, Theriault RL, Hortobagyi GN, Simeone J, Knight RD, Mellars K, et al. Pamidronate prevents skeletal complications and is effective palliative treatment in women with breast carcinoma and osteolytic bone metastases - Long term follow-up of two randomized, placebo-controlled trials. Cancer. 2000;88:1082-90.
[213] Berenson JR, Rosen LS, Howell A, Porter L, Coleman RE, Morley W, et al. Zoledronic acid reduces skeletal-related events in patients with osteolytic metastases. Cancer. 2001;91:1191-200.
[214] Saad F. Clinical benefit of zoledronic acid for the prevention of skeletal complications in advanced prostate cancer. Clinical prostate cancer. 2005;4:31-7.
[215] Rogers MJ, Crockett JC, Coxon FP, Monkkonen J. Biochemical and molecular mechanisms of action of bisphosphonates. Bone. 2011;49:34-41.
[216] Hellstein JW, Marek CL. Bisphosphonate Osteochemonecrosis (Bis-Phossy Jaw): Is This Phossy Jaw of the 21st Century? Journal of Oral and Maxillofacial Surgery. 2005;63:682-9.
[217] Russell RG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2008;19:733-59.
[218] Hollick RJ, Reid DM. Role of bisphosphonates in the management of postmenopausal osteoporosis: an update on recent safety anxieties. Menopause Int. 2011;17:66-72.
[219] Basso U, Maruzzo M, Roma A, Camozzi V, Luisetto G, Lumachi F. Malignant hypercalcemia. Curr Med Chem. 2011;18:3462-7.
[220] Hadji P. Clinical considerations for the use of antiresorptive agents in the treatment of metastatic bone disease. Critical Reviews in Oncology Hematology. 2011;80:301-13.

[221] Jin Y, An X, Cai YC, Cao Y, Cai XY, Xia Q, et al. Zoledronic acid combined with chemotherapy bring survival benefits to patients with bone metastases from nasopharyngeal carcinoma. J Cancer Res Clin. 2011;137:1545-51.
[222] Pichardo SE, van Merkesteyn JP. Bisphosphonate related osteonecrosis of the jaws: spontaneous or dental origin? Oral surgery, oral medicine, oral pathology and oral radiology. 2013;116:287-92.
[223] Wysowski DK. Reports of Esophageal Cancer with Oral Bisphosphonate Use. New England Journal of Medicine. 2009;360:89-90.
[224] Ibrahim T, Barbanti F, Giorgio-Marrano G, Mercatali L, Ronconi S, Vicini C, et al. Osteonecrosis of the jaw in patients with bone metastases treated with bisphosphonates: A retrospective study. Oncologist. 2008;13:330-6.
[225] Bagan JV, Jimenez Y, Murillo J, Hernandez S, Poveda R, Sanchis JM, et al. Jaw osteonecrosis associated with bisphosphonates: Multiple exposed areas and its relationship to teeth extractions. Study of 20 cases. Oral Oncology. 2006;42:327-9.
[226] Marx RE. Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis of the jaws: a growing epidemic. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2003;61:1115-7.
[227] Migliorati CA. Bisphosphanates and oral cavity avascular bone necrosis. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2003;21:4253-4.
[228] Wang J, Goodger NM, Pogrel MA. Osteonecrosis of the jaws associated with cancer chemotherapy. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2003;61:1104-7.
[229] Rosenberg TJ, Ruggiero S. Osteonecrosis of the jaws associated with the use of bisphosphonates. Journal of Oral and Maxillofacial Surgery. 2003;61:60.
[230] Ruggiero SL, Mehrotra B, Rosenberg TJ, Engroff SL. Osteonecrosis of the jaws associated with the use of bisphosphonates: A review of 63 cases. Journal of Oral and Maxillofacial Surgery. 2004;62:527-34.
[231] Ruggiero SL, Fantasia J, Carlson E, Park H. Bisphosphonate-related osteonecrosis of the jaw: background and guidelines for diagnosis, staging and management. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontics. 2006;102:433-41.
[232] Greenberg MS. Intravenous bisphosphonates and osteonecrosis. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2004;98:259-60.
[233] Gibbs SD, O'Grady J, Seymour JF, Prince HM. Bisphosphonate-induced osteonecrosis of the jaw requires early detection and intervention. The Medical journal of Australia. 2005;183:549-50.
[234] Mehrotra B, Ruggiero SL. Bisphosphonate Related Osteonecrosis (BRON) of the Jaw: Single Institutional Update. ASH Annual Meeting Abstracts. 2005;106:291-.
[235] Melo MD, Obeid G. Osteonecrosis of the jaws in patients with a history of receiving bisphosphonate therapy: strategies for prevention and early recognition. Journal of the American Dental Association (1939). 2005;136:1675-81.
[236] Migliorati CA, Schubert MM, Peterson DE, Seneda LM. Bisphosphonate-associated osteonecrosis of mandibular and maxillary bone: an emerging oral complication of supportive cancer therapy. Cancer. 2005;104:83-93.
[237] Purcell PM, Boyd IW. Bisphosphonates and osteonecrosis of the jaw. The Medical journal of Australia. 2005;182:417-8.
[238] Vannucchi AM, Ficarra G, Antonioli E, Bosi A. Osteonecrosis of the jaw associated with zoledronate therapy in a patient with multiple myeloma. Br J Haematol. 2005;128:738.
[239] Bilezikian JP. Osteonecrosis of the Jaw — Do Bisphosphonates Pose a Risk? New England Journal of Medicine. 2006;355:2278-81.

[240] Van Poznak C, Ward BB. Osteonecrosis of the jaw. Current Opinion in Orthopaedics. 2006;17:462-8 10.1097/01.bco.0000244040.40165.6c.
[241] Agrillo A, Filiaci F, Ramieri V, Riccardi E, Quarato D, Rinna C, et al. Bisphosphonate-related osteonecrosis of the jaw (BRONJ): 5 year experience in the treatment of 131 cases with ozone therapy. Eur Rev Med Pharmacol Sci. 2012;16:1741-7.
[242] Silverman SL, Landesberg R. Osteonecrosis of the jaw and the role of bisphosphonates: a critical review. Am J Med. 2009;122:S33-45.
[243] Bagan J, Scully C, Sabater V, Jimenez Y. Osteonecrosis of the jaws in patients treated with intravenous bisphosphonates (BRONJ): A concise update. Oral Oncol. 2009;45:551-4.
[244] Brozoski MA, Traina AA, Deboni MCZ, Marques MM, Naclério-Homem MdG. Osteonecrose maxilar associada ao uso de bisfosfonatos. Rev Bras Reumatol. 2012;52:265-70.
[245] American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws. Journal of Oral and Maxillofacial Surgery. 2007;65:369-76.
[246] Migliorati CA, Siegel MA, Elting LS. Bisphosphonate-associated osteonecrosis: a long-term complication of bisphosphonate treatment. The Lancet Oncology. 2006;7:508-14.
[247] Reid IR. Osteonecrosis of the jaw: who gets it, and why? Bone. 2009;44:4-10.
[248] Marunick M, Miller R, Gordon S. Adverse oral sequelae to bisphosphonate administration. The Journal of the Michigan Dental Association. 2005;87:44-9.
[249] Lerman MA, Xie W, Treister NS, Richardson PG, Weller EA, Woo S-B. Conservative management of bisphosphonate-related osteonecrosis of the jaws: Staging and treatment outcomes. Oral Oncology. 2013;49:977-83.
[250] Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaw - 2009 update. Australian endodontic journal : the journal of the Australian Society of Endodontology Inc. 2009;35:119-30.
[251] Magopoulos C, Karakinaris G, Telioudis Z, Vahtsevanos K, Dimitrakopoulos I, Antoniadis K, et al. Osteonecrosis of the jaws due to bisphosphonate use. A review of 60 cases and treatment proposals. Am J Otolaryng. 2007;28:158-63.
[252] Bedogni A, Saia G, Ragazzo M, Bettini G, Capelli P, D'Alessandro E, et al. Bisphosphonate-associated osteonecrosis can hide jaw metastases. Bone. 2007;41:942-5.
[253] Marx RE, Cillo JE, Ulloa JJ. Oral bisphosphonate-induced osteonecrosis: Risk factors, prediction of risk using serum CTX testing, prevention, and treatment. Journal of Oral and Maxillofacial Surgery. 2007;65:2397-410.
[254] Vogel F, Scholz H, al-Nawas B, Elies W, Kresken M, Lode H, et al. [Rational use of oral antibiotics. Findings of an expert commission of the Paul Ehrlich Society for Chemotherapy]. Medizinische Monatsschrift fur Pharmazeuten. 2002;25:193-204.
[255] Nocini PF, Saia G, Bettini G, Ragazzo M, Blandamura S, Chiarini L, et al. Vascularized fibula flap reconstruction of the mandible in bisphosphonate-related osteonecrosis. Ejso-Eur J Surg Onc. 2009;35:373-9.
[256] Freiberger JJ, Padilla-Burgos R, Chhoeu AH, Kraft KH, Boneta O, Moon RE, et al. Hyperbaric oxygen treatment and bisphosphonate-induced osteonecrosis of the jaw: a case series. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2007;65:1321-7.
[257] Vescovi P, Merigo E, Meleti M, Manfredi M. Bisphosphonate-associated osteonecrosis (BON) of the jaws: a possible treatment? Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2006;64:1460-2.
[258] Curi MM, Cossolin GS, Koga DH, Araujo SR, Feher O, dos Santos MO, et al. Treatment of avascular osteonecrosis of the mandible in cancer patients with a history of bisphosphonate therapy by combining bone resection and autologous platelet-rich plasma:

Report of 3 cases. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2007;65:349-55.
[259] Adornato MC, Morcos I, Rozanski J. The treatment of bisphosphonate-associated osteonecrosis of the jaws with bone resection and autologous platelet-derived growth factors. Journal of the American Dental Association (1939). 2007;138:971-7.
[260] Cella L, Oppici A, Arbasi M, Moretto M, Piepoli M, Vallisa D, et al. Autologous bone marrow stem cell intralesional transplantation repairing bisphosphonate related osteonecrosis of the jaw. Head Face Med. 2011;7:16.
[261] Bashutski JD, Eber RM, Kinney JS, Benavides E, Maitra S, Braun TM, et al. Teriparatide and Osseous Regeneration in the Oral Cavity. New England Journal of Medicine. 2010;363:2396-405.
[262] Epstein MS, Wicknick FW, Epstein JB, Berenson JR, Gorsky M. Management of bisphosphonate-associated osteonecrosis: pentoxifylline and tocopherol in addition to antimicrobial therapy. An initial case series. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontology. 2010;110:593-6.
[263] Petrucci MT, Gallucci C, Agrillo A, Mustazza MC, Foa R. Role of ozone therapy in the treatment of osteonecrosis of the jaws in multiple myeloma patients. Haematol-Hematol J. 2007;92:1289-90.
[264] Moher D, Liberati A, Tetzlaff J, Altman DG, Grp P. Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. J Clin Epidemiol. 2009;62:1006-12.
[265] Ruggiero SL, Mehrotra B. Bisphosphonate-related osteonecrosis of the jaw: diagnosis, prevention, and management. Annual review of medicine. 2009;60:85-96.
[266] Ruggiero SL. Bisphosphonate-related osteonecrosis of the jaw: an overview. Annals of the New York Academy of Sciences. 2011;1218:38-46.
[267] Rugani P, Acham S, Truschnegg A, Obermayer-Pietsch B, Jakse N. Bisphosphonate-associated osteonecrosis of the jaws: surgical treatment with ErCrYSGG-laser. Case report. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontology. 2010;110:E1-E6.
[268] Romeo U, Galanakis A, Marias C, Vecchio AD, Tenore G, Palaia G, et al. Observation of pain control in patients with bisphosphonate-induced osteonecrosis using low level laser therapy: preliminary results. Photomed Laser Surg. 2011;29:447-52.
[269] Mortensen M, Lawson W, Montazem A. Osteonecrosis of the jaw associated with bisphosphonate use: Presentation of seven cases and literature review. Laryngoscope. 2007;117:30-4.
[270] Dannemann C, Zwahlen R, Gratz KW. Clinical experiences with bisphopsphonate induced osteochemonecrosis of the jaws. Swiss medical weekly. 2006;136:504-9.
[271] Vescovi P, Merigo E, Meleti M, Manfredi M, Guidotti R, Nammour S. Bisphosphonates-related osteonecrosis of the jaws: a concise review of the literature and a report of a single-centre experience with 151 patients. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2012;41:214-21.
[272] Curi MM, Cossolin GSI, Koga DH, Zardetto C, Christianini S, Feher O, et al. Bisphosphonate-Related Osteonecrosis of the Jaws–An Initial Case Series Report of Treatment Combining Partial Bone Resection and Autologous Platelet-Rich Plasma. Journal of Oral and Maxillofacial Surgery. 2011;69:2465-72.
[273] Junquera L, Gallego L, Cuesta P, Pelaz A, de Vicente JC. Clinical experiences with bisphosphonate-associated osteonecrosis of the jaws: analysis of 21 cases. American journal of otolaryngology. 2009;30:390-5.

[274] Vescovi P, Merigo E, Meleti M, Fornaini C, Nammour S, Manfredi M. Nd:YAG laser biostimulation of bisphosphonate-associated necrosis of the jawbone with and without surgical treatment. British Journal of Oral and Maxillofacial Surgery. 2007;45:628-32.
[275] Agrillo A, Ungari C, Filiaci F, Priore P, Iannetti G. Ozone therapy in the treatment of avascular bisphosphonate-related jaw osteonecrosis. J Craniofac Surg. 2007;18:1071-5.
[276] Longobardi G, Boniello R, Gasparini G, Pagano I, Pelo S. Surgical therapy for osteonecotic lesions of the jaws in patients in therapy with bisphosphonates. J Craniofac Surg. 2007;18:1012-7.
[277] Chiu CT, Chiang WF, Chuang CY, Chang SW. Resolution of oral bisphosphonate and steroid-related osteonecrosis of the jaw--a serial case analysis. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2010;68:1055-63.
[278] Wutzl A, Eisenmenger G, Hoffmann M, Czerny C, Moser D, Pietschmann P, et al. Osteonecrosis of the jaws and bisphosphonate treatment in cancer patients. Wiener klinische Wochenschrift. 2006;118:473-8.
[279] Park W, Kim NK, Kim MY, Rhee YM, Kim HJ. Osteonecrosis of the jaw induced by oral administration of bisphosphonates in Asian population: five cases. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2010;21:527-33.
[280] Petra R, Astrid T, Stephan A, Barbara K, Norbert J. Use of Photodynamic Therapy in Treatment of Bisphosphonate-related Osteonecrosis of the Jaws: Literature Review and Case Series. 2013;- 0:-.
[281] Kwon YD, Lee DW, Choi BJ, Lee JW, Kim DY. Short-term teriparatide therapy as an adjunctive modality for bisphosphonate-related osteonecrosis of the jaws. Osteoporosis International. 2012;23:2721-5.
[282] Cicciu M, Herford AS, Juodzbalys G, Stoffella E. Recombinant human bone morphogenetic protein type 2 application for a possible treatment of bisphosphonates-related osteonecrosis of the jaw. J Craniofac Surg. 2012;23:784-8.
[283] Blus C, Szmukler-Moncler S, Giannelli G, Denotti G, Orru G. Use of Ultrasonic Bone Surgery (Piezosurgery) to Surgically Treat Bisphosphonate-Related Osteonecrosis of the Jaws (BRONJ). A Case Series Report with at Least 1 Year of Follow-Up. The open dentistry journal. 2013;7:94-101.
[284] Lemound J, Eckardt A, Kokemuller H, von See C, Voss PJ, Tavassol F, et al. Bisphosphonate-associated osteonecrosis of the mandible: reliable soft tissue reconstruction using a local myofascial flap. Clin Oral Invest. 2012;16:1143-52.
[285] Lazarovici TS, Yahalom R, Taicher S, Schwartz-Arad D, Peleg O, Yarom N. Bisphosphonate-related osteonecrosis of the jaw associated with dental implants. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2010;68:790-6.
[286] Narvaez J, Narvaez JA, Gomez-Vaquero C, Nolla JM. Lack of response to teriparatide therapy for bisphosphonate-associated osteonecrosis of the jaw. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2013;24:731-3.
[287] Nomura T, Shibahara T, Uchiyama T, Yamamoto N, Shibui T, Yakushiji T, et al. Bisphosphonate-related osteonecrosis of jaw (BRONJ) in Japanese population: a case series of 13 patients at our clinic. The Bulletin of Tokyo Dental College. 2013;54:117-25.
[288] Jabbour Z, El-Hakim M, Mesbah-Ardakani P, Henderson JE, Albuquerque R, Jr. The outcomes of conservative and surgical treatment of stage 2 bisphosphonate-related osteonecrosis of the jaws: a case series. Int J Oral Maxillofac Surg. 2012;41:1404-9.

[289] Vescovi P, Merigo E, Manfredi M, Meleti M, Fornaini C, Bonanini M, et al. Nd : YAG laser biostimulationin the treatment of bisphosphonate-associated osteonecrosis of the jaw: Clinical experience in 28 cases. Photomedicine and Laser Surgery. 2008;26:37-46.
[290] Hanasono MM, Militsakh ON, Richmon JD, Rosenthal EL, Wax MK. Mandibulectomy and free flap reconstruction for bisphosphonate-related osteonecrosis of the jaws. JAMA otolaryngology-- head & neck surgery. 2013;139:1135-42.
[291] Coviello V, Peluso F, Dehkhargani SZ, Verdugo F, Raffaelli L, Manicone PF, et al. Platelet-rich plasma improves wound healing in multiple myeloma bisphosphonate-associated osteonecrosis of the jaw patients. Journal of biological regulators and homeostatic agents. 2012;26:151-5.
[292] Ferlito S, Puzzo S, Palermo F, Verzi P. Treatment of bisphosphonate-related osteonecrosis of the jaws: presentation of a protocol and an observational longitudinal study of an Italian series of cases. Br J Oral Maxillofac Surg. 2012;50:425-9.
[293] Anavi-Lev K, Anavi Y, Chaushu G, Alon DM, Gal G, Kaplan I. Bisphosphonate related osteonecrosis of the jaws: clinico-pathological investigation and histomorphometric analysis. Oral surgery, oral medicine, oral pathology and oral radiology. 2013;115:660-6.
[294] Ripamonti CI, Cislaghi E, Mariani L, Maniezzo M. Efficacy and safety of medical ozone (O(3)) delivered in oil suspension applications for the treatment of osteonecrosis of the jaw in patients with bone metastases treated with bisphosphonates: Preliminary results of a phase I-II study. Oral Oncol. 2011;47:185-90.
[295] Freiberger JJ, Padilla-Burgos R, McGraw T, Suliman HB, Kraft KH, Stolp BW, et al. What is the role of hyperbaric oxygen in the management of bisphosphonate-related osteonecrosis of the jaw: a randomized controlled trial of hyperbaric oxygen as an adjunct to surgery and antibiotics. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2012;70:1573-83.
[296] Ripamonti CI, Maniezzo M, Boldini S, Pessi MA, Mariani L, Cislaghi E. Efficacy and tolerability of medical ozone gas insufflations in patients with osteonecrosis of the jaw treated with bisphosphonates—Preliminary data. Journal of Bone Oncology.1:81-7.
[297] Holzinger D, Seemann R, Klug C, Ewers R, Millesi G, Baumann A, et al. Long-term success of surgery in bisphosphonate-related osteonecrosis of the jaws (BRONJs). Oral Oncol. 2013;49:66-70.
[298] Beninati F, Pruneti R, Ficarra G. Bisphosphonate-related osteonecrosis of the jaws (Bronj). Medicina oral, patologia oral y cirugia bucal. 2013;18:e752-8.
[299] Fortuna G, Ruoppo E, Pollio A, Aria M, Adamo D, Leuci S, et al. Multiple myeloma vs. breast cancer patients with bisphosphonates-related osteonecrosis of the jaws: a comparative analysis of response to treatment and predictors of outcome. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology. 2012;41:222-8.
[300] Williamson RA. Surgical management of bisphosphonate induced osteonecrosis of the jaws. Int J Oral Maxillofac Surg. 2010;39:251-5.
[301] Moretti F, Pelliccioni GA, Montebugnoli L, Marchetti C. A prospective clinical trial for assessing the efficacy of a minimally invasive protocol in patients with bisphosphonate-associated osteonecrosis of the jaws. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2011;112:777-82.
[302] Alsehimy MM. Efficacy of a nonsurgical treatment regimen in patients with bisphosphonate-related osteonecrosis of the jaws in Saudi Arabia. SAGE Open Medicine. 2014;2.
[303] Wutzl A, Pohl S, Sulzbacher I, Seemann R, Lauer G, Ewers R, et al. Factors influencing surgical treatment of bisphosphonate-related osteonecrosis of the jaws. Head & neck. 2012;34:194-200.

[304] Scoletta M, Arduino PG, Reggio L, Dalmasso P, Mozzati M. Effect of low-level laser irradiation on bisphosphonate-induced osteonecrosis of the jaws: preliminary results of a prospective study. Photomed Laser Surg. 2010;28:179-84.
[305] Bocanegra-Perez S, Vicente-Barrero M, Knezevic M, Castellano-Navarro JM, Rodriguez-Bocanegra E, Rodriguez-Millares J, et al. Use of platelet-rich plasma in the treatment of bisphosphonate-related osteonecrosis of the jaw. Int J Oral Maxillofac Surg. 2012;41:1410-5.
[306] Scoletta M, Arduino PG, Dalmasso P, Broccoletti R, Mozzati M. Treatment outcomes in patients with bisphosphonate-related osteonecrosis of the jaws: a prospective study. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2010;110:46-53.
[307] Schubert M, Klatte I, Linek W, Muller B, Doring K, Eckelt U, et al. The saxon bisphosphonate register - therapy and prevention of bisphosphonate-related osteonecrosis of the jaws. Oral Oncol. 2012;48:349-54.
[308] Thumbigere-Math V, Tu L, Huckabay S, Dudek AZ, Lunos S, Basi DL, et al. A retrospective study evaluating frequency and risk factors of osteonecrosis of the jaw in 576 cancer patients receiving intravenous bisphosphonates. American journal of clinical oncology. 2012;35:386-92.
[309] Martins MA, Martins MD, Lascala CA, Curi MM, Migliorati CA, Tenis CA, et al. Association of laser phototherapy with PRP improves healing of bisphosphonate-related osteonecrosis of the jaws in cancer patients: a preliminary study. Oral Oncol. 2012;48:79-84.
[310] Angiero F, Sannino C, Borloni R, Crippa R, Benedicenti S, Romanos GE. Osteonecrosis of the jaws caused by bisphosphonates: evaluation of a new therapeutic approach using the Er:YAG laser. Lasers Med Sci. 2009;24:849-56.
[311] Vescovi P, Merigo E, Meleti M, Manfredi M, Fornaini C, Nammour S, et al. Conservative surgical management of stage I bisphosphonate-related osteonecrosis of the jaw. Int J Dent. 2014;2014:107690.
[312] Hoefert S, Eufinger H. Relevance of a prolonged preoperative antibiotic regime in the treatment of bisphosphonate-related osteonecrosis of the jaw. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2011;69:362-80.
[313] Vescovi P, Campisi G, Fusco V, Mergoni G, Manfredi M, Merigo E, et al. Surgery-triggered and non surgery-triggered Bisphosphonate-related Osteonecrosis of the Jaws (BRONJ): A retrospective analysis of 567 cases in an Italian multicenter study. Oral Oncology. 2011;47:191-4.
[314] Seth R, Futran ND, Alam DS, Knott PD. Outcomes of vascularized bone graft reconstruction of the mandible in bisphosphonate-related osteonecrosis of the jaws. Laryngoscope. 2010;120:2165-71.
[315] Saussez S, Javadian R, Hupin C, Magremanne M, Chantrain G, Loeb I, et al. Bisphosphonate-related osteonecrosis of the jaw and its associated risk factors: a Belgian case series. Laryngoscope. 2009;119:323-9.
[316] Alons K, Kuijpers SC, de Jong E, van Merkesteyn JP. Treating low- and medium-potency bisphosphonate-related osteonecrosis of the jaws with a protocol for the treatment of chronic suppurative osteomyelitis: report of 7 cases. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2009;107:e1-7.
[317] Yarom N, Yahalom R, Shoshani Y, Hamed W, Regev E, Elad S. Osteonecrosis of the jaw induced by orally administered bisphosphonates: incidence, clinical features, predisposing factors and treatment outcome. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2007;18:1363-70.
[318] Stanton DC, Balasanian E. Outcome of surgical management of bisphosphonate-related osteonecrosis of the jaws: review of 33 surgical cases. Journal of oral and maxillofacial

surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2009;67:943-50.
[319] Estilo CL, Van Poznak CH, Wiliams T, Bohle GC, Lwin PT, Zhou Q, et al. Osteonecrosis of the maxilla and mandible in patients with advanced cancer treated with bisphosphonate therapy. Oncologist. 2008;13:911-20.
[320] Dimitrakopoulos I, Magopoulos C, Karakasis D. Bisphosphonate-induced avascular osteonecrosis of the jaws: a clinical report of 11 cases. Int J Oral Maxillofac Surg. 2006;35:588-93.
[321] Mercer E, Norton T, Woo S, Treister N, Dodson TB, Solomon DH. Ninety-one osteoporosis patients affected with bisphosphonate-related osteonecrosis of the jaw: a case series. Calcified tissue international. 2013;93:241-8.
[322] Abu-Id MH, Warnke PH, Gottschalk J, Springer I, Wiltfang J, Acil Y, et al. "Bis-phossy jaws" - high and low risk factors for bisphosphonate-induced osteonecrosis of the jaw. Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery. 2008;36:95-103.
[323] Pozzi S, Marcheselli R, Sacchi S, Baldini L, Angrilli F, Pennese E, et al. Bisphosphonate-associated osteonecrosis of the jaw: a review of 35 cases and an evaluation of its frequency in multiple myeloma patients. Leukemia & lymphoma. 2007;48:56-64.
[324] Kos M, Brusco D, Kuebler J, Engelke W. Clinical comparison of patients with osteonecrosis of the jaws, with and without a history of bisphosphonates administration. Int J Oral Maxillofac Surg. 2010;39:1097-102.
[325] Manfredi M, Merigo E, Guidotti R, Meleti M, Vescovi P. Bisphosphonate-related osteonecrosis of the jaws: a case series of 25 patients affected by osteoporosis. International Journal of Oral and Maxillofacial Surgery. 2011;40:277-84.
[326] Maurer P, Sandulescu T, Kriwalsky MS, Rashad A, Hollstein S, Stricker I, et al. Bisphosphonate-related osteonecrosis of the maxilla and sinusitis maxillaris. Int J Oral Maxillofac Surg. 2011;40:285-91.
[327] Hong JW, Nam W, Cha IH, Chung SW, Choi HS, Kim KM, et al. Oral bisphosphonate-related osteonecrosis of the jaw: the first report in Asia. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2010;21:847-53.
[328] Mozzati M, Gallesio G, Arata V, Pol R, Scoletta M. Platelet-rich therapies in the treatment of intravenous bisphosphonate-related osteonecrosis of the jaw: A report of 32 cases. Oral Oncology. 2012;48:469-74.
[329] Urade M, Tanaka N, Furusawa K, Shimada J, Shibata T, Kirita T, et al. Nationwide survey for bisphosphonate-related osteonecrosis of the jaws in Japan. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2011;69:e364-71.
[330] Badros A, Weikel D, Salama A, Goloubeva O, Schneider A, Rapoport A, et al. Osteonecrosis of the jaw in multiple myeloma patients: clinical features and risk factors. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2006;24:945-52.
[331] Atalay B, Yalcin S, Emes Y, Aktas I, Aybar B, Issever H, et al. Bisphosphonate-related osteonecrosis: laser-assisted surgical treatment or conventional surgery? Lasers Med Sci. 2011;26:815-23.
[332] Wilde F, Heufelder M, Winter K, Hendricks J, Frerich B, Schramm A, et al. The role of surgical therapy in the management of intravenous bisphosphonates-related osteonecrosis of the jaw. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2011;111:153-63.

[333] Vescovi P, Manfredi M, Merigo E, Meleti M, Fornaini C, Rocca JP, et al. Surgical approach with Er:YAG laser on osteonecrosis of the jaws (ONJ) in patients under bisphosphonate therapy (BPT). Lasers Med Sci. 2010;25:101-13.
[334] Kim KM, Park W, Oh SY, Kim HJ, Nam W, Lim SK, et al. Distinctive role of 6-month teriparatide treatment on intractable bisphosphonate-related osteonecrosis of the jaw. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2014;25:1625-32.
[335] Kim S-K, Kwon T-G. Clinical investigation of bisphosphonate-related osteonecrosis of the jaws in patients with malignant tumors. J Korean Assoc Oral Maxillofac Surg. 2012;38:152-9.
[336] Stubinger S, Dissmann JP, Pinho NC, Saldamli B, Seitz O, Sader R. A preliminary report about treatment of bisphosphonate related osteonecrosis of the jaw with Er:YAG laser ablation. Lasers in surgery and medicine. 2009;41:26-30.
[337] Elad S, Yarom N, Hamed W, Ayalon S, Yahalom R, Regev E. Osteomylelitis and necrosis of the jaw in patients treated with bisphosphonates: a comparative study focused on multiple myeloma. Clinical and laboratory haematology. 2006;28:393-8.
[338] Van den Wyngaert T, Claeys T, Huizing MT, Vermorken JB, Fossion E. Initial experience with conservative treatment in cancer patients with osteonecrosis of the jaw (ONJ) and predictors of outcome. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2009;20:331-6.
[339] Hansen PJ, Knitschke M, Draenert FG, Irle S, Neff A. Incidence of bisphosphonate-related osteonecrosis of the jaws (BRONJ) in patients taking bisphosphonates for osteoporosis treatment - a grossly underestimated risk? Clin Oral Invest. 2013;17:1829-37.
[340] Lerman MA, Xie W, Treister NS, Richardson PG, Weller EA, Woo SB. Conservative management of bisphosphonate-related osteonecrosis of the jaws: staging and treatment outcomes. Oral Oncol. 2013;49:977-83.
[341] Nicolatou-Galitis O, Papadopoulou E, Sarri T, Boziari P, Karayianni A, Kyrtsonis MC, et al. Osteonecrosis of the jaw in oncology patients treated with bisphosphonates: prospective experience of a dental oncology referral center. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2011;112:195-202.
[342] Vescovi P, Campisi G, Fusco V, Mergoni G, Manfredi M, Merigo E, et al. Surgery-triggered and non surgery-triggered Bisphosphonate-related Osteonecrosis of the Jaws (BRONJ): A retrospective analysis of 567 cases in an Italian multicenter study. Oral Oncol. 2011;47:191-4.
[343] Rugani P, Truschnegg A, Acham S, Kirnbauer B, N J. Use of Photodynamic Therapy in Treatment of Bisphosphonate-related Osteonecrosis of the Jaws: Literature Review and Case Series. 2013;- 0:-.
[344] Woo SB, Hellstein JW, Kalmar JR. Narrative [corrected] review: bisphosphonates and osteonecrosis of the jaws. Annals of internal medicine. 2006;144:753-61.
[345] Mavrokokki T, Cheng A, Stein B, Goss A. Nature and frequency of bisphosphonate-associated osteonecrosis of the jaws in Australia. Journal of Oral and Maxillofacial Surgery. 2007;65:415-23.
[346] Dannemann C, Gratz KW, Riener MO, Zwahlen RA. Jaw osteonecrosis related to bisphosphonate therapy: a severe secondary disorder. Bone. 2007;40:828-34.
[347] Olutayo J, Agbaje JO, Jacobs R, Verhaeghe V, Velde FV, Vinckier F. Bisphosphonate-Related Osteonecrosis of the Jaw Bone: Radiological Pattern and the Potential Role of CBCT in Early Diagnosis. J Oral Maxillofac Res. 2010;1:e3.
[348] Dunstan CR, Felsenberg D, Seibel MJ. Therapy insight: the risks and benefits of bisphosphonates for the treatment of tumor-induced bone disease. Nature clinical practice Oncology. 2007;4:42-55.

[349] Dimopoulos MA, Kastritis E, Anagnostopoulos A, Melakopoulos I, Gika D, Moulopoulos LA, et al. Osteonecrosis of the jaw in patients with multiple myeloma treated with bisphosphonates: evidence of increased risk after treatment with zoledronic acid. Haematologica. 2006;91:968-71.
[350] Dimopoulos MA, Kastritis E, Moulopoulos LA, Melakopoulos I, Anagnostopoulos A, Gika D, et al. The incidence of osteonecrosis of the jaw (ONJ) in patients with multiple myeloma who receive biphosphonates depends on the type of biphosphonate. Blood. 2005;106:189a-a.
[351] Fleisch H. Bisphosphonates: mechanisms of action. Endocrine reviews. 1998;19:80-100.
[352] Lo JC, O'Ryan FS, Gordon NP, Yang J, Hui RL, Martin D, et al. Prevalence of osteonecrosis of the jaw in patients with oral bisphosphonate exposure. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2010;68:243-53.
[353] Osteonecrosis of the Jaw and Bisphosphonates. New England Journal of Medicine. 2005;353:99-102.
[354] Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws--2009 update. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2009;67:2-12.
[355] Cafro AM, Barbarano LA, Andriani A, D'Avanzo G, Nichelatti M, Gaglioti D, et al. Osteonecrosis of the Jaw Associated with Chronic Bisphosphonates Therapy: An Italian Experience. ASH Annual Meeting Abstracts. 2005;106:5152-.
[356] King AE, Umland EM. Osteonecrosis of the jaw in patients receiving intravenous or oral bisphosphonates. Pharmacotherapy. 2008;28:667-77.
[357] Cavanna L, Berte R, Arcari A, Mordenti P, Pagani R, Vallisa D. Osteonecrosis of the jaw. A newly emerging site-specific osseous pathology in patients with cancer treated with bisphosphonates. Report of five cases and review of the literature. European Journal of Internal Medicine. 2007;18:417-22.
[358] Badros A, Terpos E, Katodritou E, Goloubeva O, Kastritis E, Verrou E, et al. Natural history of osteonecrosis of the jaw in patients with multiple myeloma. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008;26:5904-9.
[359] Filleul O, Crompot E, Saussez S. Bisphosphonate-induced osteonecrosis of the jaw: a review of 2,400 patient cases. Journal of cancer research and clinical oncology. 2010;136:1117-24.
[360] Lugassy G, Shaham R, Nemets A, Ben-Dor D, Nahlieli O. Severe osteomyelitis of the jaw in long-term survivors of multiple myeloma: A new clinical entity. Am J Med.117:440-1.
[361] Pires FR, Miranda A, Cardoso ES, Cardoso AS, Fregnani ER, Pereira CM, et al. Oral avascular bone necrosis associated with chemotherapy and biphosphonate therapy. Oral Dis. 2005;11:365-9.
[362] Saad F, Brown JE, Van Poznak C, Ibrahim T, Stemmer SM, Stopeck AT, et al. Incidence, risk factors, and outcomes of osteonecrosis of the jaw: integrated analysis from three blinded active-controlled phase III trials in cancer patients with bone metastases. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2012;23:1341-7.
[363] Migliorati CA, Epstein JB, Abt E, Berenson JR. Osteonecrosis of the jaw and bisphosphonates in cancer: a narrative review. Nature reviews Endocrinology. 2011;7:34-42.
[364] Hoff AO, Toth B, Hu M, Hortobagyi GN, Gagel RF. Epidemiology and risk factors for osteonecrosis of the jaw in cancer patients. Annals of the New York Academy of Sciences. 2011;1218:47-54.

[365] Ziebart T, Pabst A, Klein MO, Kammerer P, Gauss L, Brullmann D, et al. Bisphosphonates: restrictions for vasculogenesis and angiogenesis: inhibition of cell function of endothelial progenitor cells and mature endothelial cells in vitro. Clin Oral Invest. 2011;15:105-11.
[366] Baqain ZH, Sawair FA, Tamimi Z, Bsoul N, Al Edwan G, Almasad JK, et al. Osteonecrosis of jaws related to intravenous bisphosphonates: the experience of a Jordanian teaching hospital. Annals of the Royal College of Surgeons of England. 2010;92:489-94.
[367] Hoff AO, Toth BB, Altundag K, Guarneri V, Adamus A, Nooka AK, et al. Osteonecrosis of the jaw in patients receiving intravenous bisphosphonate therapy. Journal of Clinical Oncology. 2006;24:475s-s.
[368] Schiffrin EJ, Morley JE, Donnet-Hughes A, Guigoz Y. The inflammatory status of the elderly: the intestinal contribution. Mutation research. 2010;690:50-6.
[369] Johnson TE. Recent results: biomarkers of aging. Experimental gerontology. 2006;41:1243-6.
[370] Misawa Y, Kageyama T, Moriyama K, Kurihara S, Yagasaki H, Deguchi T, et al. Effect of age on alveolar bone turnover adjacent to maxillary molar roots in male rats: A histomorphometric study. Archives of oral biology. 2007;52:44-50.
[371] Semba I, Funakoshi K, Kitano M. Histomorphometric analysis of age changes in the human inferior alveolar artery. Archives of oral biology. 2001;46:13-21.
[372] Kulikov VY, Fridman YM, Fomin AN. Role of oxidative stress in mechanisms of premature aging in shift labor workers. Alaska medicine. 2007;49:81-4.
[373] Maines E, Monti E, Doro F, Morandi G, Cavarzere P, Antoniazzi F. Children and adolescents treated with neridronate for osteogenesis imperfecta show no evidence of any osteonecrosis of the jaw. Journal of Bone and Mineral Metabolism. 2012;30:434-8.
[374] Dental management of patients receiving oral bisphosphonate therapy - Expert panel recommendations. Journal of the American Dental Association. 2006;137:1144-50.
[375] Malden NJ, Pai AY. Oral bisphosphonate associated osteonecrosis of the jaws: three case reports. Br Dent J. 2007;203:93-7.
[376] Khamaisi M, Regev E, Yarom N, Avni B, Leitersdorf E, Raz I, et al. Possible association between diabetes and bisphosphonate-related jaw osteonecrosis. The Journal of clinical endocrinology and metabolism. 2007;92:1172-5.
[377] Mehanna P, Goddard R. Bisphosphonate associated osteonecrosis: an unusual case. Aust Dent J. 2010;55:311-3.
[378] Favia G, Pilolli GP, Maiorano E. Osteonecrosis of the Jaw Correlated to Bisphosphonate Therapy in Non-oncologic Patients: Clinicopathological Features of 24 Patients. Journal of Rheumatology. 2009;36:2780-7.
[379] Sedghizadeh PP, Stanley K, Caligiuri M, Hofkes S, Lowry B, Shuler CF. Oral bisphosphonate use and the prevalence of osteonecrosis of the jaw An institutional inquiry. Journal of the American Dental Association. 2009;140:61-6.
[380] Shin EY, Kwon YH, Herr Y, Shin SI, Chung JH. Implant failure associated with oral bisphosphonate-related osteonecrosis of the jaw. Journal of periodontal & implant science. 2010;40:90-5.
[381] Markenson JA. Worldwide Trends in the Socioeconomic Impact and Long-Term Prognosis of Rheumatoid-Arthritis. Semin Arthritis Rheu. 1991;21:4-12.
[382] Bedogni A, Blandamura S, Lokmic Z, Palumbo C, Ragazzo M, Ferrari F, et al. Bisphosphonate-associated jawbone osteonecrosis: a correlation between imaging techniques and histopathology. Oral Surgery Oral Medicine Oral Pathology Oral Radiology and Endodontology. 2008;105:358-64.
[383] Green JR. Bisphosphonates: preclinical review. Oncologist. 2004;9 Suppl 4:3-13.

[384] Milner RJ, Farese J, Henry CJ, Selting K, Fan TM, de Lorimier LP. Bisphosphonates and cancer. Journal of veterinary internal medicine / American College of Veterinary Internal Medicine. 2004;18:597-604.
[385] Vincenzi B, Santini D, Dicuonzo G, Battistoni F, Gavasci M, La Cesa A, et al. Zoledronic acid-related angiogenesis modifications and survival in advanced breast cancer patients. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research. 2005;25:144-51.
[386] Wood J, Bonjean K, Ruetz S, Bellahcene A, Devy L, Foidart JM, et al. Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. The Journal of pharmacology and experimental therapeutics. 2002;302:1055-61.
[387] Marx RE. Osteoradionecrosis: a new concept of its pathophysiology. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 1983;41:283-8.
[388] Chuah C, Barnes DJ, Kwok M, Corbin A, Deininger MW, Druker BJ, et al. Zoledronate inhibits proliferation and induces apoptosis of imatinib-resistant chronic myeloid leukaemia cells. Leukemia. 2005;19:1896-904.
[389] Gronqvist A, Wistrom J, Axner O, Monsen TJ. Bactericidal effect of pulsed 1,064 nm Nd:YAG laser light on Staphylococcus epidermidis is of photothermal origin: an in vitro study. Lasers in surgery and medicine. 2000;27:336-40.
[390] Mitsimponas K, Sereti M, Semergidis T. O.130 Bisphosphonate associated osteonecrosis: role of surgery. Journal of Craniomaxillofacial Surgery.36:S33.
[391] Eckert AW, Maurer P, Meyer L, Kriwalsky MS, Rohrberg R, Schneider D, et al. Bisphosphonate-related jaw necrosis--severe complication in maxillofacial surgery. Cancer treatment reviews. 2007;33:58-63.
[392] Millesi G, Wutzl A, Biedermann E, Karschigijew G, Ewers R. Bisphosphonate induced osteonecrosis of the JAWS (BIONJ): six months follow up. International journal of oral and maxillofacial surgery. 2007;36:1043.
[393] Otto S, Hafner S, Groetz KA. The Role of Inferior Alveolar Nerve Involvement in Bisphosphonate-Related Osteonecrosis of the Jaw. Journal of Oral and Maxillofacial Surgery. 2009;67:589-92.
[394] Gomez Font R, Martinez Garcia ML, Olmos Martinez JM. Osteochemonecrosis of the jaws due to bisphosphonate treatments. Update. Medicina oral, patologia oral y cirugia bucal. 2008;13:E318-24.
[395] Nase JB, Suzuki JB. Osteonecrosis of the jaw and oral bisphosphonate treatment. Journal of the American Dental Association (1939). 2006;137:1115-9; quiz 69-70.
[396] Lee CY, David T, Nishime M. Use of platelet-rich plasma in the management of oral biphosphonate-associated osteonecrosis of the jaw: a report of 2 cases. The Journal of oral implantology. 2007;33:371-82.
[397] Arora NS, Ramanayake T, Ren YF, Romanos GE. Platelet-rich plasma: a literature review. Implant dentistry. 2009;18:303-10.
[398] Lopez-Vidriero E, Goulding KA, Simon DA, Sanchez M, Johnson DH. The use of platelet-rich plasma in arthroscopy and sports medicine: optimizing the healing environment. Arthroscopy : the journal of arthroscopic & related surgery : official publication of the Arthroscopy Association of North America and the International Arthroscopy Association. 2010;26:269-78.
[399] Plachokova AS, Nikolidakis D, Mulder J, Jansen JA, Creugers NH. Effect of platelet-rich plasma on bone regeneration in dentistry: a systematic review. Clinical oral implants research. 2008;19:539-45.
[400] Bocci V. Does ozone therapy normalize the cellular redox balance? Implications for therapy of human immunodeficiency virus infection and several other diseases. Med Hypotheses. 1996;46:150-4.

[401] Hernandez F, Menendez S, Wong R. Decrease of blood cholesterol and stimulation of antioxidative response in cardiopathy patients treated with endovenous ozone therapy. Free radical biology & medicine. 1995;19:115-9.
[402] Csonka C, Pataki T, Kovacs P, Muller SL, Schroeter ML, Tosaki A, et al. Effects of oxidative stress on the expression of antioxidative defense enzymes in spontaneously hypertensive rat hearts. Free radical biology & medicine. 2000;29:612-9.
[403] Bocci V. Ozone as Janus: this controversial gas can be either toxic or medically useful. Mediators of inflammation. 2004;13:3-11.
[404] Reth M. Hydrogen peroxide as second messenger in lymphocyte activation. Nature immunology. 2002;3:1129-34.
[405] Babior BM, Takeuchi C, Ruedi J, Gutierrez A, Wentworth P, Jr. Investigating antibody-catalyzed ozone generation by human neutrophils. Proceedings of the National Academy of Sciences of the United States of America. 2003;100:3031-4.
[406] Freiberger JJ. Utility of hyperbaric oxygen in treatment of bisphosphonate-related osteonecrosis of the jaws. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2009;67:96-106.
[407] Boykin JV, Jr., Baylis C. Hyperbaric oxygen therapy mediates increased nitric oxide production associated with wound healing: a preliminary study. Advances in skin & wound care. 2007;20:382-8.
[408] Rubin MR, Bilezikian JP. The anabolic effects of parathyroid hormone therapy. Clinics in geriatric medicine. 2003;19:415-32.
[409] Quattrocchi E, Kourlas H. Teriparatide: a review. Clinical therapeutics. 2004;26:841-54.
[410] Pleiner-Duxneuner J, Zwettler E, Paschalis E, Roschger P, Nell-Duxneuner V, Klaushofer K. Treatment of Osteoporosis with Parathyroid Hormone and Teriparatide. Calcified tissue international. 2009;84:159-70.
[411] McClung MR, San Martin J, Miller PD, Civitelli R, Bandeira F, Omizo M, et al. Opposite bone remodeling effects of teriparatide and alendronate in increasing bone mass. Archives of internal medicine. 2005;165:1762-8.
[412] Chen P, Satterwhite JH, Licata AA, Lewiecki EM, Sipos AA, Misurski DM, et al. Early changes in biochemical markers of bone formation predict BMD response to teriparatide in postmenopausal women with osteoporosis. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2005;20:962-70.
[413] Lindsay R, Cosman F, Zhou H, Bostrom MP, Shen VW, Cruz JD, et al. A novel tetracycline labeling schedule for longitudinal evaluation of the short-term effects of anabolic therapy with a single iliac crest bone biopsy: early actions of teriparatide. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2006;21:366-73.
[414] Ettinger B, San Martin J, Crans G, Pavo I. Differential effects of teriparatide on BMD after treatment with raloxifene or alendronate. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2004;19:745-51.
[415] Ma YL, Bryant HU, Zeng Q, Schmidt A, Hoover J, Cole HW, et al. New bone formation with teriparatide [human parathyroid hormone-(1-34)] is not retarded by long-term pretreatment with alendronate, estrogen, or raloxifene in ovariectomized rats. Endocrinology. 2003;144:2008-15.
[416] Harper RP, Fung E. Resolution of Bisphosphonate-Associated Osteonecrosis of the Mandible: Possible Application for Intermittent Low-Dose Parathyroid Hormone [rhPTH(1-34)]. Journal of Oral and Maxillofacial Surgery. 2007;65:573-80.
[417] Ohbayashi Y, Miyake M, Sawai F, Minami Y, Iwasaki A, Matsui Y. Adjunct teriparatide therapy with monitoring of bone turnover markers and bone scintigraphy for

bisphosphonate-related osteonecrosis of the jaw. Oral surgery, oral medicine, oral pathology and oral radiology. 2013;115:e31-7.
[418] Rogers MJ. From Molds and Macrophages to Mevalonate: A Decade of Progress in Understanding the Molecular Mode of Action of Bisphosphonates. Calcified tissue international. 2004;75:451-61.
[419] Ruggiero SL, Drew SJ. Osteonecrosis of the jaws and bisphosphonate therapy. Journal of dental research. 2007;86:1013-21.
[420] Bonadio J, Cunningham ML. Genetic Approaches to Craniofacial Tissue Repair. Annals of the New York Academy of Sciences. 2002;961:48-57.
[421] Dai J, Rabie AB, Hagg U, Xu R. Alternative gene therapy strategies for the repair of craniofacial bone defects. Current gene therapy. 2004;4:469-85.
[422] Kademani D, Mardini S, Moran SL. Reconstruction of head and neck defects: a systematic approach to treatment. Seminars in plastic surgery. 2008;22:141-55.
[423] Katari RS, Peloso A, Orlando G. Tissue engineering. Advances in surgery. 2014;48:137-54.
[424] Bonadio J. Tissue engineering via local gene delivery: update and future prospects for enhancing the technology. Advanced drug delivery reviews. 2000;44:185-94.
[425] Rios HF, Lin Z, Oh B, Park CH, Giannobile WV. Cell- and gene-based therapeutic strategies for periodontal regenerative medicine. Journal of periodontology. 2011;82:1223-37.
[426] Chatterjee A, Singh N, Saluja M. Gene therapy in periodontics. Journal of Indian Society of Periodontology. 2013;17:156-61.
[427] Edwards PC, Mason JM. Gene-enhanced tissue engineering for dental hard tissue regeneration: (2) dentin-pulp and periodontal regeneration. Head Face Med. 2006;2:16.
[428] Shilpashree HS, Sarapur S. Gene therapy in dentistry: a review. The New York state dental journal. 2013;79:60-4.
[429] Mahale S, Dani N, Ansari SS, Kale T. Gene therapy and its implications in Periodontics. Journal of Indian Society of Periodontology. 2009;13:1-5.
[430] Peters JL, Sutton AJ, Jones DR, Rushton L, Abrams KR. A systematic review of systematic reviews and meta-analyses of animal experiments with guidelines for reporting. Journal of environmental science and health Part B, Pesticides, food contaminants, and agricultural wastes. 2006;41:1245-58.
[431] Moher D, Liberati A, Tetzlaff J, Altman DG. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. International journal of surgery (London, England). 2010;8:336-41.
[432] Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS biology. 2010;8:e1000412.
[433] Schwarz F, Iglhaut G, Becker J. Quality assessment of reporting of animal studies on pathogenesis and treatment of peri-implant mucositis and peri-implantitis. A systematic review using the ARRIVE guidelines. Journal of clinical periodontology. 2012;39 Suppl 12:63-72.
[434] Sena E, van der Worp HB, Howells D, Macleod M. How can we improve the pre-clinical development of drugs for stroke? Trends in Neurosciences. 2007;30:433-9.
[435] Macleod MR, O'Collins T, Howells DW, Donnan GA. Pooling of animal experimental data reveals influence of study design and publication bias. Stroke; a journal of cerebral circulation. 2004;35:1203-8.
[436] Cohen J. Statistical Power Analysis for the Behavioral Sciences. . Hillsdale, NJ: L. Erlbaum Associates.; 1988.
[437] Higgins JP, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ (Clinical research ed). 2003;327:557-60.

[438] ZR A, Chang P-C, Cirelli JA, Jin Q, Sugai J, Giannobile WV. PDGF Gene Therapy to Promote Oral Implant Osseointegration European cells & materials. 2007;13:43.
[439] Alden TD, Beres EJ, Laurent JS, Engh JA, Das S, London SD, et al. The use of bone morphogenetic protein gene therapy in craniofacial bone repair. J Craniofac Surg. 2000;11:24-30.
[440] Ashinoff RL, Cetrulo CL, Jr., Galiano RD, Dobryansky M, Bhatt KA, Ceradini DJ, et al. Bone morphogenic protein-2 gene therapy for mandibular distraction osteogenesis. Annals of plastic surgery. 2004;52:585-90; discussion 91.
[441] Chang PC, Cirelli JA, Jin Q, Seol YJ, Sugai JV, D'Silva NJ, et al. Adenovirus encoding human platelet-derived growth factor-B delivered to alveolar bone defects exhibits safety and biodistribution profiles favorable for clinical use. Human gene therapy. 2009;20:486-96.
[442] Chang PC, Seol YJ, Cirelli JA, Pellegrini G, Jin Q, Franco LM, et al. PDGF-B gene therapy accelerates bone engineering and oral implant osseointegration. Gene Ther. 2010;17:95-104.
[443] Chen JC, Winn SR, Gong X, Ozaki WH. rhBMP-4 gene therapy in a juvenile canine alveolar defect model. Plastic and reconstructive surgery. 2007;120:1503-9.
[444] Cirelli JA, Park CH, MacKool K, Taba M, Jr., Lustig KH, Burstein H, et al. AAV2/1-TNFR:Fc gene delivery prevents periodontal disease progression. Gene Ther. 2008;16:426-36.
[445] Dunn CA, Jin Q, Taba M, Jr., Franceschi RT, Bruce Rutherford R, Giannobile WV. BMP gene delivery for alveolar bone engineering at dental implant defects. Molecular therapy : the journal of the American Society of Gene Therapy. 2005;11:294-9.
[446] Jin QM, Anusaksathien O, Webb SA, Rutherford RB, Giannobile WV. Gene therapy of bone morphogenetic protein for periodontal tissue engineering. Journal of periodontology. 2003;74:202-13.
[447] Jin Q, Anusaksathien O, Webb SA, Printz MA, Giannobile WV. Engineering of tooth-supporting structures by delivery of PDGF gene therapy vectors. Molecular therapy : the journal of the American Society of Gene Therapy. 2004;9:519-26.
[448] Yu H, Li Q, Herbert B, Zinna R, Martin K, Junior CR, et al. Anti-inflammatory effect of MAPK phosphatase-1 local gene transfer in inflammatory bone loss. Gene Ther. 2011;18:344-53.
[449] Cao Y, Liu Z, Xie Y, Hu J, Wang H, Fan Z, et al. Adenovirus-mediated transfer of hepatocyte growth factor gene to human dental pulp stem cells under good manufacturing practice improves their potential for periodontal regeneration in swine. Stem cell research & therapy. 2015;6:249.
[450] Dai J, Rabie AB. Direct AAV-mediated gene delivery to the temporomandibular joint. Frontiers in bioscience : a journal and virtual library. 2007;12:2212-20.
[451] Hu J, Qi MC, Zou SJ, Li JH, Luo E. Callus formation enhanced by BMP-7 ex vivo gene therapy during distraction osteogenesis in rats. J Orthop Res. 2007;25:241-51.
[452] Jiang X, Zhao J, Wang S, Sun X, Zhang X, Chen J, et al. Mandibular repair in rats with premineralized silk scaffolds and BMP-2-modified bMSCs. Biomaterials. 2009;30:4522-32.
[453] Jiang X, Zou S, Ye B, Zhu S, Liu Y, Hu J. bFGF-Modified BMMSCs enhance bone regeneration following distraction osteogenesis in rabbits. Bone. 2010;46:1156-61.
[454] Jiang XQ, Sun XJ, Lai HC, Zhao J, Wang SY, Zhang ZY. Maxillary sinus floor elevation using a tissue-engineered bone complex with beta-TCP and BMP-2 gene-modified bMSCs in rabbits. Clinical oral implants research. 2009;20:1333-40.
[455] Lai QG, Sun SL, Zhou XH, Zhang CP, Yuan KF, Yang ZJ, et al. Adipose-derived stem cells transfected with pEGFP-OSX enhance bone formation during distraction osteogenesis. Journal of Zhejiang University Science B. 2014;15:482-90.
[456] Lai QG, Yuan KF, Xu X, Li DR, Li GJ, Wei FL, et al. Transcription factor osterix modified bone marrow mesenchymal stem cells enhance callus formation during distraction

osteogenesis. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2011;111:412-9.
[457] Li J, Li Y, Ma S, Gao Y, Zuo Y, Hu J. Enhancement of bone formation by BMP-7 transduced MSCs on biomimetic nano-hydroxyapatite/polyamide composite scaffolds in repair of mandibular defects. Journal of biomedical materials research Part A. 2010;95:973-81.
[458] Li YF, Yan FH, Zhong Q, Zhao X. [Effect of hBMP-7 gene modified bone marrow stromal cells on periodontal tissue regeneration]. Zhonghua yi xue za zhi. 2010;90:1427-30.
[459] Long J, Li P, Du HM, Liu L, Zheng XH, Lin YF, et al. Effects of bone morphogenetic protein 2 gene therapy on new bone formation during mandibular distraction osteogenesis at rapid rate in rabbits. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2011;112:50-7.
[460] Rabie AB, Dai J, Xu R. Recombinant AAV-mediated VEGF gene therapy induces mandibular condylar growth. Gene Ther. 2007;14:972-80.
[461] Su F, Liu SS, Ma JL, Wang DS, E LL, Liu HC. Enhancement of periodontal tissue regeneration by transplantation of osteoprotegerin-engineered periodontal ligament stem cells. Stem cell research & therapy. 2015;6:22.
[462] Sun JJ, Zheng XH, Wang LY, Liu L, Jing W, Lin YF, et al. New bone formation enhanced by ADSCs overexpressing hRunx2 during mandibular distraction osteogenesis in osteoporotic rabbits. J Orthop Res. 2014;32:709-20.
[463] Sun M, Tan W, Wang K, Dong Z, Peng H, Wei F. Effects of Allogenous Periosteal-Derived Cells Transfected With Adenovirus-Mediated BMP-2 on Repairing Defects of the Mandible in Rabbits. Journal of Oral and Maxillofacial Surgery. 2013;71:1789-99.
[464] Sun QF, Zhu XM, Yang PS, Liu Y, Du F. [Gene therapy of bone morphogenetic protein-2 for periodontal tissue regeneration in vivo]. Shanghai kou qiang yi xue = Shanghai journal of stomatology. 2007;16:211-4.
[465] Tan Z, Zhao Q, Gong P, Wu Y, Wei N, Yuan Q, et al. Research on promoting periodontal regeneration with human basic fibroblast growth factor-modified bone marrow mesenchymal stromal cell gene therapy. Cytotherapy. 2009;11:317-25.
[466] Tang Y, Tang W, Lin Y, Long J, Wang H, Liu L, et al. Combination of bone tissue engineering and BMP-2 gene transfection promotes bone healing in osteoporotic rats. Cell Biol Int. 2008;32:1150-7.
[467] Tang YC, Tang W, Tian WD, Chen XZ, Li SW. [A study on repairing mandibular defect by means of tissue-engineering and human bone morphogenetic protein-2 gene transfection in osteoporotic rats]. Zhonghua kou qiang yi xue za zhi = Zhonghua kouqiang yixue zazhi = Chinese journal of stomatology. 2006;41:430-1.
[468] Wang L, Zhao Y, Cao J, Yang X, Lei D. Mesenchymal stem cells modified with nerve growth factor improve recovery of the inferior alveolar nerve after mandibular distraction osteogenesis in rabbits. Br J Oral Maxillofac Surg. 2015;53:279-84.
[469] Wen Y, Lan J, Huang H, Yu M, Cui J, Liang J, et al. Application of eGFP to label human periodontal ligament stem cells in periodontal tissue engineering. Archives of oral biology. 2012;57:1241-50.
[470] Ye ZC, Wei FC, Wang KT, Sun SZ, Zhao HQ, Li GJ. [Repair of mandibular central fissures in rabbits with hBMP-2 gene modified tissue engineered bone]. Shanghai kou qiang yi xue = Shanghai journal of stomatology. 2006;15:42-7.
[471] Zhang Y, Miron RJ, Li S, Shi B, Sculean A, Cheng X. Novel MesoPorous BioGlass/silk scaffold containing adPDGF-B and adBMP7 for the repair of periodontal defects in beagle dogs. Journal of clinical periodontology. 2015;42:262-71.
[472] Zhang Y, Shi B, Li C, Wang Y, Chen Y, Zhang W, et al. The synergetic bone-forming effects of combinations of growth factors expressed by adenovirus vectors on

chitosan/collagen scaffolds. Journal of controlled release : official journal of the Controlled Release Society. 2009;136:172-8.
[473] Zhang Y, Song J, Shi B, Wang Y, Chen X, Huang C, et al. Combination of scaffold and adenovirus vectors expressing bone morphogenetic protein-7 for alveolar bone regeneration at dental implant defects. Biomaterials. 2007;28:4635-42.
[474] Zhao J, Hu J, Wang S, Sun X, Xia L, Zhang X, et al. Combination of beta-TCP and BMP-2 gene-modified bMSCs to heal critical size mandibular defects in rats. Oral Dis. 2010;16:46-54.
[475] Zhou W, Mei L. Effect of autologous bone marrow stromal cells transduced with osteoprotegerin on periodontal bone regeneration in canine periodontal window defects. The International journal of periodontics & restorative dentistry. 2012;32:e174-81.
[476] Zhou W, Zhao CH, Mei LX. [Effect of the compound of poly lactic-co-glycolic acid and bone marrow stromal cells modified by osteoprotegerin gene on the periodontal regeneration in Beagle dog periodontal defects]. Hua xi kou qiang yi xue za zhi = Huaxi kouqiang yixue zazhi = West China journal of stomatology. 2010;28:324-9.
[477] Zou D, He J, Zhang K, Dai J, Zhang W, Wang S, et al. The bone-forming effects of HIF-1alpha-transduced BMSCs promote osseointegration with dental implant in canine mandible. PLoS One. 2012;7:e32355.
[478] Chen YL, Chen PK, Jeng LB, Huang CS, Yang LC, Chung HY, et al. Periodontal regeneration using ex vivo autologous stem cells engineered to express the BMP-2 gene: an alternative to alveolaplasty. Gene Ther. 2008;15:1469-77.
[479] Chen R, Chiba M, Mori S, Fukumoto M, Kodama T. Periodontal gene transfer by ultrasound and nano/microbubbles. Journal of dental research. 2009;88:1008-13.
[480] Kanzaki H, Chiba M, Arai K, Takahashi I, Haruyama N, Nishimura M, et al. Local RANKL gene transfer to the periodontal tissue accelerates orthodontic tooth movement. Gene Ther. 2006;13:678-85.
[481] Iglesias-Linares A, Moreno-Fernandez AM, Yanez-Vico R, Mendoza-Mendoza A, Gonzalez-Moles M, Solano-Reina E. The use of gene therapy vs. corticotomy surgery in accelerating orthodontic tooth movement. Orthodontics & craniofacial research. 2011;14:138-48.
[482] Park J, Ries J, Gelse K, Kloss F, von der Mark K, Wiltfang J, et al. Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes. Gene Ther. 2003;10:1089-98.
[483] Lattanzi W, Parrilla C, Fetoni A, Logroscino G, Straface G, Pecorini G, et al. Ex vivo-transduced autologous skin fibroblasts expressing human Lim mineralization protein-3 efficiently form new bone in animal models. Gene Ther. 2008;15:1330-43.
[484] Park SY, Kim KH, Gwak EH, Rhee SH, Lee JC, Shin SY, et al. Ex vivo bone morphogenetic protein 2 gene delivery using periodontal ligament stem cells for enhanced re-osseointegration in the regenerative treatment of peri-implantitis. J Biomed Mater Res A. 2015;103:38-47.
[485] Chang SC, Chuang HL, Chen YR, Chen JK, Chung HY, Lu YL, et al. Ex vivo gene therapy in autologous bone marrow stromal stem cells for tissue-engineered maxillofacial bone regeneration. Gene Ther. 2003;10:2013-9.
[486] Jiang X, Gittens SA, Chang Q, Zhang X, Chen C, Zhang Z. The use of tissue-engineered bone with human bone morphogenetic protein-4-modified bone-marrow stromal cells in repairing mandibular defects in rabbits. Int J Oral Maxillofac Surg. 2006;35:1133-9.
[487] Kroczek A, Park J, Birkholz T, Neukam FW, Wiltfang J, Kessler P. Effects of osteoinduction on bone regeneration in distraction: results of a pilot study. Journal of cranio-maxillo-facial surgery : official publication of the European Association for Cranio-Maxillo-Facial Surgery. 2010;38:334-44.

[488] Steinhardt Y, Aslan H, Regev E, Zilberman Y, Kallai I, Gazit D, et al. Maxillofacial-derived stem cells regenerate critical mandibular bone defect. Tissue engineering Part A. 2008;14:1763-73.
[489] Sun XJ, Xia LG, Chou LL, Zhong W, Zhang XL, Wang SY, et al. Maxillary sinus floor elevation using a tissue engineered bone complex with BMP-2 gene modified bMSCs and a novel porous ceramic scaffold in rabbits. Archives of oral biology. 2010;55:195-202.
[490] Wei F, Song T, Ding G, Xu J, Liu Y, Liu D, et al. Functional tooth restoration by allogeneic mesenchymal stem cell-based bio-root regeneration in swine. Stem cells and development. 2013;22:1752-62.
[491] Yang H, Aprecio RM, Zhou X, Wang Q, Zhang W, Ding Y, et al. Therapeutic effect of TSG-6 engineered iPSC-derived MSCs on experimental periodontitis in rats: a pilot study. PLoS One. 2014;9:e100285.
[492] Zhao N, Liu Y, Kanzaki H, Liang W, Ni J, Lin J. Effects of local osteoprotegerin gene transfection on orthodontic root resorption during retention: an in vivo micro-CT analysis. Orthodontics & craniofacial research. 2012;15:10-20.
[493] Kuboki T, Nakanishi T, Kanyama M, Sonoyama W, Fujisawa T, Kobayashi K, et al. Direct adenovirus-mediated gene delivery to the temporomandibular joint in guinea-pigs. Archives of oral biology. 1999;44:701-9.
[494] Iglesias-Linares A, Yanez-Vico RM, Moreno-Fernandez AM, Mendoza-Mendoza A, Solano-Reina E. Corticotomy-assisted orthodontic enhancement by bone morphogenetic protein-2 administration. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2012;70:e124-32.
[495] Abbayya K, Zope SA, Naduwinmani S, Pisal A, Puthanakar N. Cell- and Gene- Based Therapeutics for Periodontal Regeneration. International journal of preventive medicine. 2015;6:110.
[496] Aghaloo T, Jiang X, Soo C, Zhang Z, Zhang X, Hu J, et al. A study of the role of nell-1 gene modified goat bone marrow stromal cells in promoting new bone formation. Molecular therapy : the journal of the American Society of Gene Therapy. 2007;15:1872-80.
[497] Delgado-Ruiz RA, Calvo-Guirado JL, Romanos GE. Critical size defects for bone regeneration experiments in rabbit calvariae: systematic review and quality evaluation using ARRIVE guidelines. Clinical oral implants research. 2015;26:915-30.
[498] Gupta K, Singh S, Garg KN. Gene therapy in dentistry: tool of genetic engineering. Revisited. Archives of oral biology. 2015;60:439-46.
[499] Betz VM, Betz OB, Harris MB, Vrahas MS, Evans CH. Bone tissue engineering and repair by gene therapy. Frontiers in bioscience : a journal and virtual library. 2008;13:833-41.
[500] Friedmann T. Human gene therapy--an immature genie, but certainly out of the bottle. Nature medicine. 1996;2:144-7.
[501] Fischer J, Kolk A, Wolfart S, Pautke C, Warnke PH, Plank C, et al. Future of local bone regeneration – Protein versus gene therapy. Journal of Cranio-Maxillofacial Surgery. 2011;39:54-64.
[502] Franceschi RT, Yang S, Rutherford RB, Krebsbach PH, Zhao M, Wang D. Gene therapy approaches for bone regeneration. Cells, tissues, organs. 2004;176:95-108.
[503] Luo J, Sun MH, Kang Q, Peng Y, Jiang W, Luu HH, et al. Gene therapy for bone regeneration. Current gene therapy. 2005;5:167-79.
[504] Jane JA, Jr., Dunford BA, Kron A, Pittman DD, Sasaki T, Li JZ, et al. Ectopic osteogenesis using adenoviral bone morphogenetic protein (BMP)-4 and BMP-6 gene transfer. Molecular therapy : the journal of the American Society of Gene Therapy. 2002;6:464-70.
[505] Blum JS, Barry MA, Mikos AG, Jansen JA. In vivo evaluation of gene therapy vectors in ex vivo-derived marrow stromal cells for bone regeneration in a rat critical-size calvarial defect model. Human gene therapy. 2003;14:1689-701.

[506] Hsieh SC, Graves DT. Pulse application of platelet-derived growth factor enhances formation of a mineralizing matrix while continuous application is inhibitory. Journal of cellular biochemistry. 1998;69:169-80.
[507] Viggeswarapu M, Boden SD, Liu Y, Hair GA, Louis-Ugbo J, Murakami H, et al. Adenoviral delivery of LIM mineralization protein-1 induces new-bone formation in vitro and in vivo. The Journal of bone and joint surgery American volume. 2001;83-A:364-76.
[508] Mali S. Delivery systems for gene therapy. Indian journal of human genetics. 2013;19:3-8.
[509] Gardlik R, Palffy R, Hodosy J, Lukacs J, Turna J, Celec P. Vectors and delivery systems in gene therapy. Medical science monitor : international medical journal of experimental and clinical research. 2005;11:RA110-21.
[510] Guan X, Goddard MA, Mack DL, Childers MK. Gene therapy in monogenic congenital myopathies. Methods (San Diego, Calif). 2016;99:91-8.
[511] Tilemann L, Ishikawa K, Weber T, Hajjar RJ. Gene Therapy for Heart Failure. Circulation research. 2012;110:777-93.
[512] Stacey GN, Merten OW. Host cells and cell banking. Methods in molecular biology (Clifton, NJ). 2011;737:45-88.
[513] Schucht R, Coroadinha AS, Zanta-Boussif MA, Verhoeyen E, Carrondo MJ, Hauser H, et al. A new generation of retroviral producer cells: predictable and stable virus production by Flp-mediated site-specific integration of retroviral vectors. Molecular therapy : the journal of the American Society of Gene Therapy. 2006;14:285-92.
[514] Carr DJ, Wallace JM, Aitken RP, Milne JS, Martin JF, Zachary IC, et al. Peri- and Postnatal Effects of Prenatal Adenoviral VEGF Gene Therapy in Growth-Restricted Sheep. Biology of reproduction. 2016.
[515] Teos LY, Zheng CY, Liu X, Swaim WD, Goldsmith CM, Cotrim AP, et al. Adenovirus-mediated hAQP1 expression in irradiated mouse salivary glands causes recovery of saliva secretion by enhancing acinar cell volume decrease. Gene Ther. 2016.
[516] Song K, Rao NJ, Chen ML, Huang ZJ, Cao YG. Enhanced bone regeneration with sequential delivery of basic fibroblast growth factor and sonic hedgehog. Injury. 2011;42:796-802.
[517] Kaur H, Uludag H, El-Bialy T. Effect of nonviral plasmid delivered basic fibroblast growth factor and low intensity pulsed ultrasound on mandibular condylar growth: a preliminary study. BioMed research international. 2014;2014:426710.
[518] Apaolaza PS, Del Pozo-Rodriguez A, Torrecilla J, Rodriguez-Gascon A, Rodriguez JM, Friedrich U, et al. Solid lipid nanoparticle-based vectors intended for the treatment of X-linked juvenile retinoschisis by gene therapy: In vivo approaches in Rs1h-deficient mouse model. Journal of controlled release : official journal of the Controlled Release Society. 2015;217:273-83.
[519] Kain SR, Ganguly S. Overview of genetic reporter systems. Current protocols in molecular biology / edited by Frederick M Ausubel [et al]. 2001;Chapter 9:Unit9 6.
[520] Thibodeau SA, Fang R, Joung JK. High-throughput beta-galactosidase assay for bacterial cell-based reporter systems. BioTechniques. 2004;36:410-5.
[521] Phippard D, Manning AM. Screening for inhibitors of transcription factors using luciferase reporter gene expression in transfected cells. Methods in molecular biology (Clifton, NJ). 2003;225:19-23.
[522] Tarassoli P, Khan WS, Hughes A, Heidari N. A review of techniques for gene therapy in bone healing. Current stem cell research & therapy. 2013;8:201-9.
[523] Balmayor ER, van Griensven M. Gene therapy for bone engineering. Frontiers in bioengineering and biotechnology. 2015;3:9.
[524] Evans CH. Gene therapy for bone healing. Expert reviews in molecular medicine. 2010;12:e18.

[525] Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. Journal of dental research. 2009;88:792-806.
[526] Sheyn D, Mizrahi O, Benjamin S, Gazit Z, Pelled G, Gazit D. Genetically modified cells in regenerative medicine and tissue engineering. Advanced drug delivery reviews. 2010;62:683-98.
[527] Park JS, Suryaprakash S, Lao YH, Leong KW. Engineering mesenchymal stem cells for regenerative medicine and drug delivery. Methods (San Diego, Calif). 2015;84:3-16.
[528] Estrela C, Alencar AH, Kitten GT, Vencio EF, Gava E. Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration. Braz Dent J. 2011;22:91-8.
[529] Tracy CJ, Sanders DN, Bryan JN, Jensen CA, Castaner LJ, Kirk MD, et al. Intravitreal Implantation of Genetically Modified Autologous Bone Marrow-Derived Stem Cells for Treating Retinal Disorders. Advances in experimental medicine and biology. 2016;854:571-7.
[530] Yin C, Chen J, Chen Z, Zeng Z, Qiu J. hBMP-2 and hTGF-beta1 expressed in implanted BMSCs synergistically promote the repairing of segmental bone defects. J Orthop Sci. 2015;20:717-27.
[531] Xie Q, Wang Z, Zhou H, Yu Z, Huang Y, Sun H, et al. The role of miR-135-modified adipose-derived mesenchymal stem cells in bone regeneration. Biomaterials. 2016;75:279-94.
[532] Yang LY, Zheng JK, Hui GZ, Guo LH. [Adipose tissue-derived stromal cells as vector for gene therapy in central nervous system]. Sichuan da xue xue bao Yi xue ban = Journal of Sichuan University Medical science edition. 2004;35:463-5.
[533] Watanabe N, Ohashi K, Tatsumi K, Utoh R, Shim IK, Kanegae K, et al. Genetically modified adipose tissue-derived stem/stromal cells, using simian immunodeficiency virus-based lentiviral vectors, in the treatment of hemophilia B. Human gene therapy. 2013;24:283-94.
[534] Mi HW, Lee MC, Fu E, Chow LP, Lin CP. Highly efficient multipotent differentiation of human periodontal ligament fibroblasts induced by combined BMP4 and hTERT gene transfer. Gene Ther. 2011;18:452-61.
[535] Nakashima M, Iohara K, Ishikawa M, Ito M, Tomokiyo A, Tanaka T, et al. Stimulation of reparative dentin formation by ex vivo gene therapy using dental pulp stem cells electrotransfected with growth/differentiation factor 11 (Gdf11). Human gene therapy. 2004;15:1045-53.
[536] Krebsbach PH, Gu K, Franceschi RT, Rutherford RB. Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone in vivo. Human gene therapy. 2000;11:1201-10.
[537] Palmer G, Pascher A, Gouze E, Gouze JN, Betz O, Spector M, et al. Development of gene-based therapies for cartilage repair. Critical reviews in eukaryotic gene expression. 2002;12:259-73.
[538] Cotrim AP, Mineshiba F, Sugito T, Samuni Y, Baum BJ. Salivary gland gene therapy. Dental clinics of North America. 2006;50:157-73, vii.
[539] Feurer E, Chapurlat R. Emerging drugs for osteoporosis. Expert Opinion on Emerging Drugs. 2014;19:385-95.
[540] Boyle WJ, Simonet WS, Lacey DL. Osteoclast differentiation and activation. Nature. 2003;423:337-42.
[541] Russell RG. Pharmacological diversity among drugs that inhibit bone resorption. Current opinion in pharmacology. 2015;22:115-30.
[542] Van den Wyngaert T, Huizing MT, Fossion E, Vermorken JB. Bisphosphonates in oncology: rising stars or fallen heroes. Oncologist. 2009;14:181-91.
[543] Fleisch H. Development of bisphosphonates. Breast cancer research : BCR. 2002;4:30-4.

[544] Hanley DA, Adachi JD, Bell A, Brown V. Denosumab: mechanism of action and clinical outcomes. International journal of clinical practice. 2012;66:1139-46.
[545] Russell RG. Bisphosphonates: the first 40 years. Bone. 2011;49:2-19.
[546] Reszka AA, Rodan GA. Mechanism of action of bisphosphonates. Curr Osteoporos Rep. 2003;1:45-52.
[547] Moen MD, Keam SJ. Denosumab: a review of its use in the treatment of postmenopausal osteoporosis. Drugs & aging. 2011;28:63-82.
[548] Baron R, Ferrari S, Russell RG. Denosumab and bisphosphonates: different mechanisms of action and effects. Bone. 2011;48:677-92.
[549] Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, et al. Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. The Journal of clinical investigation. 1991;88:2095-105.
[550] Hughes DE, Wright KR, Uy HL, Sasaki A, Yoneda T, Roodman GD, et al. Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 1995;10:1478-87.
[551] Miller SC, Jee WS. The effect of dichloromethylene diphosphonate, a pyrophosphate analog, on bone and bone cell structure in the growing rat. The Anatomical record. 1979;193:439-62.
[552] Flanagan AM, Chambers TJ. Inhibition of bone resorption by bisphosphonates: interactions between bisphosphonates, osteoclasts, and bone. Calcified tissue international. 1991;49:407-15.
[553] Coxon FP, Helfrich MH, Van't Hof R, Sebti S, Ralston SH, Hamilton A, et al. Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2000;15:1467-76.
[554] Hughes DE, MacDonald BR, Russell RG, Gowen M. Inhibition of osteoclast-like cell formation by bisphosphonates in long-term cultures of human bone marrow. The Journal of clinical investigation. 1989;83:1930-5.
[555] Russell RG, Rogers MJ. Bisphosphonates: from the laboratory to the clinic and back again. Bone. 1999;25:97-106.
[556] Ebetino FH FM, Rogers MJ, Russell RG. Etidronate. Mechanisms of action of etidronate and other bisphosphonates. Reviews in Contemporary Pharmacotherapy. 1998;9:233-43.
[557] Thompson K, Rogers MJ, Coxon FP, Crockett JC. Cytosolic entry of bisphosphonate drugs requires acidification of vesicles after fluid-phase endocytosis. Molecular pharmacology. 2006;69:1624-32.
[558] Yamashita J, McCauley LK. Antiresorptives and osteonecrosis of the jaw. The journal of evidence-based dental practice. 2012;12:233-47.
[559] Collin-Osdoby P. Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. Circulation research. 2004;95:1046-57.
[560] Lewiecki EM. Treatment of osteoporosis with denosumab. Maturitas. 2010;66:182-6.
[561] Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, et al. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proceedings of the National Academy of Sciences of the United States of America. 1999;96:3540-5.
[562] Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, et al. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell. 1998;93:165-76.

[563] Burgess TL, Qian Y, Kaufman S, Ring BD, Van G, Capparelli C, et al. The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. The Journal of cell biology. 1999;145:527-38.
[564] Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell. 1997;89:309-19.
[565] Schwarz EM, Ritchlin CT. Clinical development of anti-RANKL therapy. Arthritis research & therapy. 2007;9 Suppl 1:S7.
[566] Bekker PJ, Holloway D, Nakanishi A, Arrighi M, Leese PT, Dunstan CR. The effect of a single dose of osteoprotegerin in postmenopausal women. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2001;16:348-60.
[567] Uyanne J, Calhoun CC, Le AD. Antiresorptive drug-related osteonecrosis of the jaw. Dental clinics of North America. 2014;58:369-84.
[568] Prolia Product Monograph. Amgen Canada (October 2011).
[569] Kennel KA, Drake MT. Adverse effects of bisphosphonates: implications for osteoporosis management. Mayo Clinic proceedings. 2009;84:632-7; quiz 8.
[570] Papapetrou PD. Bisphosphonate-associated adverse events. Hormones (Athens, Greece). 2009;8:96-110.
[571] Lipton A, Fizazi K, Stopeck AT, Henry DH, Brown JE, Yardley DA, et al. Superiority of denosumab to zoledronic acid for prevention of skeletal-related events: a combined analysis of 3 pivotal, randomised, phase 3 trials. European journal of cancer (Oxford, England : 1990). 2012;48:3082-92.
[572] Landesberg R, Woo V, Cremers S, Cozin M, Marolt D, Vunjak-Novakovic G, et al. Potential pathophysiological mechanisms in osteonecrosis of the jaw. Annals of the New York Academy of Sciences. 2011;1218:62-79.
[573] Wimalawansa SJ. Insight into bisphosphonate-associated osteomyelitis of the jaw: pathophysiology, mechanisms and clinical management. Expert opinion on drug safety. 2008;7:491-512.
[574] Yoneda T, Hagino H, Sugimoto T, Ohta H, Takahashi S, Soen S, et al. Bisphosphonate-related osteonecrosis of the jaw: position paper from the Allied Task Force Committee of Japanese Society for Bone and Mineral Research, Japan Osteoporosis Society, Japanese Society of Periodontology, Japanese Society for Oral and Maxillofacial Radiology, and Japanese Society of Oral and Maxillofacial Surgeons. J Bone Miner Metab. 2010;28:365-83.
[575] Chapurlat RD, Arlot M, Burt-Pichat B, Chavassieux P, Roux JP, Portero-Muzy N, et al. Microcrack frequency and bone remodeling in postmenopausal osteoporotic women on long-term bisphosphonates: a bone biopsy study. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2007;22:1502-9.
[576] Stepan JJ, Burr DB, Pavo I, Sipos A, Michalska D, Li J, et al. Low bone mineral density is associated with bone microdamage accumulation in postmenopausal women with osteoporosis. Bone. 2007;41:378-85.
[577] Pazianas M. Osteonecrosis of the jaw and the role of macrophages. Journal of the National Cancer Institute. 2011;103:232-40.
[578] Reid IR, Cornish J. Epidemiology and pathogenesis of osteonecrosis of the jaw. Nature reviews Rheumatology. 2012;8:90-6.
[579] Ristow O, Gerngross C, Schwaiger M, Hohlweg-Majert B, Kehl V, Jansen H, et al. Effect of antiresorptive drugs on bony turnover in the jaw: denosumab compared with bisphosphonates. Br J Oral Maxillofac Surg. 2014;52:308-13.
[580] Pickett FA. Bisphosphonate-associated osteonecrosis of the jaw: a literature review and clinical practice guidelines. Journal of dental hygiene : JDH / American Dental Hygienists' Association. 2006;80:10.

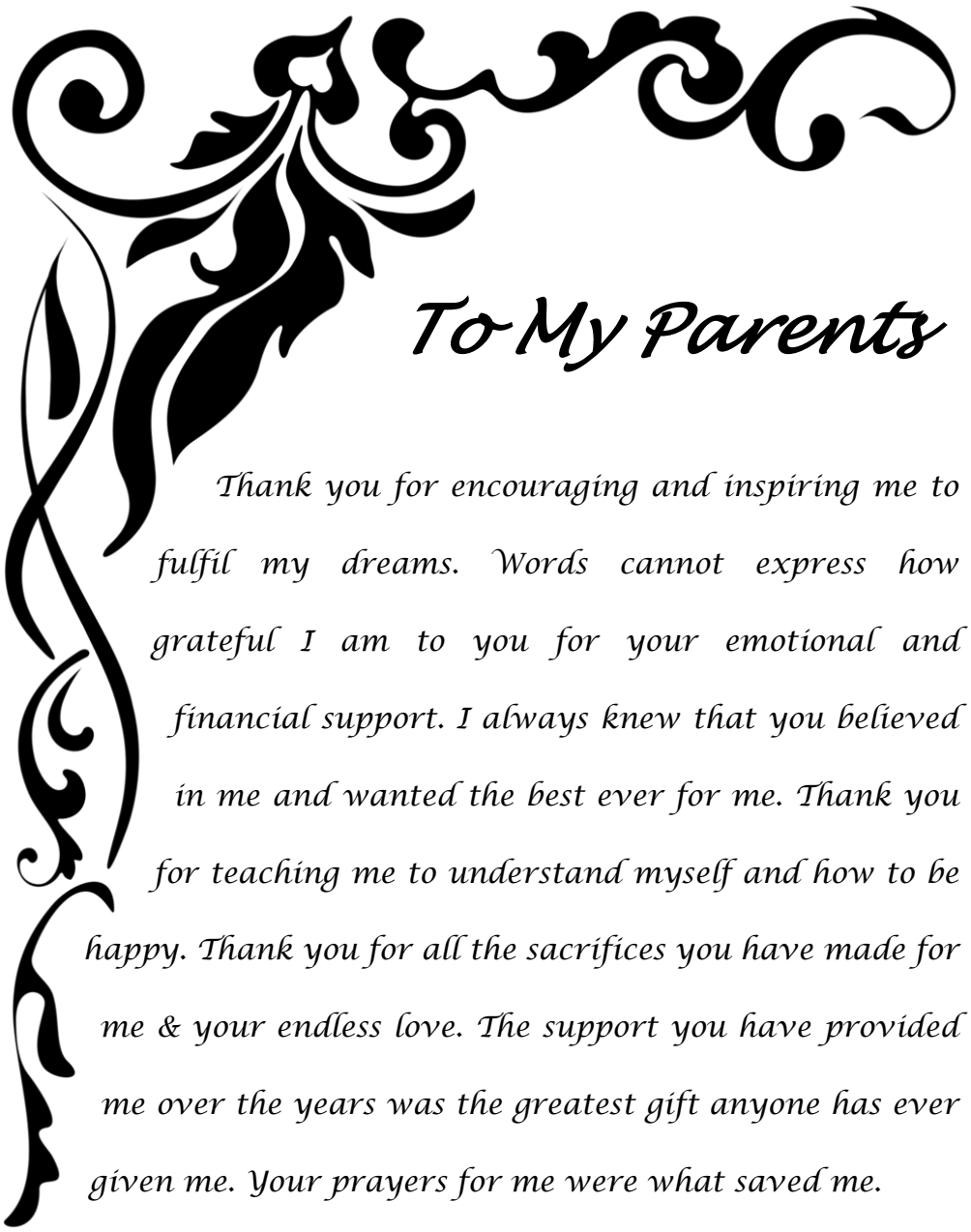
[581] Fournier P, Boissier S, Filleur S, Guglielmi J, Cabon F, Colombel M, et al. Bisphosphonates inhibit angiogenesis in vitro and testosterone-stimulated vascular regrowth in the ventral prostate in castrated rats. Cancer research. 2002;62:6538-44.
[582] Wehrhan F, Stockmann P, Nkenke E, Schlegel KA, Guentsch A, Wehrhan T, et al. Differential impairment of vascularization and angiogenesis in bisphosphonate-associated osteonecrosis of the jaw-related mucoperiosteal tissue. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics. 2011;112:216-21.
[583] Scheller EL, Baldwin CM, Kuo S, D'Silva NJ, Feinberg SE, Krebsbach PH, et al. Bisphosphonates inhibit expression of p63 by oral keratinocytes. Journal of dental research. 2011;90:894-9.
[584] Roelofs AJ, Thompson K, Gordon S, Rogers MJ. Molecular mechanisms of action of bisphosphonates: current status. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006;12:6222s-30s.
[585] Koch FP, Walter C, Hansen T, Jager E, Wagner W. Osteonecrosis of the jaw related to sunitinib. Oral and maxillofacial surgery. 2011;15:63-6.
[586] Misso G, Porru M, Stoppacciaro A, Castellano M, De Cicco F, Leonetti C, et al. Evaluation of the in vitro and in vivo antiangiogenic effects of denosumab and zoledronic acid. Cancer biology & therapy. 2012;13:1491-500.
[587] Compston J. Pathophysiology of atypical femoral fractures and osteonecrosis of the jaw. Osteoporosis international : a journal established as result of cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA. 2011;22:2951-61.
[588] Badel T, Pavicin IS, Carek AJ, Rosin-Grget K, Grbesa D. Pathophysiology of osteonecrosis of the jaw in patients treated with bisphosphonate. Collegium antropologicum. 2013;37:645-51.
[589] Cornish J, Bava U, Callon KE, Bai J, Naot D, Reid IR. Bone-bound bisphosphonate inhibits growth of adjacent non-bone cells. Bone. 2011;49:710-6.
[590] Melani C, Sangaletti S, Barazzetta FM, Werb Z, Colombo MP. Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. Cancer research. 2007;67:11438-46.
[591] Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, et al. Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer research. 2007;67:7450-7.
[592] Fiore F, Castella B, Nuschak B, Bertieri R, Mariani S, Bruno B, et al. Enhanced ability of dendritic cells to stimulate innate and adaptive immunity on short-term incubation with zoledronic acid. Blood. 2007;110:921-7.
[593] Sato K, Kimura S, Segawa H, Yokota A, Matsumoto S, Kuroda J, et al. Cytotoxic effects of gammadelta T cells expanded ex vivo by a third generation bisphosphonate for cancer immunotherapy. International journal of cancer Journal international du cancer. 2005;116:94-9.
[594] Roodman GD. Mechanisms of bone metastasis, pathophysiology of osteonecrosis of the jaw, and integrins, platelets and bone metastasis: meeting report from skeletal complications of malignancy V. IBMS BoneKEy. 2008;5:294-6.
[595] Ikebe T. Pathophysiology of BRONJ: Drug-related osteoclastic disease of the jaw. Oral Science International. 2013;10:1-8.
[596] Aghaloo TL, Kang B, Sung EC, Shoff M, Ronconi M, Gotcher JE, et al. Periodontal disease and bisphosphonates induce osteonecrosis of the jaws in the rat. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2011;26:1871-82.

[597] Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons Position Paper on Medication-Related Osteonecrosis of the Jaw-2014 Update. Journal of Oral and Maxillofacial Surgery. 2014;72:1938-56.
[598] Bonnet N, Lesclous P, Saffar JL, Ferrari S. Zoledronate effects on systemic and jaw osteopenias in ovariectomized periostin-deficient mice. PLoS One. 2013;8:e58726.
[599] Lopez-Jornet P, Camacho-Alonso F, Martinez-Canovas A, Molina-Minano F, Gomez-Garcia F, Vicente-Ortega V. Perioperative antibiotic regimen in rats treated with pamidronate plus dexamethasone and subjected to dental extraction: a study of the changes in the jaws. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2011;69:2488-93.
[600] Abtahi J, Agholme F, Aspenberg P. Prevention of osteonecrosis of the jaw by mucoperiosteal coverage in a rat model. Int J Oral Maxillofac Surg. 2013;42:632-6.
[601] McLeod NM, Patel V, Kusanale A, Rogers SN, Brennan PA. Bisphosphonate osteonecrosis of the jaw: a literature review of UK policies versus international policies on the management of bisphosphonate osteonecrosis of the jaw. Br J Oral Maxillofac Surg. 2011;49:335-42.
[602] Grötz KA. PJ, Al-Nawas Bilal. Bisphosphonatassoziierte Kiefemekrose (BP-ONJ) und andere Medikamenten-assoziierte Kiefemekrosen.: AWMF; 2012.
[603] Erkan M, Bilgi O, Mutluoglu M, Uzun G. Bisphosphonate-related osteonecrosis of the jaw in cancer patients and hyperbaric oxygen therapy. JOP. 2009;10:579-80; author reply 81-2.
[604] Ficarra G, Beninati F. Bisphosphonate-related osteonecrosis of the jaws: an update on clinical, pathological and management aspects. Head and neck pathology. 2007;1:132-40.
[605] Mulliken JB, Glowacki J. Induced osteogenesis for repair and construction in the craniofacial region. Plastic and reconstructive surgery. 1980;65:553-60.
[606] Bostrom R MA. Tissue engineering of bone. In: Atala Anthony DJM, editor. Synthetic Biodegradable Polymer Scaffolds. Boston: Birkhäuser 2013. p. 258.
[607] Gonzalvez-Garcia M, Rodriguez-Lozano FJ, Villanueva V, Segarra-Fenoll D, Rodriguez-Gonzalez MA, Onate-Sanchez R, et al. Mesenchymal stem cells and bisphosphonate-related osteonecrosis of the jaw: the future? Oral Dis. 2012;18:823-4.
[608] Langer R, Vacanti JP. Tissue engineering. Science (New York, NY). 1993;260:920-6.
[609] Koh CJ, Atala A. Tissue engineering, stem cells, and cloning: opportunities for regenerative medicine. Journal of the American Society of Nephrology : JASN. 2004;15:1113-25.
[610] Robey PG. Cell sources for bone regeneration: the good, the bad, and the ugly (but promising). Tissue engineering Part B, Reviews. 2011;17:423-30.
[611] Mao JJ CF. Stem Cells: Sources, Therapies and the Dental Professional.
[612] Mao JJ. Stem cells and the future of dental care. The New York state dental journal. 2008;74:20-4.
[613] Leventhal A, Chen G, Negro A, Boehm M. The benefits and risks of stem cell technology. Oral Dis. 2012;18:217-22.
[614] JB R. Stem cells: emerging medical and dental therapies for the dental professional.
[615] Nedel F, Andre Dde A, de Oliveira IO, Cordeiro MM, Casagrande L, Tarquinio SB, et al. Stem cells: therapeutic potential in dentistry. The journal of contemporary dental practice. 2009;10:90-6.
[616] Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science (New York, NY). 2007;318:1917-20.

[617] Nakahara H, Bruder SP, Haynesworth SE, Holecek JJ, Baber MA, Goldberg VM, et al. Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. Bone. 1990;11:181-8.
[618] Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America. 2000;97:13625-30.
[619] Romanov YA, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem cells (Dayton, Ohio). 2003;21:105-10.
[620] Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet. 2004;364:149-55.
[621] Pountos I, Giannoudis PV. Biology of mesenchymal stem cells. Injury. 2005;36 Suppl 3:S8-S12.
[622] Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nature medicine. 2007;13:1219-27.
[623] Zannettino AC, Paton S, Arthur A, Khor F, Itescu S, Gimble JM, et al. Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. Journal of cellular physiology. 2008;214:413-21.
[624] Krebsbach PH, Robey PG. Dental and skeletal stem cells: potential cellular therapeutics for craniofacial regeneration. Journal of dental education. 2002;66:766-73.
[625] Gronthos S, Graves SE, Ohta S, Simmons PJ. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. Blood. 1994;84:4164-73.
[626] Warren SM, Fong KD, Chen CM, Loboa EG, Cowan CM, Lorenz HP, et al. Tools and techniques for craniofacial tissue engineering. Tissue Eng. 2003;9:187-200.
[627] Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, et al. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nature biotechnology. 2004;22:560-7.
[628] Warnke PH, Springer IN, Wiltfang J, Acil Y, Eufinger H, Wehmoller M, et al. Growth and transplantation of a custom vascularised bone graft in a man. Lancet. 2004;364:766-70.
[629] Schantz JT, Machens HG, Schilling AF, Teoh SH. Regenerative medicine: implications for craniofacial surgery. J Craniofac Surg. 2012;23:530-6.
[630] Thesleff I. The genetic basis of tooth development and dental defects. American journal of medical genetics Part A. 2006;140:2530-5.
[631] Cordero DR, Brugmann S, Chu Y, Bajpai R, Jame M, Helms JA. Cranial neural crest cells on the move: their roles in craniofacial development. American journal of medical genetics Part A. 2011;155A:270-9.
[632] Chung IH, Yamaza T, Zhao H, Choung PH, Shi S, Chai Y. Stem cell property of postmigratory cranial neural crest cells and their utility in alveolar bone regeneration and tooth development. Stem cells (Dayton, Ohio). 2009;27:866-77.
[633] Sanchez-Lara PA, Warburton D. Impact of stem cells in craniofacial regenerative medicine. Frontiers in physiology. 2012;3:188.
[634] Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. Proceedings of the National Academy of Sciences of the United States of America. 2002;99:8932-7.
[635] Schmitt A, van Griensven M, Imhoff AB, Buchmann S. Application of stem cells in orthopedics. Stem Cells Int. 2012;2012:394962.
[636] Rosenthal N. Prometheus's vulture and the stem-cell promise. The New England journal of medicine. 2003;349:267-74.

[637] Sun Y, Feng Y, Zhang C. The effect of bone marrow mononuclear cells on vascularization and bone regeneration in steroid-induced osteonecrosis of the femoral head. Joint Bone Spine. 2009;76:685-90.
[638] Iohara K, Nakashima M, Ito M, Ishikawa M, Nakasima A, Akamine A. Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2. Journal of dental research. 2004;83:590-5.
[639] Ueda M, Yamada Y, Ozawa R, Okazaki Y. Clinical case reports of injectable tissue-engineered bone for alveolar augmentation with simultaneous implant placement. The International journal of periodontics & restorative dentistry. 2005;25:129-37.
[640] Sauerbier S, Rickert D, Gutwald R, Nagursky H, Oshima T, Xavier SP, et al. Bone marrow concentrate and bovine bone mineral for sinus floor augmentation: a controlled, randomized, single-blinded clinical and histological trial--per-protocol analysis. Tissue engineering Part A. 2011;17:2187-97.
[641] Handschel J, Meyer U. Infection, vascularization, remodelling--are stem cells the answers for bone diseases of the jaws? Head Face Med. 2011;7:5.
[642] Kikuiri T, Kim I, Yamaza T, Akiyama K, Zhang Q, Li Y, et al. Cell-based immunotherapy with mesenchymal stem cells cures bisphosphonate-related osteonecrosis of the jaw-like disease in mice. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research. 2010;25:1668-79.
[643] Li Y, Xu J, Mao L, Liu Y, Gao R, Zheng Z, et al. Allogeneic mesenchymal stem cell therapy for bisphosphonate-related jaw osteonecrosis in Swine. Stem cells and development. 2013;22:2047-56.
[644] Elad S, Zadik Y, Yarom N, Or R, Shapira MY. Hematopoietic stem cells and bisphosphonate-related osteonecrosis of the jaw. Oral Dis. 2013;19:530.
[645] Cross M, Dexter TM. Growth factors in development, transformation, and tumorigenesis. Cell. 1991;64:271-80.
[646] Lee K, Silva EA, Mooney DJ. Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. Journal of the Royal Society, Interface / the Royal Society. 2011;8:153-70.
[647] Reddi AH. Cartilage morphogenetic proteins: role in joint development, homoeostasis, and regeneration. Annals of the rheumatic diseases. 2003;62 Suppl 2:ii73-8.
[648] Tsumaki N, Tanaka K, Arikawa-Hirasawa E, Nakase T, Kimura T, Thomas JT, et al. Role of CDMP-1 in skeletal morphogenesis: promotion of mesenchymal cell recruitment and chondrocyte differentiation. The Journal of cell biology. 1999;144:161-73.
[649] Gruber R, Mayer C, Schulz W, Graninger W, Peterlik M, Watzek G, et al. Stimulatory effects of cartilage-derived morphogenetic proteins 1 and 2 on osteogenic differentiation of bone marrow stromal cells. Cytokine. 2000;12:1630-8.
[650] Wozney JM. The bone morphogenetic protein family and osteogenesis. Molecular reproduction and development. 1992;32:160-7.
[651] Boyne PJ. Application of bone morphogenetic proteins in the treatment of clinical oral and maxillofacial osseous defects. The Journal of bone and joint surgery American volume. 2001;83-A Suppl 1:S146-50.
[652] Cetiner S, Sucak GT, Kahraman SA, Aki SZ, Kocakahyaoglu B, Gultekin SE, et al. Osteonecrosis of the jaw in patients with multiple myeloma treated with zoledronic acid. J Bone Miner Metab. 2009;27:435-43.
[653] Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (P-PRP) to leucocyte- and platelet-rich fibrin (L-PRF). Trends in biotechnology. 2009;27:158-67.
[654] Marx RE. Platelet-rich plasma: evidence to support its use. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2004;62:489-96.

[655] Oliver R. Bisphosphonates and oral surgery. Oral Surgery. 2009;2:56-63.
[656] Tischler M. Platelet rich plasma. The use of autologous growth factors to enhance bone and soft tissue grafts. The New York state dental journal. 2002;68:22-4.
[657] Carlson NE, Roach RB, Jr. Platelet-rich plasma: clinical applications in dentistry. Journal of the American Dental Association (1939). 2002;133:1383-6.
[658] Nikolidakis D, Jansen JA. The biology of platelet-rich plasma and its application in oral surgery: literature review. Tissue engineering Part B, Reviews. 2008;14:249-58.
[659] Scoletta M, Arata V, Arduino PG, Lerda E, Chiecchio A, Gallesio G, et al. Tooth extractions in intravenous bisphosphonate-treated patients: a refined protocol. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2013;71:994-9.
[660] Curi MM, Cossolin GS, Koga DH, Zardetto C, Christianini S, Feher O, et al. Bisphosphonate-related osteonecrosis of the jaws--an initial case series report of treatment combining partial bone resection and autologous platelet-rich plasma. Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons. 2011;69:2465-72.
[661] Ziebart T, Koch F, Klein MO, Guth J, Adler J, Pabst A, et al. Geranylgeraniol - a new potential therapeutic approach to bisphosphonate associated osteonecrosis of the jaw. Oral Oncol. 2011;47:195-201.



To My Parents

Thank you for encouraging and inspiring me to fulfil my dreams. Words cannot express how grateful I am to you for your emotional and financial support. I always knew that you believed in me and wanted the best ever for me. Thank you for teaching me to understand myself and how to be happy. Thank you for all the sacrifices you have made for me & your endless love. The support you have provided me over the years was the greatest gift anyone has ever given me. Your prayers for me were what saved me.

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It has been a pleasure working with these colleagues and lab mates in particular: ***Paolo Alberton*** who spent countless hours in the lab explaining and showing me how to do everything and for sacrificing his time to help me. Thank you and the best of luck in your future. I’m also thankful to***: Li Ping, Feng-koo Hseih, Chi Fen, Wu Fan, Lin Dasheng*** and all the current and former lab members that I know for sharing the literature and invaluable assistance.

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For the ***newer PhD students*** of the program “Oral Science”, best wishes! Keep your enthusiasm if possible. And good luck to you all. Hope you get what you want!

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PERSONAL INFORMATION

Date of Birth: 16 /01/1982.

Place of Birth: Alexandria, Egypt.

Nationality: Egyptian.

Gender: Female.

Marital Status: Single.

OBJECTIVES

Ambitious to have an academic career where I can extend my teaching and research experience based on strong research skills gained through studies so far as a clinical researcher in one of the leading research centre in Germany.

EDUCATION

Start/End Date	Name of Institution	Degree
Sep 2000-May 2005	***Alexandria University*** Alexandria Egypt	**BSc** of Oral medicine and Dental surgery
Feb 2008-Feb 2011	***Alexandria University*** Alexandria Egypt	**MSc** of Oral and Maxillofacial Surgery
Oct 2013-till now	***Ludwig-Maximilians University*** Munich Germany	**PhD** of Oral Sciences and Medical Research

ACADEMIC POSITIONS

Date	Title	Department	Name of Institution
July 2007-March 2011	Demonstrator	Oral and Maxillofacial Surgery	Faculty of Dentistry ***Alexandria University*** Egypt
May 2011-till now	Assistant Lecturer	Oral and Maxillofacial Surgery	Faculty of Dentistry ***Alexandria University*** Egypt

RESEARCH EXPERIENCE

Date	Field of Research	Institution	Professor
2008-2012	Bone Tissue Engineering	***Tissue Engineering Laboratories***, Faculty of Dentistry, Alexandria, University, Egypt.	Prof Dr. Mona Marei
2012-till now	Bone Tissue Engineering	Experimental surgery and regenerative medicine laboratory (***Experimed***), Surgery Clinic, LMU, Munich, Germany.	Prof Dr. Matthias Schieker PD Dr.Dr. Sven Otto

RESEARCH INTERESTS

- Gene therapy
- Vascularized Tissue Engineering
- Cartilage Tissue Engineering
- Systematic Reviews and Meta-analysis
- Osteonecrosis of the jaw

PROFESSIONAL QUALIFICATIONS

Languages

Mother tongue: Arabic

Other languages:

- English: Fluently spoken.
- German: A2 level.
- French: Good.

Technological Skills

- Photoshop CS3
- Microsoft Office
- GraphPad Prism
- RevMan 5.3
- EndNote

LAB SKILLS

Familiar with a range of practical laboratory techniques including

Skill Name	Skill Level	Last Used/Experience
Cell culture	Experienced	Currently used/3 years
Isolation of Stem cells from bone marrow	Experienced	Currently used/3 years
Isolation of Stem cells from adipose tissue	Experienced	Currently used/3 years
Isolation and culture of osteoblasts	Experienced	Currently used/3 years
Isolation and culture of osteoclasts	Experienced	Currently used/3 years
Lineage differentiation of stem cells	Experienced	Currently used/3 years
PCR techniques	Experienced	Currently used/3 years
Western Blotting	High	Currently used/3 years
Immunofluorescence	High	Currently used/3 years
Immunohistochemistry	Intermediate	Currently used/3 years
RNA extraction	Experienced	Currently used/3 years
cDNA synthesis	Experienced	Currently used/3 years
Gel electrophoresis	Experienced	Currently used/3 years
Light microscopy	High	Currently used/3 years
Medium preparation	Experienced	Currently used/3 years
Staining and quantification	Experienced	Currently used/3 years
Cell Counts	Experienced	Currently used/3 years

FORMALLY SUPERVISED TRAINEES

Date	Title of Persons	Name of Institution
June 2015	Two undergraduate students	Harvard University

GRANTS

Pending: Investigations into BRONJ development in a wild type and Tenomodulin knock-out mice model. In:Anti-osteoclastic drugs and their impact on maxillofacial and orthopedic bone biology, disease, diagnosis, prevention, surgery, and treatment modalities (ARONJ). AOCMF *Jan* 2016.

Expired: Large animal model for antiresorptive drug induced osteonecrosis of the jaw. AOCMF-14-070O. Otto S, Tröltzsch M, Voss P, Poxleitner P, Ziebart T, Wilke M. Ludwig-Maximilians-University, Munich (Germany).

MEMBERSHIPS/SCIENTIFIC SOCIETIES

Date	Name of Society
May 2016	European Society for Gene and Cell Therapy
May 2016	AOCMF Foundation

PUBLICATIONS

BOOK CHAPTERS

- ***Riham Fliefel*** and Sven Otto. Pathogenesis of antiresorptive drug-related osteonecrosis of the jaw. In: Kenneth E Fleisher, Risto Kontio, Sven Otto. Antiresorptive Drug-related Osteonecrosis of the Jaw (ARONJ)—a Guide to Research. Switzerland: AOCMF; 2016. p 64. ISBN: 978-3-905363-10-4.

- ***Riham Fliefel*** and Pit Voss. New and Innovative Treatment Strategies for Medication-Related Osteonecrosis of the Jaw. In: Sven Otto. Medication-Related Osteonecrosis of the Jaws: Bisphosphonates, Denosumab, and New Agents. Heidelberg: Springer; 2015. p 220. ISBN: 978-3-662-43732-2.

JOURNAL PUBLICATIONS

- ***Riham Fliefel***, Cvetan Popov, Matthias Tröltzsch, Jan Kühnisch, Michael Ehrenfeld, Sven Otto. Mesenchymal stem cell proliferation and mineralization but not osteogenic differentiation are strongly affected by extracellular pH. J Craniomaxillofacial Surgery 2016; 44(6): 715–24.

- Sven Otto, Oliver Ristow, Christoph Pache, Matthias Tröltzsch, ***Riham Fliefel***, Michael Ehrenfeld, Christoph Pautke. Fluorescence-guided surgery for the treatment of medication-related osteonecrosis of the jaw: a prospective cohort study. J Craniomaxillofacial Surgery 2016. In Press Accepted.

- ***Riham Fliefel***, Matthias Tröltzsch, Jan Kühnisch, Michael Ehrenfeld, Sven Otto. Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. International Journal of Oral and Maxillofacial Surgery 2015; 44(5): 568–85.

- Sappasith Panya, ***Riham Fliefel***, Florian Probst, Matthias Tröltzsch, Michael Ehrenfeld, Sören Schubert, Sven Otto. Role of microbiological culture and PCR in Medication- related osteonecrosis of the jaw (MRONJ). Under review in J Craniomaxillofacial Surgery 2016.

- ***Riham Fliefel***, Jan Kühnisch, Michael Ehrenfeld, Sven Otto. Gene therapy for bone defects in Oral and Maxillofacial Surgery: a Systematic review and Meta-analysis. Under review in Stem Cells and Development 2016.

- Benjamin Palla, Egon Burian, John Richard Klecker, ***Riham Fliefel***, Sven Otto. Systematic review of oral ulceration with bone sequestration. J Craniomaxillofacial Surgery 2015; 44(3): 257–64.

- Florian Probst, Sven Otto, Matthias Cornelsen, ***Riham Fliefel***, Egon Burian, M. Seitz, M. Berger, Michael Ehrenfeld. Custom-made vitalized scaffolds for bone tissue reconstruction in craniomaxillofacial surgery. International Journal of Oral and Maxillofacial Surgery 2013; 42(10): 1375.

- Florian Probst, Egon Burian, ***Riham Fliefel***, Michael Ehrenfeld, Sven Otto. Zukünftige Optionen zur Rekonstruktion bei ausgedehnten knöchernen Defekten im Kiefer-, Gesichts- und Schädelbereich mittels CAD/CAM-gefertigter bioaktiver Leitschienen. OP-Journal 2014; 29(02):200-4.

- Sven Otto, Robert E Marx, Matthias Tröltzsch, Oliver Ristow, Thomas Ziebart, Bilal Al-Nawas, Knut A Groetz, Michael Ehrenfeld, Valeria Mercadante, Stephen Porter, Alberto Bedogni, Giuseppina Campisi, Vittorio Fusco, Ezher Dayisoylu, ***Riham Fliefel***, Bente Brokstad Herlofson, Christoph Pautke, Tae-Geon Kwon, Stefano Fedele: Comments on "Diagnosis and Management of Osteonecrosis of the Jaw: A Systematic Review and International Consensus": LETTER TO THE EDITOR. Journal of bone and mineral research 2015; 30(6):1113–5.

RESEARCH PROJECTS

Date	Title of Project	Name of Principal investigator
Jan 2013	Regeneration of Critical-sized defects in oral and maxillofacial surgery in minipigs	Dr. Florian Probst
May 2014	Large animal model for antiresorptive drug induced osteonecrosis of the jaw	PD Dr. Dr. Sven Otto

REVIEWER

Reviewer in Journal of Craniomaxillofacial Surgery.

PROFESSIONAL DEVELOPMENT

Date	Name of Conference/ Seminar/Presentation	Name of Institution
Nov 2015	GraphPad Prism	Poliklinik Für Zahnerhaltung und Paradontologie, LMU, Munich, Germany
Jun 2016	RevMan	Poliklinik Für Zahnerhaltung und Paradontologie, LMU, Munich, Germany

REFEREES

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APPENDIX

Journal of Cranio-Maxillo-Facial Surgery 44 (2016) 715–724

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Journal of Cranio-Maxillo-Facial Surgery

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Mesenchymal stem cell proliferation and mineralization but not osteogenic differentiation are strongly affected by extracellular pH

Riham Fliefel [a, b, c, *], Cvetan Popov [a], Matthias Tröltzsch [b], Jan Kühnisch [d], Michael Ehrenfeld [b], Sven Otto [b]

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ABSTRACT

Osteomyelitis is a serious complication in oral and maxillofacial surgery affecting bone healing. Bone remodeling is not only controlled by cellular components but also by ionic and molecular composition of the extracellular fluids in which calcium phosphate salts are precipitated in a pH dependent manner.
Objective: To determine the effect of pH on self-renewal, osteogenic differentiation and matrix mineralization of mesenchymal stem cells (MSCs).
Methods: We selected three different pH values; acidic (6.3, 6.7), physiological (7.0–8.0) and severe alkaline (8.5). MSCs were cultured at different pH ranges, cell viability measured by WST-1, apoptosis detected by JC-1, senescence was analyzed by β-galactosidase whereas mineralization was detected by Alizarin Red and osteogenic differentiation analyzed by Real-time PCR.
Results: Self-renewal was affected by pH as well as matrix mineralization in which pH other than physiologic inhibited the deposition of extracellular matrix but did not affect MSCs differentiation as osteoblast markers were upregulated. The expression of osteocalcin and alkaline phosphatase activity was upregulated whereas osteopontin was downregulated under acidic pH.
Conclusion: pH affected MSCs self-renewal and mineralization without influencing osteogenic differentiation. Thus, future therapies, based on shifting acid-base balance toward the alkaline direction might be beneficial for prevention or treatment of osteomyelitis.

1. Introduction

Osteomyelitis (OM) of the jaw is a debilitating disease (Sanchez et al., 2013) in which severe bone infection leads to dysfunction, progressive inflammatory destruction, marked bone resorption at sites of infection, and abnormal bone formation (Teitelbaum et al., 1997; Sax and Lew 1999). It occurs more frequently in the mandible than in the maxilla (Singh et al., 2010) with *Staphylococcus aureus* creating an acidic environment, decreasing the pH to 5.5–7.0 (Ma et al., 2010) as a result of massive infiltration of neutrophils and macrophages (Issekutz and Bhimji, 1982; Spector et al., 2001; Otto et al., 2010; Pavlukhina et al., 2010; Hatzenbuehler and Pulling, 2011; Humber et al., 2011). It is well known that infection and inflammation interfere with the process of bone healing and regeneration by excessive bone resorption as well as impaired bone formation by activation of several cell populations producing inflammatory cytokines with an impact on bone remodeling (Marriott et al., 2004; Romas and Gillespie, 2006; Thomas and Puleo, 2011, Redlich and Smolen, 2012).

Bone remodeling is controlled not only by osteoblasts and osteoclasts (Eriksen, 2010) but also by the ionic and molecular composition of the extracellular fluids in which calcium phosphate salts are precipitated in a pH-dependent manner (Chakkalakal et al., 1994; Kohn et al., 2002; Iyemere et al., 2006). Osteoblasts are the most affected cells by pH and acidity of the extracellular microenvironment (Arnett and Dempster, 1990; Chakkalakal et al., 1994; Green, 1994; Wu et al., 1997). On a cellular level, even modest

* Corresponding author. Experimental Surgery and Regenerative Medicine (ExperiMed), Ludwig-Maximilians-University, Nußbaumstr. 20, 80336, Munich, Germany. Tel.: +49 89 440055487; fax: +49 89 440055814.
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reduction in extracellular pH has an effect on osteoblast mineralization and energy metabolism, as it was suggested that changes in acid–base balance in the extracellular microenvironment can direct bone formation and resorption (Chakkalakal et al., 1994; Green 1994; Ramp et al., 1994; Kaysinger and Ramp, 1998). It was shown that alkaline pH enhances mineralization of osteoblasts and decreases the activity of osteoclasts, whereas acidic surroundings can activate osteoclasts as well as impair osteoblast differentiation and in severe cases can cause osteoblast death (Muzylak et al., 2007; Han et al., 2009; Shen et al., 2012).

MSCs are adult stem cells originating from the mesoderm, possessing self-renewal ability and multi-lineage differentiation into mesoderm lineages such as chondrocytes, osteocytes, and adipocytes, and also ectodermic and endodermic cells (Wei et al., 2013). MSCs exist in almost all tissues including bone marrow, adipose tissue, synovium, periosteum, and perichondrium, as well as cartilage (Larsen and Jensen, 1989). They have the ability to migrate into sites of injury, releasing trophic and growth factors and differentiated toward terminally committed cells, making them prime candidates for use in regenerative medicine (Pereira et al., 1995; Pittenger et al., 1999; Bianco et al., 2001; Kim et al., 2005; Nuschke et al., 2014). Recently, MSCs have shown great potential in clinical practice upon activation by biological or pharmacological means, leading to improvement in bone healing by modulating their differentiation into osteoblasts (Knight and Hankenson, 2013; Qin et al., 2014). The chemical and physical environment of MSCs has a strong influence on their behavior, in which matrix acidity is a crucial factor (Moore and Lemischka, 2006; Wuertz et al., 2008). The effect of the pH of the tissue microenvironment on bone mineralization and repair has been previously reported (Swenson and Claff, 1946; Arnett and Dempster, 1986; Newman et al., 1987). However, the mechanisms underlying pH-related destruction of bone in osteomyelitis and osteogenic differentiation of human mesenchymal stem cells under various pH conditions have not been discussed. As tissue engineering becomes more of a clinical reality through the ongoing bench-to-bedside transition, research in this field must focus on addressing relevant clinical situations. Although most in vivo work in the area of bone tissue engineering focuses on bone regeneration within sterile, surgically created defects, there is a growing need for investigation of bone tissue engineering approaches within contaminated or scarred wound beds, such as those that may be encountered following traumatic injury or during delayed reconstruction/regeneration (Nair et al., 2011). Our study is novel and of importance when considering bone infections, as it might be used in future clinical applications for prevention and treatment of some bone infections or diseases. It explains what happens in the bone microenvironment during pH changes, which could be a key study not only for bone infection/disease but also adds an important facet to the linkage between pH and other hard tissue mineralization. Thus, in the present study, we aimed to determine the effect of pH on viability and proliferation of human MSCs, and to investigate the role of the pH on human MSC–mediated osteogenesis, expression of osteoblast markers, and matrix mineralization. This may contribute to understanding how changing pH modulates biological and biochemical processes during bone healing in osteomyelitis.

2. Material and methods

2.1. Cell culture

All experiments were performed with commercially available human MSCs (hMSCs; Lonza, Basel, Switzerland). Cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Life Technologies, California, USA), supplemented with 10% fetal bovine serum (FBS; Life Technologies, California, USA), 1% penicillin/streptomycin (GE Healthcare, Little Chalfont, UK) at 37 °C in a humidified atmosphere of 5% CO_2. Cells between passages 5 and 10 were used from three donors for the experiments.

2.2. Preparation of pH culture media

The pH of the culture medium was adjusted to one of six values (6.3, 6.7, 7.0, 7.4, 8.0, or 8.5) by adding an appropriate amount of 6M HCl or 10M NaOH to the supplemented DMEM. Before resuspending the cells, the culture media were kept in the incubator for 24 hours under culture conditions to allow the desired pH value to equilibrate (CO_2-dependent). After incubation, a small adjustment in pH was occasionally required to create the desired final pH. The pH was monitored with a pH meter (Mettler Toledo GmbH, Giessen, Germany). The pH media were filtered using a syringe driven through a 0.22-μm sterile filter and stored at 4 °C to be used later. For pH experiments, normal medium was replaced with various pH media 24 hours upon cell plating and was kept throughout the experiment.

2.3. Self-renewal analysis and WST-1 assay

Long-term cell growth was evaluated by calculation of increased cell number as described previously (Alberton et al., 2012). The effect of pH on hMSCs proliferation in monolayer culture was evaluated over a 5-day time course. Cells were plated into 35-mm dishes at a density of 3.0×10^4 and incubated in different pH media. At each time point, cell yield was divided by the number of cells plated at the start of the experiment to obtain a fold-change in cell number. The experiment was repeated twice.

Cell viability was assessed with WST-1 assay (Roche Diagnostics, Risch-Rotkreuz, Switzerland) as previously described (Kohler et al., 2013). Cells were seeded at a density of 1.7×10^3 cells per well in 96-well plates and incubated with different pH media for 3 days. The WST-1 was mixed with the fresh complete medium, added to the wells, and incubated for 4 hours at 37 °C in 5% CO_2. WST-1 was quantified by measuring the absorbance at 450 nm using Multiskan FC microplate plate reader (Thermo Scientific, Massachusetts, USA). Each experiment was repeated at least twice with two different donors to obtain the mean values.

2.4. JC-1 staining for apoptosis detection

One of the hallmarks of apoptosis is mitochondrial disruption, which is characterized by changes in the mitochondrial membrane potential. These changes were detected by using the fluorescent dye 5,5′,6,6'-tetrachloro-1,1′,3,3'-tetraethylimidacarbocyanine iodide (JC-1; Life Technologies, California, USA), a membrane-permeable dye that accumulates in mitochondria in a membrane potential–dependent manner. To ascertain whether pH induced apoptosis, slides were coated with collagen, hMSCs (7.0×10^3 cells) were cultured in different pH media for 24 hours. They were stained with JC-1 at 37 °C for 60 minutes, and Hoechst 33342 (Thermo Scientific, Massachusetts, USA) was used as the counterstain (Popov et al., 2011). Cells were mounted on slides and pictured with Axio Observer.Z1 fluorescence microscope (Zeiss, Oberkochen, Germany). The positive control was cells treated with hydrogen peroxide for 5 minutes, and the negative control was cells cultured in normal media.

2.5. Detection and quantification of senescent cells

Senescence-associated β-galactosidase (SA β-Gal; Sigma Aldrich, Missouri, USA) staining was used to detect senescent cells as previously described (Kohler et al., 2013). Cells were seeded at a density of 3.0×10^4 in 35-mm dishes and cultured at different pH media for

72 hours. Fresh staining mixture was added and incubated at 37 °C overnight. The cells were observed under an Axiovert 40 CFL microscope (Zeiss, Oberkochen, Germany). The percentage of blue cells expressing β-galactosidase (senescent cells) was calculated. The proportion of cells positive for SA-βgal activity was determined by counting the number of blue cells in the total population.

2.6. Osteogenic differentiation of hMSCs

Osteogenic differentiation was performed (Alberton et al., 2012). Shortly, cells were counted and plated at density of 3.2×10^4 on 35 mm dishes. After 24 hours, normal media were replaced with pH-adjusted osteogenic media, and cells were cultured for 21 days. The osteogenic media consisted of DMEM supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, and 150 μM ascorbic-2-phosphates (Sigma Aldrich, Missouri, USA). Media were changed twice per week. As a control, hMSCs were cultured at different pH media without osteogenic reagents.

Alizarin Red staining was performed on day 21. Mineralized nodules were visualized and photographed with Axiovert 40 CFL microscope (Zeiss, Oberkochen, Germany). An osteogenic quantification kit was used for quantification of the staining (Merck Millipore, Darmstadt, Germany). The osteogenic differentiation was calculated versus standard curve, and the absorbance was measured at 405 nm using Multiskan FC microplate reader plate reader (Thermo Scientific, Massachusetts, USA).

2.7. Alkaline phosphatase activity and mineralization

The differentiation of cells to osteoblasts was evaluated as a function of alkaline phosphatase (ALP) activity. The ALP assay was performed on days 0, 2, 5, 7, 10 and 14 of culture. For this, cells were seeded in 35-mm dishes and cultured at different pH media. The media were changed twice per week. ALP released from the cells was measured with a commercially available ALP assay kit (StemTAG; Cell Biolabs, California, USA). The amount of enzyme released by the cells was quantified by comparison with a standard curve. The experiment was repeated twice with two different donors. The enzyme activities expressed as nanamoles (nmol) of protein.

2.8. Reverse transcription–polymerase chain reaction analysis of osteogenic genes

Reverse transcription–polymerase chain reaction (RT-PCR) was used to evaluate the osteogenic differentiation at different pH after 21 days. RNA was isolated as previously described (Alberton et al., 2012) by QIAzol reagent (Qiagen, Hilden, Germany). RNA concentration and quality was analyzed by NanoDrop (Thermo Scientific, Massachusetts, USA). Reverse transcription of RNA into complementary DNA (cDNA) was done using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). RT-PCR was used to analyze the expression of the osteogenic genes. The primers for the target genes used and PCR conditions are shown in Table 1. The gel electrophoresis was visualized and photographed using gel imager (Vilber Lourmat, Eberhardzell, Germany). Bands were quantitatively analyzed by ImageJ (http://imagej.nih.gov/ij/). Gene expression was calculated as the ration to the housekeeping gene (GAPDH).

2.9. Statistical analysis

All of the experiments were repeated at least two times with three different donors each, and the results were expressed as means ± standard deviations. Statistical analysis was performed by using GraphPad Prism (GraphPad, California, USA) using one-way analysis of variance, followed by the Tukey test to determine the statistical significance among the different groups. Levels of significance were indicated at $P < 0.05$, $P < 0.01$, and $P < 0.0001$.

3. Results

3.1. hMSC self-renewal under different pH conditions

First, we analyzed hMSC self-renewal by examining the effect of pH on the cell proliferation and viability. For this, we cultured hMSCs in the six different pH conditions for 5 days. We found that the exposure of hMSCs to pH (6.3, 6.7, and 8.5) had a negative effect on proliferation capability in comparison to physiologic pH (7.0, 7.4, and 8.0), indicating that the latter pHs are optimal for cell growth (Fig. 1A). Then we analyzed cell activity by measuring the enzymatic catabolism of formazan to WST-1. Our results showed that, similarly to proliferation, the viability of hMSCs was influenced by pH, and more viable cells were observed at physiologic pH (7.0, 7.4, and 8.0) while cell viability at pH (6.3, 6.7, and 8.5) decreased (Fig. 1B).

These findings suggested that the physiological pH (7.0, 7.4, and 8.0) was suitable for hMSC growth. Since the cell viability at pH 8.5 was severely decreased, this result indicated that alkaline environment up to a certain limit was advantageous for cell growth.

3.2. pH effect on hMSCs apoptosis and senescence

Observing the fact that pH (6.3, 6.7, and 8.5) resulted in less self-renewal of hMSCs, we next investigated the reasons for this. We checked whether the cells had undergone apoptosis or senescence. Apoptosis was inspected using JC-1 staining that shows the loss of the mitochondrial membrane potential. In healthy cells, the dye stains the mitochondria bright red, whereas in apoptotic cells, the mitochondrial membrane potential collapses and JC-1 stains the cells green. The results showed that cells cultured in different pH media appeared orange-red and the green cells were the positive control, suggesting that pH did not induce apoptosis in cells (Fig. 2A).

In addition, we tested whether different pH would trigger senescence. We found that treatment of hMSCs with different pH

Table 1
Sequences of the polymerase chain reaction primers with the annealing temperatures and the expected sizes of the amplified products.

Gene	Name	Primer sequence (F, R, 5′-3′)	$T_{annealing}$ (°C)	Product size (bp)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	F: CAA CTA CAT GGT TTA CAT GTT C R: GCC AGT GGA CTC CAC GAC	50 °C	181
RunX2	Runt-related transcription factor 2	F: TCT TCA CAAATC CTC CCC R: TGG ATT AAA AGG ACT TGG TG	55 °C	230
OCN	Osteocalcin	F: GGC ACA AAG AAG CCG TAC TC R: CAC TGG GCA GAC AGT CAG AA	56 °C	242
OPN	Osteopontin	F: CTG ATG AAC TGG TCA CTG ATT TTC R: CCG CTT ATA TAA TCT GGA CTG CTT	60 °C	347
Col1α1	Collagen 1alpha 1	F: AGG GCT CCA ACG AGA TCG AGA TCC G R: TAC AGG AAG CAG ACA GGG CCA ACG TCG	54 °C	223

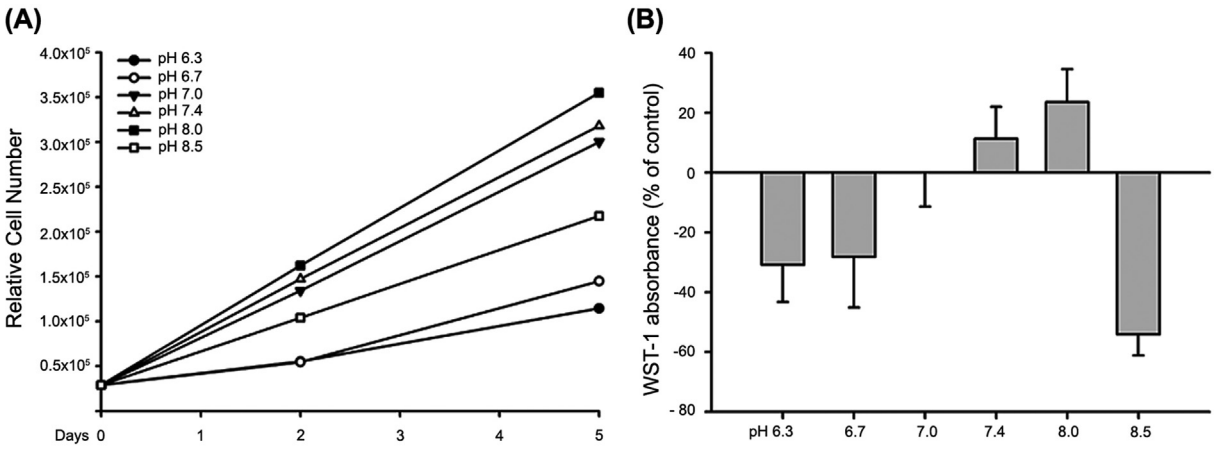

Fig. 1. Effect of pH on proliferation and viability of human bone marrow stem cells (hMSCs). (A) Proliferation of human bone marrow stem cells (hMSCs) in different pH media from day 0 to day 5. hMSCs grown in pH 97.0, 7.4, and 8.0 showed the highest proliferation rate compared with those grown in pH 6.3, 6.7, and 8.5. (B) Effect of pH on viability of hMSCs cultured at different pH for 3 days was measured at the indicated time points using WST-1 assay and expressed as optical density at 450 nm (A_{450}) as described in Material and Methods. Error bars represent standard deviations (n = 2).

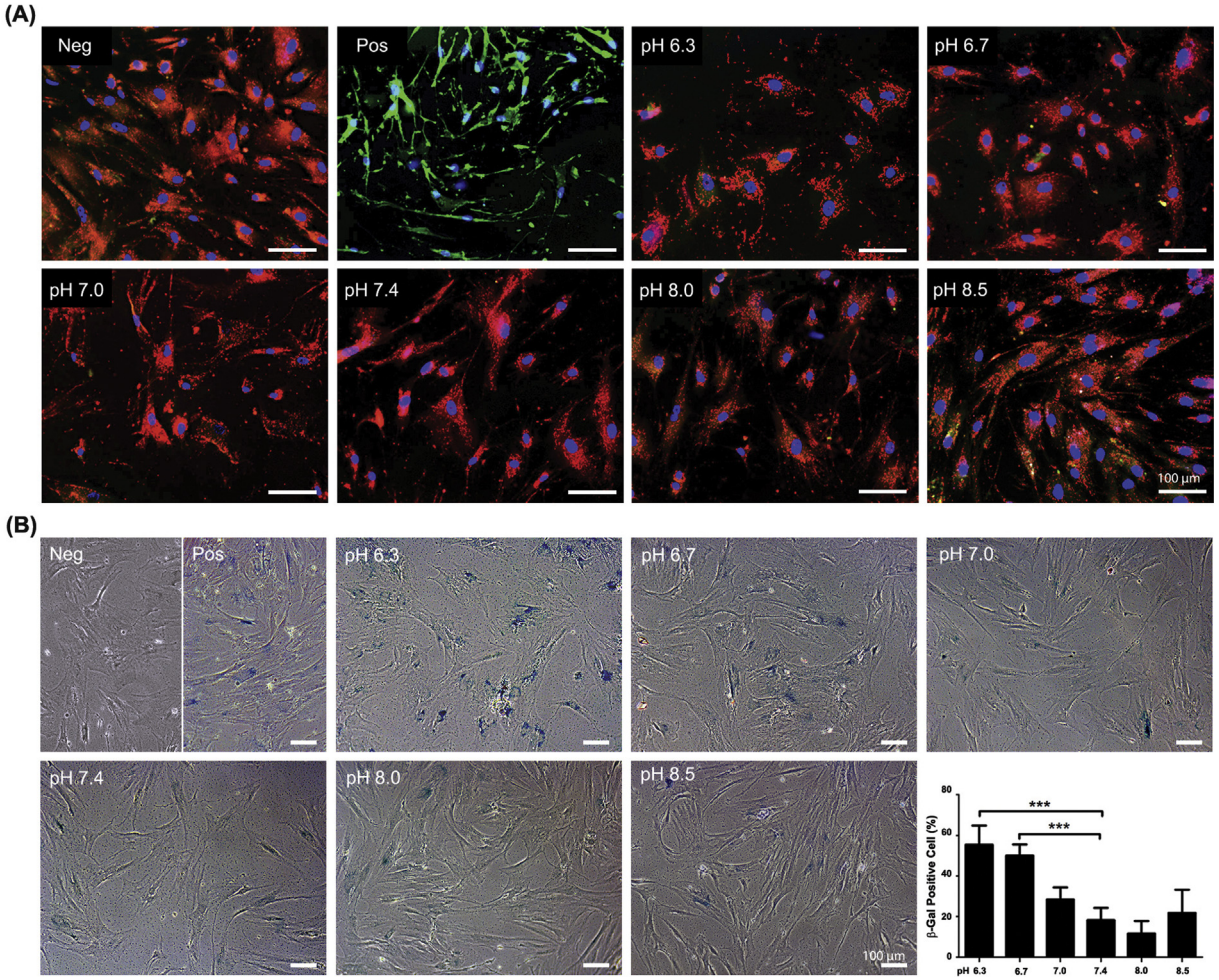

Fig. 2. Apoptosis and senescence of human bone marrow stem cells (hMSCs) at different pH. (A) Morphological observation of JC-1 and Hoechst 33342 staining of cells treated at different pH examined with fluorescence microscope at ×10 magnification; scale bar represents 100 µm. The experiments were performed in two different donors. Cells at different pH appeared orange-red, whereas the positive control (hydrogen peroxide–treated cells) showed strong green fluorescence and indicated typical apoptotic morphology. (B) hMSCS senescence at different pH conditions measured by SA β-Gal activity assay. The nuclei of senescent cells are surrounded by cyan dye; a significant increase in cell size was detected at pH 6.3, 6.7, and 8.5. Staining was quantified by positive cell count. Error bars represent the means ± SD; n = 2. $P < 0.0001$.

media for 3 days resulted in senescent cells in cultures. Cells incubated at pH (6.3, 6.7, and 8.5) appeared flattened and were more positive for β-gal staining, whereas at physiologic pH (7.0, 7.4, and 8.0), cells maintained their spindle shape and only a few stained blue (Fig. 2B). Quantification of β-gal staining demonstrated that the staining frequency of hMSCs was approximately 58% blue-positive at pH 6.3, 56% at pH 6.7, and 25% for pH 8.5. In contrast, the frequency for pH 7.0 was 30%, whereas at pH 7.4, it was 18% and at pH 8.0 it was about 15%, which nearly lacked detectable β-gal activity (Fig. 2B).

3.3. Osteogenic differentiation of hMSCs and mineralization assay

We performed osteogenic differentiation of hMSCs in different pH osteogenic media (OD) or control media. At day 21, Alizarin Red staining confirmed osteogenic differentiation and matrix mineralization of hMSCs. Cells grown in OD exhibited red staining at pH (7.0, 7.4, and 8.0); among them, pH 8.0 showed the strongest staining. At pH other than physiologic, the cells showed weaker or no mineralization (Fig. 3A and B).

3.4. Quantitative estimation of ALP activity and RT-PCR of osteogenic genes

To validate the defected mineralization under various pH conditions, we first investigated the changes in ALP activity. Our results showed no significant differences at different pH conditions at days 0, 2, 5, and 7. However, from day 10 to day 14, ALP activity showed a significant difference, as its activity increased proportionally at

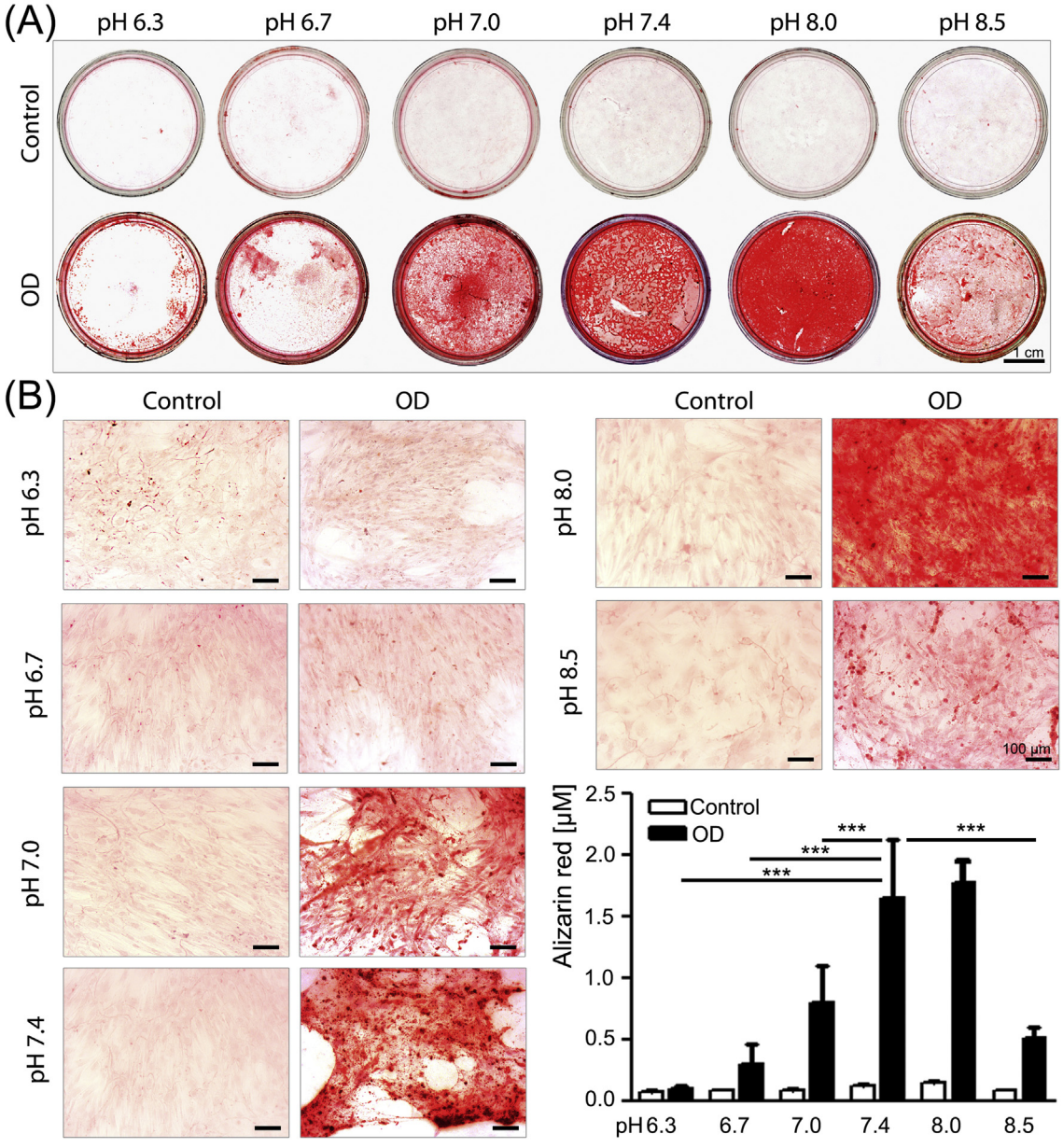

Fig. 3. Osteogenic differentiation of human bone marrow stem cells (hMSCs) and quantification of Alizarin Red staining. (A) Osteogenic differentiation of hMSCs stained with Alizarin Red; scale bars represent 1 cm. (B) Morphology of hMSCs grown in control or osteogenic medium (OD) at different pH (magnification ×10, scale bars = 100 µm). Cells were incubated for 21 days in DMEM containing 10% fetal bovine serum and observed under a phase contrast microscope with Alizarin Red staining quantification. Osteogenic differentiation showed significant difference of the amount of soluble Alizarin Red. The average absorbance value at 405 nm. Error bars represent standard deviations; n = 2. $P < 0.0001$.

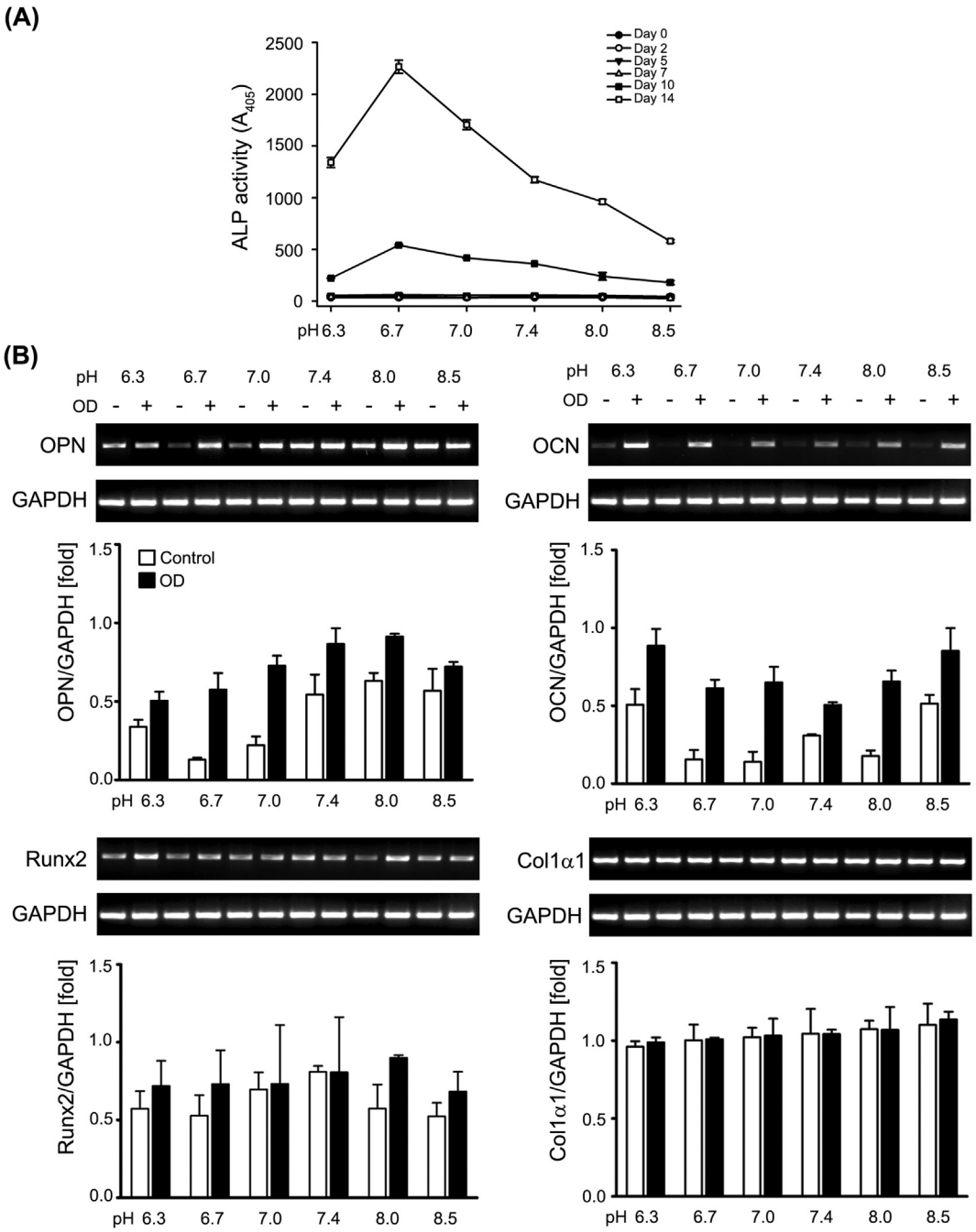

Fig. 4. Alkaline phosphatase (ALP) activity of the osteogenic differentiated mesenchymal stem cells at different pH, with the expression level of bone-related markers (OPN, OCN, Runx2, and Col1α1) of hMSCs cultured in control and osteogenic media (OD) at different pH values for 21 days. (A) ALP activity was measured during the course of osteogenic differentiation from day 0 to day 14 and showed that it was inversely proportional to the pH: when the pH increased, the ALP activity increased, and vice versa. (B) Reverse transcription–polymerase chain reaction data of OPN, OCN, Runx2, and Col1α1 representative of three independent experiments from three different donors were combined together and analyzed. Runx2 codes for major osteogenic transcription factors; Col1α1 is an early marker of osteogenic differentiation; OCN and OPN are markers of late stages of osteogenesis. GAPDH was used as the control housekeeping gene for this study. Graphs representing mean values of relative optical densities of polymerase chain reaction results are shown in the mRNA expression patterns of osteogenic marker genes in cells at day 21; the results are expressed as the fold change relative to the respective control.

lower pH (6.3 and 6.7) (Fig. 4A). Additionally, our RT-PCR analysis showed that all of the important osteogenic markers were expressed by the cells in comparison to control media. From the assessed genes, pH media had an effect on OPN and OCN, whereas Col1α1 and Runx2 was pH independent. OPN increased gradually with increasing the pH of the media until pH 8.0 and then down-regulated at pH 8.5. The expression of OPN in osteogenic differentiated cells was always higher compared to control media. In contrast to OPN, OCN had an opposite correlation whereby pH (6.3 and 6.7) showed higher expression, followed by pH 8.5 and then the physiologic pHs (Fig. 4B).

4. Discussion

In this study, we confirmed that hMSCs are sensitive to pH as their self-renewal and mineralization were significantly affected. Our study provides new insight into the mechanism underlying pH-related bone destruction and adds an important facet to the

linkage between pH and bone infections that might be used clinically in the future to treat osteomyelitis of the jaw. We have selected pH values in accordance to their relevance in vivo as follows: pH 6.3 to 6.7 is common in infection (Otto et al., 2010) and in cultures with high cell numbers but limited nutrients (Naciri et al., 2008); pH 7.0 to 7.4 is commonly used in cell culture (Mackenzie et al., 1961) and a typical value in the bloodstream (Arnett, 2008); and pH 8.0 to 8.5 is recommended for greater production of osteocytes (Moghadam et al., 2014). An in vitro approach was used to answer two clinically important questions: First, what is the effect of pH on self-renewal and differentiation? Second, how can we make use of this knowledge for preventing or treating osteomyelitis of the jaw?

Osteomyelitis is prevalent in the facial skeleton associated with abnormal bone remodeling and massive bone resorption. It also presents a major complication ensuing orthopedic and maxillofacial surgeries as well as routine dental extractions (Uskokovic et al., 2013). There is increased formation and activity of osteoclasts in osteomyelitis, together with the elimination of the osteoblasts responsible for new bone matrix deposition following infection (Mori et al., 2007). Infection causes some essential changes in the extracellular milieu. On these occasions, the pH of the bone tissue environment often falls below pH 7.0, whereas in healthy tissues this pH value varies in the range 7.35–7.45 (Kinnari et al., 2009).

During early embryonic development, pH regulation is critical for cell metabolism, intracellular ionic signaling, differentiation, quiescence, and proliferation (Taylor and Hodson, 1984; Musgrove et al., 1987). pH controlled self-renewal (proliferation and viability) as well as expression of extracellular matrix proteins, not only in fibroblasts but also in several cell types by affecting the cytoskeleton and cell adhesion molecules in addition to arresting the cell cycle at the G1 phase (Laffey et al., 2000; Wuertz et al., 2009; Teo et al., 2014). Our results demonstrate that changes in pH other than the physiologic can negatively influence cell proliferation and viability of MSCs, which might be caused by several factors such as apoptosis or senescence.

It is not clear what determines whether cells undergo senescence or apoptosis. One determinant is cell type; for example, damaged fibroblasts and epithelial cells tend to senesce, whereas damaged lymphocytes tend to undergo apoptosis (Campisi and d'Adda di Fagagna, 2007). Although it is well known that pH regulates many vital cell functions (Busa and Nuccitelli, 1984), the effect of pH on apoptotic signaling is poorly defined. Loss of the mitochondrial membrane potential is a hallmark of intrinsic apoptosis, because it is associated with the release of pro-apoptotic proteins into the cytosol (Brunelle and Letai 2009). Some studies have demonstrated that severe extracellular acidification or alkalization induced a pro-apoptotic effect (D'Arcangelo et al., 2000; Cutaia et al., 2005); in addition, other studies revealed a link between acidosis and apoptosis (Webster et al., 1999; Aoyama et al., 2005), and another study showed that pH had no effect on mitochondria-mediated apoptosis in hMSCs (Brandao-Burch et al., 2005). Even though viability testing revealed a pH dependency, it was difficult to draw conclusions about apoptotic processes. Comparing the apoptotic events in our experiment, we did not find increased apoptosis throughout the different pH conditions. It is possible that this may represent a time-dependent phenomenon, and that 7 days or more may be required to observe an enhancement in hMSC apoptosis. Cellular senescence occurs in response to various cellular stresses with the loss of proliferative capacity, despite continued viability and metabolic activity (Kuilman et al., 2010). From our results, we saw that the strongest senescence occurred under the acidic pH (6.3 and 6.7). Taken together, we found that the effect of pH on proliferation or viability is modulated through increased senescence.

MSCs are characterized not only by the capacity for self-renewal but also by the ability to differentiate into osteoblasts and deposition of matrix minerals in which pH plays a regulatory role in the process of mineralization and bone repair (Chakkalakal et al., 1994; Di Benedetto et al., 2015). Poor mineralization at alkaline conditions beyond pH 8.0 affected the solubility of calcium and magnesium pyrophosphate with no beneficial effect on bone mineralization (Simao et al., 2013). It was also suggested that acidic pH reduces bone mineralization via increased hydroxyapatite solubility, and that systemic alkali therapy can be used to treat osteomalacia and the bone pain associated with it (Richards et al., 1972; Disthabanchong et al., 2004; Brandao-Burch et al., 2005). The physicochemical mechanism also plays a role in matrix mineralization, based on the fact that low pH decreases calcium and phosphate tissue deposition because it increases their solubility (Larsen and Jensen, 1989, Iyemere et al., 2006). The most effective ways to destroy the ability of the nucleation core to induce mineral formation is exposure to acidic citrate buffer (Wu et al., 1993). Also, the nucleation activity and core is operative only within a very narrow pH range, between 7.4 and 7.8 (Valhmu et al., 1990). Either below or above this range, its ability to nucleate mineral formation was very much reduced. However, in studies by Wu et al (Wu et al., 2008), the pH range in which rapid mineral formation occurred was broader (pH 7.4–8.0), indicating that at pH 8.0, the nucleation core is highly stable and insoluble. In accordance with these data, our results showed that a slight elevation in pH from 7.4 to 8.0 significantly increases the mineralization, and the rise of pH to 8.5 does not further drive differentiation. This implies that small pH fluctuations will facilitate bone formation by elevating the phosphate ratio at least in the very narrow pH zone where the nucleation core is operative, up to a maximum of pH 8.0.

Since we have found defective mineralization at certain pH conditions, a question regarding the reason for the defective mineralization remained. It occurred due to impairment of osteogenic differentiation or due to the change in the extracellular environment. Therefore we performed PCR to analyze the key osteogenic markers for differentiation and mineralization. From our results, the PCR results were different from the Alizarin red staining, and late markers of osteogenesis were expressed on PCR with a lack of mineralization in the staining.

Osteoblasts arise from mesenchymal stem cells and determine the formation and structural organization of bone extracellular matrix and its mineralization (Marie, 2008). ALP is synthesized by the osteoblasts and is presumed to be involved in the calcification of bone matrix (Masrour Roudsari and Mahjoub, 2012). Some researchers have shown that pH 8.5 was optimum for ALP activity toward inorganic pyrophosphate during bone formation, whereas the activity was retained at pH 7.3 to 7.4 (Harada et al., 1986; Kaunitz and Yamaguchi, 2008). It was reported that decreasing the extracellular pH reduced the amount of collagen and ALP activity in mesenchymal stem cells, whereas others reported that alkaline pH decreased the ALP activity and could delay the differentiation of MSCs (Kohn et al., 2002; Leem et al., 2012). It was shown in the literature that a higher calcium concentration inhibits the ALP activity but stimulates the expression of OPN associated with the osteogenic differentiation (Cheng et al., 2013). ALP activity appeared to decrease during mineralization (McLean et al., 1987). In another study, it was also reported that a consistent marked loss of ALP activity occurs during mineralization. The time of onset and the extent of decline in ALP activity were found to mirror almost exactly the time of onset and the extent of calcium accumulation by the matrix vesicles (MV) (Genge et al., 1988). Our results showed that ALP was decreased at higher pH, indicating that mineralization downregulated the ALP activity.

In parallel, we also investigated the changes of the expression levels of several key osteogenic genes such as Runx2, collagen I (Col1α1), OPN, and OCN. We reported that among the analyzed genes only OPN and OCN were slightly influenced by the different pH values. In the body, OPN is normally linked to mineralization of the tissues (Kohri et al., 1993) and, similar to our data, was found to be sensitive to pH (Frick and Bushinsky, 1998; Brandao-Burch et al., 2005). The highest expression that we observed was less than pH 8.0, whereas the least was detected at acidic pH (6.3 and 6.7). The other osteogenic marker, OCN is linked to terminally differentiated osteoblasts; however, its role in bone mineralization remains unclear, because in OCN-deficient mice, it was discovered that osteocalcin does not necessarily ensure normal osteoblast function (Ducy et al., 1996). The trend in OCN expression in our hMSCs showed increased levels under lower and higher pH values (different from physiologic). Analysis of the other two osteogenic markers, collagen I and Runx2, showed no significant changes upon pH treatment. In all pH conditions during differentiation, we found strong upregulation of both genes. Collagen I is the main building protein of bone, whereas Runx2 is the master regulator of osteoblast lineage (Komori et al., 1997; Otto et al., 1997; Jonason et al., 2009) that controls expression of several osteogenic genes, among which is collagen I (Ducy et al., 1997). Expression of Runx2 and collagen I can be affected by the pH was dependent on the MSC donor (Sprague et al., 1994; Frick and Bushinsky, 1998; Brandao-Burch et al., 2005; Disthabanchong et al., 2006).

The difference between the osteogenic markers expression and the matrix mineralization can be explained by initiation of matrix vesicle–mediated mineralization followed by collagen-mediated mineralization. The matrix vesicle mineralization is characterized by an initial formation of apatite or primary nucleation intracellularly within matrix vesicles (MV) that transport hydroxyapatite (HA) crystals outside of the cells (Ali et al., 1970; Anderson, 1995; Anderson et al., 2005; Golub, 2009). During collagen-mediated mineralization (secondary nucleation), MV membranes break down and expose preformed HA to the extracellular fluid, allowing propagation of HA deposition onto the collagenous ECM (Anderson, 1995; Anderson et al., 2005), leading to mineralization by physicochemical and biochemical processes (Millan, 2013). At low pH, calcium and phosphate tissue deposition decreases by increasing HA solubility with 10-fold for each unit decrease in pH (Thylstrup and Fejerskov, 1986; Larsen and Jensen, 1989; Iyemere et al., 2006). According to our data, pH had an effect on hMSCs mineralization potential whereby induction of mineralization was more efficient at physiologic pH 7.0, 7.4, and 8.0 and much less at pH 6.3, 6.7, and 8.5.

Taken together, our study demonstrates that different pH conditions can strongly affect both cell self-renewal and mineralization. However, the same pH did not affect cell osteogenic potential, since the main lineage–specific markers were expressed.

A number of limitations of this study needed to be considered. For instance, one question still not answered is whether comparison to diseased tissue would have been advantageous to determine cell responses to alterations in the physicochemical environment. Direct comparison can often be complicated due to inherent heterogeneity of both normal and diseased tissue and the difficulty in obtaining bone samples. Another limitation is that cells from different lots or donors were used, causing variability of the results represented by large means and standard deviations. Despite these limitations, the effect of pH on the gene expression remains.

5. Conclusion

In this study, it was demonstrated that MSCs were highly sensitive to small shifts in external pH, as their viability, proliferation, and mineralization were affected. However, the osteogenic differentiation was not affected by pH. Thus, we think that at the injured sites, MSC behavior could be altered by the extracellular pH. The results of our study indicate that changing the pH of culture medium from normal to alkaline medium could improve the differentiation of MSCs to osteoblasts. There are currently various treatments clinically available for treating osteomyelitis of the jaw due to the complex nature of the infection, including the presence of microorganisms and change in pH. Future therapies for treating osteomyelitis could be based on shifting the pH of the local environment in the alkaline direction to overcome the acidic inflammatory exudates released during infection.

Conflict of interest

The authors have declared that there is no conflict of interest.

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References

Alberton P, Popov C, Pragert M, Kohler J, Shukunami C, Schieker M, et al: Conversion of human bone marrow-derived mesenchymal stem cells into tendon progenitor cells by ectopic expression of scleraxis. Stem Cells Dev 21: 846–858, 2012
Ali SY, Sajdera SW, Anderson HC: Isolation and characterization of calcifying matrix vesicles from epiphyseal cartilage. Proc Natl Acad Sci USA 67: 1513–1520, 1970
Anderson HC: Molecular biology of matrix vesicles. Clin Orthop Relat Res: 266–280, 1995
Anderson HC, Garimella R, Tague SE: The role of matrix vesicles in growth plate development and biomineralization. Front Biosci 10: 822–837, 2005
Aoyama K, Burns DM, Suh SW, Garnier P, Matsumori Y, Shiina H, et al: Acidosis causes endoplasmic reticulum stress and caspase-12-mediated astrocyte death. J Cereb Blood Flow Metab 25: 358–370, 2005
Arnett TR: Extracellular pH regulates bone cell function. J Nutr 138: 415S–418S, 2008
Arnett TR, Dempster DW: Effect of pH on bone resorption by rat osteoclasts in vitro. Endocrinology 119: 119–124, 1986
Arnett TR, Dempster DW: Protons and osteoclasts. J Bone Miner Res 5: 1099–1103, 1990
Bianco P, Riminucci M, Gronthos S, Robey PG: Bone marrow stromal stem cells: nature, biology, and potential applications. Stem Cells 19: 180–192, 2001
Brandao-Burch A, Utting JC, Orriss IR, Arnett TR: Acidosis inhibits bone formation by osteoblasts in vitro by preventing mineralization. Calcif Tissue Int 77: 167–174, 2005
Brunelle JK, Letai A: Control of mitochondrial apoptosis by the Bcl-2 family. J Cell Sci 122: 437–441, 2009
Busa WB, Nuccitelli R: Metabolic regulation via intracellular pH. Am J Physiol 246: R409–R438, 1984
Campisi J, d'Adda di Fagagna F: Cellular senescence: when bad things happen to good cells. Nat Rev Mol Cell Biol 8: 729–740, 2007
Chakkalakal DA, Mashoof AA, Novak J, Strates BS, McGuire MH: Mineralization and pH relationships in healing skeletal defects grafted with demineralized bone matrix. J Biomed Mater Res 28: 1439–1443, 1994
Cheng S, Wang W, Lin Z, Zhou P, Zhang X, Zhang W, et al: Effects of extracellular calcium on viability and osteogenic differentiation of bone marrow stromal cells in vitro. Hum Cell 26: 114–120, 2013
Cutaia M, Black AD, Cohen I, Cassai ND, Sidhu GS: Alkaline stress-induced apoptosis in human pulmonary artery endothelial cells. Apoptosis 10: 1457–1467, 2005
D'Arcangelo D, Facchiano F, Barlucchi LM, Melillo G, Illi B, Testolin L, et al: Acidosis inhibits endothelial cell apoptosis and function and induces basic fibroblast growth factor and vascular endothelial growth factor expression. Circ Res 86: 312–318, 2000
Di Benedetto A, Brunetti G, Posa F, Ballini A, Grassi FR, Colaianni G, et al: Osteogenic differentiation of mesenchymal stem cells from dental bud: role of integrins and cadherins. Stem Cell Res 15: 618–628, 2015
Disthabanchong S, Domrongkitchaiporn S, Sirikulchayanonta V, Stitchantrakul W, Karnsombut P, Rajatanavin R: Alteration of noncollagenous bone matrix proteins in distal renal tubular acidosis. Bone 35: 604–613, 2004

Disthabanchong S, Radinahamed P, Stitchantrakul W, Hongeng S, Rajatanavin R: Chronic metabolic acidosis alters osteoblast differentiation from human mesenchymal stem cells. Kidney Int 71: 201–209, 2006
Ducy P, Desbois C, Boyce B, Pinero G, Story B, Dunstan C, et al: Increased bone formation in osteocalcin-deficient mice. Nature 382: 448–452, 1996
Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G: Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell 89: 747–754, 1997
Eriksen EF: Cellular mechanisms of bone remodeling. Rev Endocr Metab Disord 11: 219–227, 2010
Frick KK, Bushinsky DA: Chronic metabolic acidosis reversibly inhibits extracellular matrix gene expression in mouse osteoblasts. Am J Physiol 275: F840–F847, 1998
Genge BR, Sauer GR, Wu LN, McLean FM, Wuthier RE: Correlation between loss of alkaline phosphatase activity and accumulation of calcium during matrix vesicle-mediated mineralization. J Biol Chem 263: 18513–18519, 1988
Golub EE: Role of matrix vesicles in biomineralization. Biochim Biophys Acta 1790: 1592–1598, 2009
Green J: Cytosolic pH regulation in osteoblasts. Miner Electrolyte Metab 20: 16–30, 1994
Han S-H, Chae S-W, Choi J-Y, Kim E-C, Chae H-J, Kim H-R: Acidic pH environments increase the expression of cathepsin B in osteoblasts: the significance of ER stress in bone physiology. Immunopharmacol Immunotoxicol 31: 428–431, 2009
Harada M, Udagawa N, Fukasawa K: Hiraoka BY and Mogi M. Inorganic pyrophosphatase activity of purified bovine pulp alkaline phosphatase at physiological pH. J Dent Res 65: 125–127, 1986
Hatzenbuehler J, Pulling TJ: Diagnosis and management of osteomyelitis. Am Fam Physician 84: 1027–1033, 2011
Humber CC, Albilia JB, Rittenberg B: Chronic osteomyelitis following an uncomplicated dental extraction. J Can Dent Assoc 77: b98, 2011
Issekutz AC, Bhimji S: Role for endotoxin in the leukocyte infiltration accompanying Escherichia coli inflammation. Infect Immun 36: 558–566, 1982
Iyemere VP, Proudfoot D, Weissberg PL, Shanahan CM: Vascular smooth muscle cell phenotypic plasticity and the regulation of vascular calcification. J Intern Med 260: 192–210, 2006
Jonason JH, Xiao G, Zhang M, Xing L, Chen D: Post-translational regulation of Runx2 in bone and cartilage. J Dent Res 88: 693–703, 2009
Kaunitz JD, Yamaguchi DTTNAP: TrAP, ecto-purinergic signaling, and bone remodeling. J Cell Biochem 105: 655–662, 2008
Kaysinger KK, Ramp WK: Extracellular pH modulates the activity of cultured human osteoblasts. J Cell Biochem 68: 83–89, 1998
Kim DH, Yoo KH, Choi KS, Choi J, Choi SY, Yang SE, et al: Gene expression profile of cytokine and growth factor during differentiation of bone marrow-derived mesenchymal stem cell. Cytokine 31: 119–126, 2005
Kinnari TJ, Esteban J, Martin-de-Hijas NZ, Sanchez-Munoz O, Sanchez-Salcedo S, Colilla M, et al: Influence of surface porosity and pH on bacterial adherence to hydroxyapatite and biphasic calcium phosphate bioceramics. J Med Microbiol 58: 132–137, 2009
Knight MN, Hankenson KD: Mesenchymal stem cells in bone regeneration. Adv Wound Care (New Rochelle) 2: 306–316, 2013
Kohler J, Popov C, Klotz B, Alberton P, Prall WC, Haasters F, et al: Uncovering the cellular and molecular changes in tendon stem/progenitor cells attributed to tendon aging and degeneration. Aging Cell 12: 988–999, 2013
Kohn DH, Sarmadi M, Helman JI, Krebsbach PH: Effects of pH on human bone marrow stromal cells in vitro: implications for tissue engineering of bone. J Biomed Mater Res 60: 292–299, 2002
Kohri K, Nomura S, Kitamura Y, Nagata T, Yoshioka K, Iguchi M, et al: Structure and expression of the mRNA encoding urinary stone protein (osteopontin). J Biol Chem 268: 15180–15184, 1993
Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, et al: Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 89: 755–764, 1997
Kuilman T, Michaloglou C, Mooi WJ, Peeper DS: The essence of senescence. Genes Dev 24: 2463–2479, 2010
Laffey JG, Engelberts D, Kavanagh BP: Injurious effects of hypocapnic alkalosis in the isolated lung. Am J Respir Crit Care Med 162: 399–405, 2000
Larsen MJ, Jensen SJ: The hydroxyapatite solubility product of human dental enamel as a function of pH in the range 4.6-7.6 at 20 degrees C. Arch Oral Biol 34: 957–961, 1989
Leem YH, Nam TS, Kim JH, Lee KS, Lee DH, Yun J, et al: The effects of extracellular pH on proliferation and differentiation of human bone marrow stem cells. Korean J Bone Metab 19: 35–46, 2012
Ma L, Liu M, Liu H, Chen J, Cui D: In vitro cytotoxicity and drug release properties of pH- and temperature-sensitive core-shell hydrogel microspheres. Int J Pharmacol 385: 86–91, 2010
Mackenzie CG, Mackenzie JB, Beck P: The effect of pH on growth, protein synthesis, and lipid-rich particles of cultured mammalian cells. J Biophys Biochem Cytol 9: 141–156, 1961
Marie PJ: Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys 473: 98–105, 2008
Marriott I, Gray DL, Tranguch SL, Fowler Jr VG, Stryjewski M, Scott Levin L, et al: Osteoblasts express the inflammatory cytokine interleukin-6 in a murine model of *Staphylococcus aureus* osteomyelitis and infected human bone tissue. Am J Pathol 164: 1399–1406, 2004
Masrour Roudsari J, Mahjoub S: Quantification and comparison of bone-specific alkaline phosphatase with two methods in normal and Paget's specimens. Caspian J Intern Med 3: 478–483, 2012
McLean FM, Keller PJ, Genge BR, Walters SA, Wuthier RE: Disposition of preformed mineral in matrix vesicles. Internal localization and association with alkaline phosphatase. J Biol Chem 262: 10481–10488, 1987
Millan JL: The role of phosphatases in the initiation of skeletal mineralization. Calcif Tissue Int 93: 299–306, 2013
Moghadam FH, Tayebi T, Dehghan M, Eslami G, Nadri H, Moradi A, et al: Differentiation of bone marrow mesenchymal stem cells into chondrocytes after short term culture in alkaline medium. Int J Hematol Oncol Stem Cell Res 8: 12–19, 2014
Moore KA, Lemischka IR: Stem cells and their niches. Science 311: 1880–1885, 2006
Mori G, Brunetti G, Colucci S, Ciccolella F, Coricciati M, Pignataro P, et al: Alteration of activity and survival of osteoblasts obtained from human periodontitis patients: role of TRAIL. J Biol Regul Homeost Agents 21: 105–114, 2007
Musgrove E, Seaman M, Hedley D: Relationship between cytoplasmic pH and proliferation during exponential growth and cellular quiescence. Exp Cell Res 172: 65–75, 1987
Muzylak M, Arnett TR, Price JS, Horton MA: The in vitro effect of pH on osteoclasts and bone resorption in the cat: implications for the pathogenesis of FORL. J Cell Physiol 213: 144–150, 2007
Naciri M, Kuystermans D, Al-Rubeai M: Monitoring pH and dissolved oxygen in mammalian cell culture using optical sensors. Cytotechnology 57: 245–250, 2008
Nair MB, Kretlow JD, Mikos AG, Kasper FK: Infection and tissue engineering in segmental bone defects—a mini review. Curr Opin Biotechnol 22: 721–725, 2011
Newman RJ, Francis MJ, Duthie RB: Nuclear magnetic resonance studies of experimentally induced delayed fracture union. Clin Orthop Relat Res: 253–261, 1987
Nuschke A, Rodrigues M, Stolz DB, Chu CT, Griffith L, Wells A: Human mesenchymal stem cells/multipotent stromal cells consume accumulated autophagosomes early in differentiation. Stem Cell Res Ther 5: 140, 2014
Otto F, Thornell AP, Crompton T, Denzel A, Gilmour KC, Rosewell IR, et al: Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell 89: 765–771, 1997
Otto S, Hafner S, Mast G, Tischer T, Volkmer E, Schieker M, et al: Bisphosphonate-related osteonecrosis of the jaw: is pH the missing part in the pathogenesis puzzle? J Oral Maxillofac Surg 68: 1158–1161, 2010
Pavlukhina S, Lu Y, Patimetha A, Libera M, Sukhishvili S: Polymer multilayers with pH-triggered release of antibacterial agents. Biomacromolecules 11: 3448–3456, 2010
Pereira RF, Halford KW, O'Hara MD, Leeper DB, Sokolov BP, Pollard MD, et al: Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. Proc Natl Acad Sci U S A 92: 4857–4861, 1995
Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al: Multilineage potential of adult human mesenchymal stem cells. Science 284: 143–147, 1999
Popov C, Radic T, Haasters F, Prall WC, Aszodi A, Gullberg D, et al: Integrins alpha2beta1 and alpha11beta1 regulate the survival of mesenchymal stem cells on collagen I. Cell Death Dis 2: e186, 2011
Qin Y, Guan J, Zhang C: Mesenchymal stem cells: mechanisms and role in bone regeneration. Postgrad Med J 90: 643–647, 2014
Ramp WK, Lenz LG, Kaysinger KK: Medium pH modulates matrix, mineral, and energy metabolism in cultured chick bones and osteoblast-like cells. Bone Miner 24: 59–73, 1994
Redlich K, Smolen JS: Inflammatory bone loss: pathogenesis and therapeutic intervention. Nat Rev Drug Discov 11: 234–250, 2012
Richards P, Chamberlain MJ, Wrong OM: Treatment of osteomalacia of renal tubular acidosis by sodium bicarbonate alone. Lancet 2: 994–997, 1972
Romas E, Gillespie MT: Inflammation-induced bone loss: can it be prevented? Rheum Dis Clin North Am 32: 759–773, 2006
Sanchez Jr CJ, Ward CL, Romano DR, Hurtgen BJ, Hardy SK, Woodbury RL, et al: *Staphylococcus aureus* biofilms decrease osteoblast viability, inhibits osteogenic differentiation, and increases bone resorption in vitro. BMC Musculoskelet Disord 14: 187, 2013
Sax H, Lew D: Osteomyelitis. Curr Infect Dis Rep 1: 261–266, 1999
Shen Y, Liu W, Wen C, Pan H, Wang T, Darvell BW, et al: Bone regeneration: importance of local pH-strontium-doped borosilicate scaffold. J Mater Chem 22: 8662–8670, 2012
Simao AM, Bolean M, Hoylaerts MF, Millan JL, Ciancaglini P: Effects of pH on the production of phosphate and pyrophosphate by matrix vesicles' biomimetics. Calcif Tissue Int 93: 222–232, 2013
Singh M, Singh S, Jain J, Singh KT: Chronic suppurative osteomyelitis of maxilla mimicking actinimycotic osteomyelitis: a rare case report. Natl J Maxillofac Surg 1: 153–156, 2010
Spector JA, Mehrara BJ, Greenwald JA, Saadeh PB, Steinbrech DS, Bouletreau PJ, et al: Osteoblast expression of vascular endothelial growth factor is modulated by the extracellular microenvironment. Am J Physiol Cell Physiol 280: C72–C80, 2001
Sprague SM, Krieger NS, Bushinsky DA: Greater inhibition of in vitro bone mineralization with metabolic than respiratory acidosis. Kidney Int 46: 1199–1206, 1994

Swenson O, Claff CL: Changes in the hydrogen ion concentration of healing fractures. Proc Soc Exp Biol Med 61: 151–154, 1946
Taylor IW, Hodson PJ: Cell cycle regulation by environmental pH. J Cell Physiol 121: 517–525, 1984
Teitelbaum SL, Tondravi MM, Ross FP: Osteoclasts, macrophages, and the molecular mechanisms of bone resorption. J Leukoc Biol 61: 381–388, 1997
Teo A, Mantalaris A, Lim M: Influence of culture pH on proliferation and cardiac differentiation of murine embryonic stem cells. Biochem Engin J 90: 8–15, 2014
Thomas MV, Puleo DA: Infection, inflammation, and bone regeneration: a paradoxical relationship. J Dent Res 90: 1052–1061, 2011
Thylstrup A, Fejerskov O: Textbook of cariology. Copenhagen: Munksgaard, 1986
Uskokovic V, Hoover C, Vukomanovic M, Uskokovic DP, Desai TA: Osteogenic and antimicrobial nanoparticulate calcium phosphate and poly-(D,L-lactide-co-glycolide) powders for the treatment of osteomyelitis. Mater Sci Eng C Mater Biol Appl 33: 3362–3373, 2013
Valhmu WB, Wu LN, Wuthier RE: Effects of Ca/Pi ratio, Ca2+ x Pi ion product, and pH of incubation fluid on accumulation of 45Ca2+ by matrix vesicles in vitro. Bone Miner 8: 195–209, 1990
Webster KA, Discher DJ, Kaiser S, Hernandez O, Sato B, Bishopric NH: Hypoxia-activated apoptosis of cardiac myocytes requires reoxygenation or a pH shift and is independent of p53. J Clin Invest 104: 239–252, 1999
Wei X, Yang X, Han Z-p, Qu F-f, Shao L, Shi Y-f: Mesenchymal stem cells: a new trend for cell therapy. Acta Pharmacol Sin 34: 747–754, 2013
Wu LN, Genge BR, Wuthier RE: Analysis and molecular modeling of the formation, structure, and activity of the phosphatidylserine-calcium-phosphate complex associated with biomineralization. J Biol Chem 283: 3827–3838, 2008
Wu LN, Wuthier MG, Genge BR, Wuthier RE: In situ levels of intracellular Ca2+ and pH in avian growth plate cartilage. Clin Orthop Relat Res: 310–324, 1997
Wu LN, Yoshimori T, Genge BR, Sauer GR, Kirsch T, Ishikawa Y, et al: Characterization of the nucleational core complex responsible for mineral induction by growth plate cartilage matrix vesicles. J Biol Chem 268: 25084–25094, 1993
Wuertz K, Godburn K, Iatridis JC: MSC response to pH levels found in degenerating intervertebral discs. Biochem Biophys Res Commun 379: 824–829, 2009
Wuertz K, Godburn K, Neidlinger-Wilke C, Urban J, Iatridis JC: Behavior of mesenchymal stem cells in the chemical microenvironment of the intervertebral disc. Spine (Phila Pa 1976) 33: 1843–1849, 2008

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Fluorescence-guided surgery for the treatment of medication-related osteonecrosis of the jaw: A prospective cohort study

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ABSTRACT

Introduction: The delineation of the necrotic bone is a crucial step in the surgical treatment of medication-related osteonecrosis of the jaw (MRONJ). Several different approaches have been described including the innovative technique of fluorescence-guided surgery. However, until now there is a lack of data regarding the outcome. Therefore, the aim of the present study is to investigate the long-term success rates of fluorescence-guided surgery in the treatment of MRONJ.

Patients and methods: 54 Patients were prospectively assigned for surgical treatment of medication-related osteonecrosis of the jaw using fluorescence-guided surgery. Patients received doxycycline 100 mg twice a day for at least seven days preoperatively. Surgical treatment of MRONJ included complete removal of necrotic bone, which was monitored using the visual enhanced lesion scope (Velscope), followed by smoothening sharp bony edges and meticulous wound closure. Procedure success was assessed as postoperative maintenance of full mucosal coverage without pain, infection or bone exposure during regular follow-up.

Results: The study included a total of 54 patients (32 female and 22 male, mean age 71.4 ± 9.2 years). In the last follow-up an intact mucosa and absence of exposed bone, pain or signs of infection was identified in 47 of 54 patients (87%) and 56 of 65 lesions (86.2%) after first surgery using fluorescence-guidance. In 4 patients with 6 lesions a second fluorescence-guided surgery was necessary to achieve complete mucosal closure. Respectively, including the case with second surgical attempt 51 of 54 patients (94.4%) and 62 of 65 lesions (95.4%) showed complete mucosal healing.

Conclusion: The study shows that fluorescence-guided surgery is a safe and successful treatment option which can be considered for all stages of MRONJ. The technique seems also promising for MRONJ cases under denosumab.

1. Introduction

There is an ongoing debate on treatment strategies for medication-related osteonecrosis of the jaw (MRONJ): namely non-surgical (conservative) versus surgical treatment. The success rates for surgical strategies in MRONJ cases under bisphosphonates are significantly higher (Pautke et al., 2011; Stockmann et al., 2010; Voss et al., 2012; Carlson and Basile, 2009) than conservative treatment regimens (Marx et al., 2005; Hoff et al., 2008; Montebugnoli et al., 2007; O'Ryan et al., 2009; Watters et al., 2013; Fliefel et al., 2015) even though a direct prospective comparison between surgical and non-surgical treatment is missing till date.

MRONJ is currently diagnosed by the presence of exposed jawbone for a period that exceeds 8 weeks (Khosla et al., 2007, 2007; Ruggiero et al., 2009). Consequently, a successful therapy

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should aim for absence of bone exposure and restoration of mucosal integrity (Carlson and Basile, 2009; Ristow et al., 2015). Due to the fact that the infected necrotic and exposed bone will not be revitalized and resurrected, MRONJ should be removed even if only small bone areas are affected. Thus, the aim of the surgical therapy should be a complete removal of the necrotic bone. But even among those who favor surgical therapy there is an uncertainty as to which surgical technique is more effective. Indeed, the challenge as well as the limitations of the MRONJ therapy is that the margins of the osteonecrosis cannot be exactly determined, and therefore a clear demarcation of the necrotic bone is difficult if not impossible (Khosla et al., 2007, 2007; Pautke et al., 2009). The complete removal of necrotic bone is of crucial importance because otherwise there is the risk of disease recurrence or progression (Mucke et al., 2011; Carlson and Basile, 2009). Furthermore, it must be avoided to unintentionally and unnecessarily remove healthy bone without signs of osteonecrosis. Still, surgical experiences supported by various imaging modalities are used to remove only as much as necessary and the least amount possible of necrotic bone (Hutchinson et al., 2010; Fabbricini et al., 2009; Dore et al., 2009; Guggenberger et al., 2013). Therefore, surgical therapy is dependent on the surgeon and can neither be comparable nor reproducibly objectified.

Fluorescence-guided bone surgery has shown promising results in the surgical MRONJ management (Assaf et al., 2014; Pautke et al., 2006; Otto et al., 2013). Providing a controllable therapeutic approach, this technique may help to define the transitions between necrotic and non-necrotic bone during the surgical procedure. Due to the fact that this surgical approach is easy to apply and reproducible it may help to objectify surgical MRONJ therapy auguring an improvement of the treatment.

Therefore, the aim of this study is to examine the success rate of fluorescence-guided surgery in MRONJ patients in terms of postoperative mucosal integrity and absence of bone exposure. Furthermore, pain, infection rates as well as disturbances of sensitivity are monitored.

2. Materials and methods

2.1. Patients

Over a period of 5 years (2010–2014), 54 patients were recruited and prospectively included in our monocentric cohort study (Department of Oral and Maxillofacial Surgery, Ludwig-Maximilians-University, Munich, Germany). 32 female and 22 male patients were enrolled with a mean age of 71.4 (standard deviation ±9.2 years; age range, 45–91 years). Inclusion criteria were: Exposed necrotic jawbone over a period of more than 8 weeks (according Ruggiero et al., 2009, 2014); with a history of antiresorptive drug treatment (bisphosphonates and/or denosumab) in the absence of radiotherapy to the head and neck region (Ruggiero et al., 2009, 2014). Exclusion criteria were a history of head and neck irradiation, metastatic bone disease of the maxillofacial region and contradictions for surgery under general anesthesia. After obtaining the approval of the institutional ethics committee (LMU 189/10), patients were informed about all treatment options and provided written informed consent.

2.2. Surgical procedure

All surgical procedures were performed by the same board-certified and specialized Oral and Maxillofacial Surgeons (SO) under general anesthesia using a nasal intubation. The surgeries were performed under sterile conditions following a standardized operation protocol (Pautke et al., 2011).

All patients received 100 mg doxycycline twice a day for at least 7 days preoperatively. Surgical procedures were performed as the fluorescence guided surgery technique described previously by our group using the VELscope® system (LED Dental, White Rock, British Columbia, Canada) to induce and visualize fluorescence of the jaw bone (Pautke et al., 2011, 2009, 2012; Otto et al., 2013). After surgical bone exposure was performed the bone fluorescence showed viable bone in a bright greenish fluorescence and necrotic bone areas showed none or only pale fluorescence. Reddish fluorescence was considered as a bacterial colonization or infection of necrotic bone parts and the respective areas were removed. Necrotic bone was removed using a burr a homogenous greenish bone fluorescence was observed as described in previous studies (Assaf et al., 2014; Pautke et al., 2009, 2010, 2011; Fleisher et al., 2008). It should be stressed that only necrotic and infected bone parts were removed and the surrounding vital bone was preserved which means that no resections including safety margins have been performed. Thereafter, sharp bony edges were smoothened using burrs and diamante burrs. A tension free wound closure was achieved using mucoperiostal flaps and simple as well as back stitches (Serafit 3-0, SERAG-Wiesner GmbH Germany). In extensive cases of the maxillary molar and premolar region (stage 2 and 3) a second layer of wound closure was achieved using the buccal fat pad before mucoperiostal closure.

All patients stayed in hospital for at least 48 h after surgery. Patients received the routine postoperative instructions and routine postoperative analgesic drug therapy; antibiotic treatment was continued using Augmentin 2.2 g or Unacid 3g intravenously three times per day for 3–5 days. In case of a penicillin allergy clindamycine 600 mg was used. In cases of severe infection (mainly stage 2 and 3) metronidazole 500 mg (1-0-1) was administered additionally. In cases of renal function disturbances the doses were adjusted accordingly. The antibiotic treatment was continued orally after discharge from hospital for 2–4 weeks orally.

2.3. Measurements

Regular clinical examinations were performed daily during inpatient treatment, weekly during the first month and monthly during first year of out-patient treatment. The surgical treatment was only considered a success if full mucosal coverage without signs of residual infection or exposed bone was achieved at the time of last follow-up. Furthermore, all patients were asked for pain and were examined for signs of sinusitis and checked for oro-antral fistula in cases of upper jaw lesions and checked for sensitivity in the lower lip area in cases of MRONJ of the lower jaw.

2.4. Statistical analysis

Descriptive statistics were computed using SPSS version 16. Results are expressed as percentages or as mean values including standard deviation and range. Means were compared by statistical testing (students t-test), where $p < 0.05$ was considered to be significant.

3. Results

3.1. Baseline characteristics

54 patients (32 female and 22 male) patients with a mean age of 71.4 years (standard deviation 9.2 years) were included in the study. The mean age of the female patients was 70.4 years (standard deviation 7.6 years), the mean age of all male patients was 72.9 years (standard deviation 7.0 years). Respectively, there was no significant difference (see Fig. 1).

45 of the patients (83.3%) suffered from an underlying malignant disease, specifically breast cancer (n = 20; 37%), prostate cancer (n = 16; 29.6%), and multiple myeloma (n = 4; 7.4%). There were also cases of metastatic thyroid cancer (n = 2), squamous cell carcinoma (n = 1), bronchial cancer (n = 1), and endometrial cancer (n = 1) in the study cohort. In the remaining 9 (16.7%) patients osteoporosis was the cause of the antiresorptive treatment. An overview is given in Fig. 2.

Of the 54 patients included, 47 were treated with nitrogen-containing bisphosphonates (87%), 3 had a history of denosumab intake (5.5%) and the remaining 4 patients (7.4%) reported a sequential intake of bisphosphonates and denosumab. The most common antiresorptive drugs within the cohort were zoledronate (n = 40; 74.1%), alendronate (n = 5; 9.3%), ibandronate (n = 2; 3.7%) and denosumab (n = 3; 5.5%) or the combination of bisphosphonate and denosumab (n = 4; 7.4%). The mean duration of intake of the antiresorptive drugs was 46.3 months (SD 31.8 months).

The 54 patients revealed 65 MRONJ lesions. 40 of the lesions (61.5%) were located in the mandible and 25 (38.5%) were located in the maxilla. The majority of the lesions referred to stage 2 (n = 42; 64.6%) and stage 3 (n = 8; 12.3%) according to Ruggiero et al. (2014). It is worth mentioning that also stage 1 lesions were included (n = 14; 21.5) and even a singular case of stage 0 (n = 1; 1.5%). The mean follow-up of the patients was 12.9 months (median 11 months; range 1–39 months).

3.2. Results of fluorescence-guided bone surgery

The first surgical intervention using fluorescence-guided bone surgery resulted in complete mucosal healing in 47/54 of the evaluated patients (87%) and 56/65 lesions (86.2%) without any kind of bone exposure and without complaints at the time of last follow-up. Typical cases are illustrated in Figs. 3 and 4.

2/54 (3.7%) patients were also free of complaints and had no bone exposure and a complete mucosal coverage of the bone. However, in these patients the lesions in the maxilla were that extensive (AAOMS stage 3) that oro-antral fistula persisted. Both patients preferred an obturator prosthesis instead of another surgical approach to close the oro-antral fistula. One of these two cases is illustrated in Fig. 5.

5/54 patients (9.3%) with 7/65 lesions (10.8%) showed stage improvement and were free of pain after first surgery but still had bone exposure present. 4 of these patients (with 6 of the 7 lesions) underwent a second surgery using fluorescence-guided bone surgery, which in all 4 patients and all 6 lesions resulted in complete mucosal healing. An overview of the treatment outcome after first surgery and including the 4 cases with second surgery is provided in Table 1.

Only in one single patient (1 lesion) the bone exposure persisted and was subsequently treated conservatively as the patients systemic condition had worsened over time caused by the underlying malignant disease. The initial stage improvement (stage 2 prior to surgery and stage 1 after surgery) gradually worsened over time back to the initial stage 2.

Taken together the results of the first and second surgery 51/54 patients (94.4%) and 62/65 lesions (95.4%) showed complete mucosal healing and no bone exposure. Two further patients were free of complaints and had no bone exposure but developed oro-antral communication. Only one patient with a single lesion showed persistent bone exposure which could not be addressed by a second surgery due to the worsened general condition of the patient.

It is worth mentioning that no continuity resection had to be performed in the mandible, whereas the removal of MRONJ in the maxilla resulted in resection-like defects in 4 cases. Two of those cases developed a persistent oro-antral fistula. None of the patients showed a recurrence of MRONJ in the respective area after complete mucosal healing in the further postoperative course. None of the patients developed a pathological fracture of the mandible.

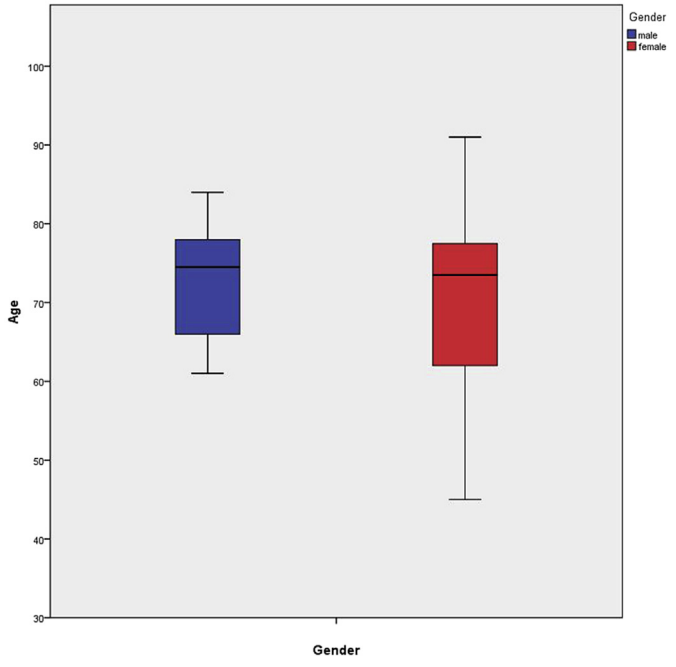

Fig. 1. Comparison of age and age range between male and female patients suffering from MRONJ.

4. Discussion

There is an ongoing debate and certainly no consensus yet regarding the management of patients with MRONJ. Moreover, there is not even consensus regarding the main treatment aim and the optimal outcome measures.

While some authors recommend conservative treatment protocols mainly aiming in relief of pain and control of infection, a number of papers have suggested that in patients with a good performance status the primary aim of treatment should be mucosal healing as this is the physiological status, rather than bone exposure without symptoms (Assaf et al., 2014; Carlson and Basile, 2009; Pautke et al., 2011; Vescovi et al., 2008; Otto, 2015). Conservative treatment cannot achieve this aim, neither considering the frequency nor the predictability especially in oncological patients who have received long term intravenous courses of nitrogen-containing bisphosphonates. In this respect Hoff et al. (2008) reported 23% healing (3/13 patients) and similarly Nicolatou-Galitis et al. reported mucosal healing in only 14.9% of BRONJ cases (7/47) managed conservatively, notably after a median time of 8 months (range 2–36 months, mean 14.7 months), while pain subsided in 80.9% (38/47) (Nicolatou-Galitis et al., 2011). It is also worth mentioning that 4 of the 7 patients who showed complete healing referred to stage 0 according to the AAOMS definition (Ruggiero et al., 2009, 2014). This in turn means that the outcome results for cases with bone exposure are even less convincing. Regardless of the type of definition or staging system applied, the vast majority of patients with BRONJ (especially oncological patients) cannot be cured using conservative measurements and have long lasting jaw bone exposure which can not only affect their

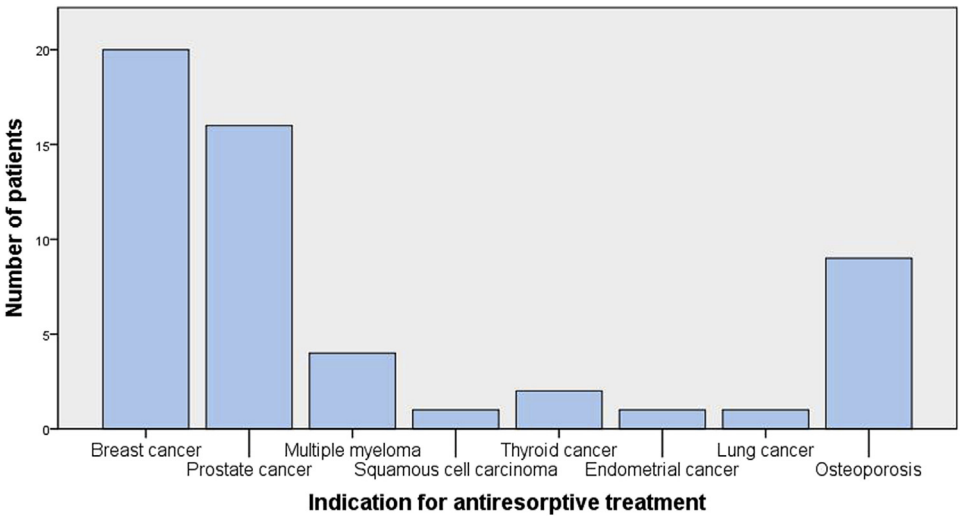

Fig. 2. Overview of the underlying diseases leading to antiresorptive treatment with bisphosphonates and denosumab in patients suffering from MRONJ.

quality of life (Kyrgidis et al., 2012), but may also limit the oncological treatment options including immuno- or chemotherapy and possibly further antiresorptive treatment with bisphosphonates or denosumab (Then et al., 2012; Otto et al., 2012). Conservative treatment might be adequate if the aim of treatment is to slow down or stop disease progression and to alleviate pain and superinfection of the exposed bone, while there is increasing evidence supporting surgical protocols if the aim of treatment is mucosal healing.

In this respect our study showed that fluorescence-guided bone surgery is a reliable and promising treatment option for patients suffering from MRONJ.

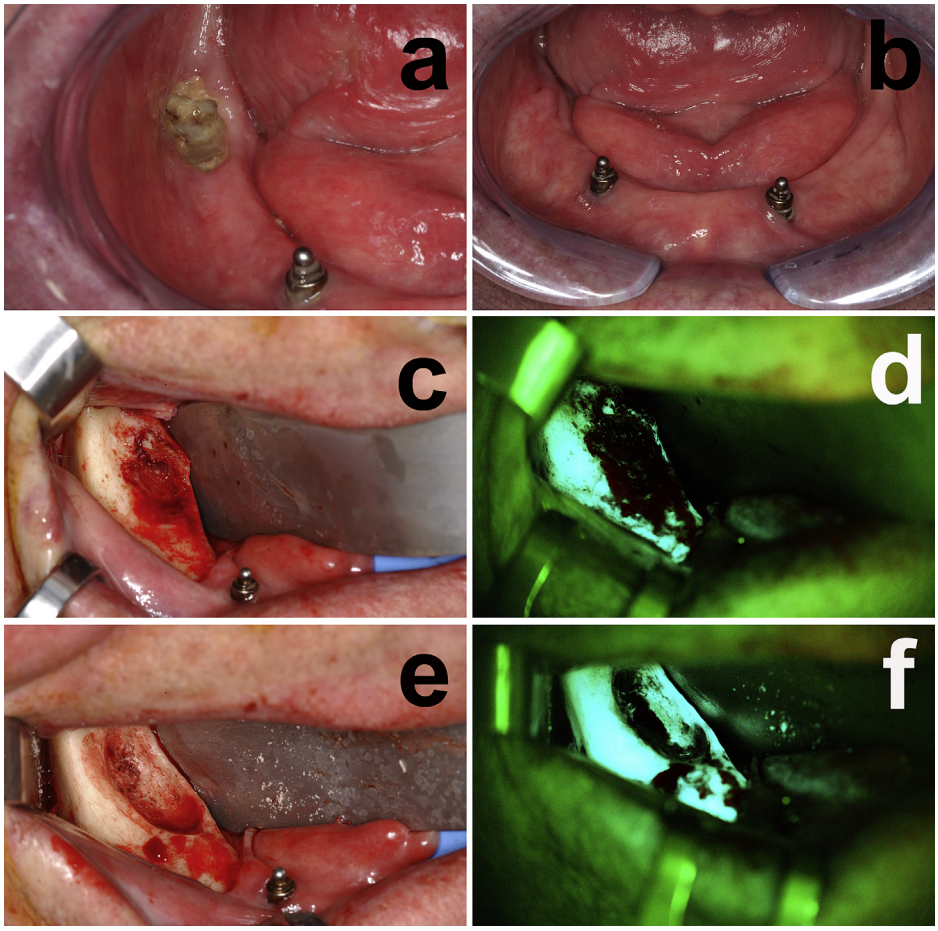

Fig. 3. Illustration of a 74-year old male patient suffering from prostate cancer who has received intravenous treatment with zoledronate over 2 years and exposed necrotic bone and putrid exudation of the right mandible (regio 47/48) according to a medication-related osteonecrosis of the jaw (regio 47/48) prior to (a) and one year after fluorescence-guided surgery (b). During surgery there was necrotic bone with diminished fluorescence in the lingual aspect of the mandible (c and d). After complete removal of the necrotic bone parts and smoothening of sharp bony edges the fluorescence was homogenously green (e and f).

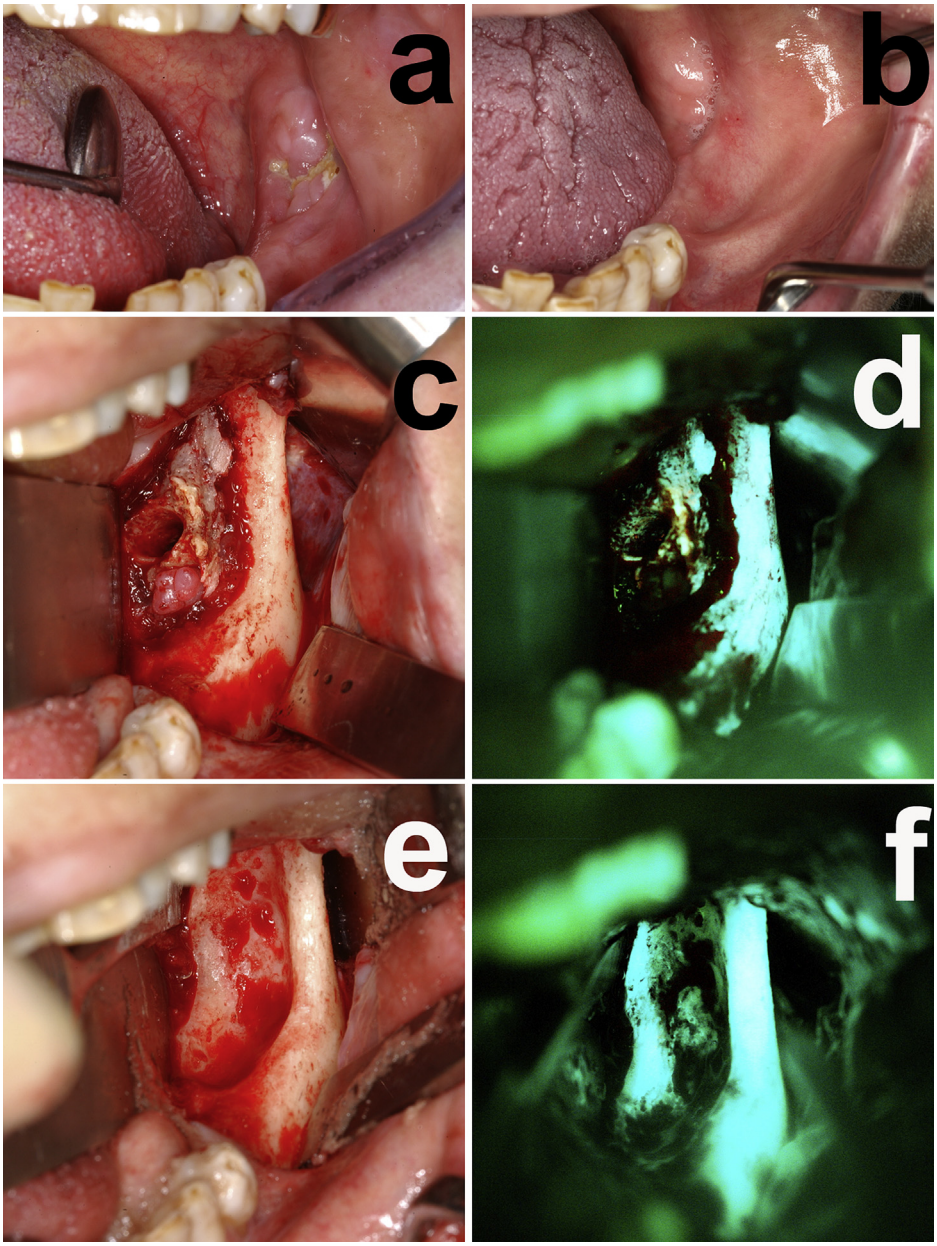

Fig. 4. Illustration of a 58-year old female patient suffering from breast cancer who received 56 months zoledronate and developed a medication-related osteonecrosis of the jaw in her left mandible (region 37/38 mainly lingual aspect). The illustration depicts the clinical intraoral situation prior to (a) and 3 months after surgery (b). The intraoperative clinical and fluorescence view prior to removal of the necrotic bone (c and d) and after the removal of necrotic bone and smoothening of sharp bony edges (e and f) are also illustrated. Note the weak green fluorescence in the lingual aspect regio 37/38 corresponding to the necrotic bone area (d) as well as the reddish fluorescence in this area corresponding to the bacterial infection of this region prior to removal of the necrotic and infected bone parts as well as the homogenous greenish fluorescence after the removal and the absence of red fluorescence after the removal of necrotic bone parts.

This is in line with the recent literature where Carlson et al. reported mucosal integrity of 92% after surgical resection in a case series of 95 patients (Carlson and Basile, 2009). Likewise, other authors stated a healing rate up to 89% (12 month follow up; n = 50) (Stockmann et al., 2010) as well as 88 % (60 weeks follow up; n = 24) after surgical treatment. Prospective case series further support the benefit of a surgical treatment of BRONJ: Bedogni et al. (2011) (n = 30) surgical treatment: 90 % healing 6 months follow up, Schubert et al. (2012) (n = 54) surgical treatment: 89 % healing (min. 3 months follow up), Jacobsen et al. (2012) (n = 64 surgical treatment: 78 % healing (7 years follow up)). It is however hard to compare the different studies because the underlying study cohorts were composed of different populations regarding the proportion of oncological and osteoporotic patients, regarding the surgical protocol applied (e.g. only removal of necrotic bone versus resection) and regarding the outcome evaluation and postoperative follow-up but the bottom line of all of the above mentioned studies was that patients suffering from MRONJ can successfully be treated using surgical treatment protocols.

Comparative studies also seem to substantiate these findings. The multivariate analysis of Mücke and co-workers showed a lower recurrence rate for surgically-treated ONJ patients when compared to conservative treatment (n = 108) (Mucke et al., 2011), as well as the multivariate analysis of (Graziani et al., 2012, 2013) (n = 347)

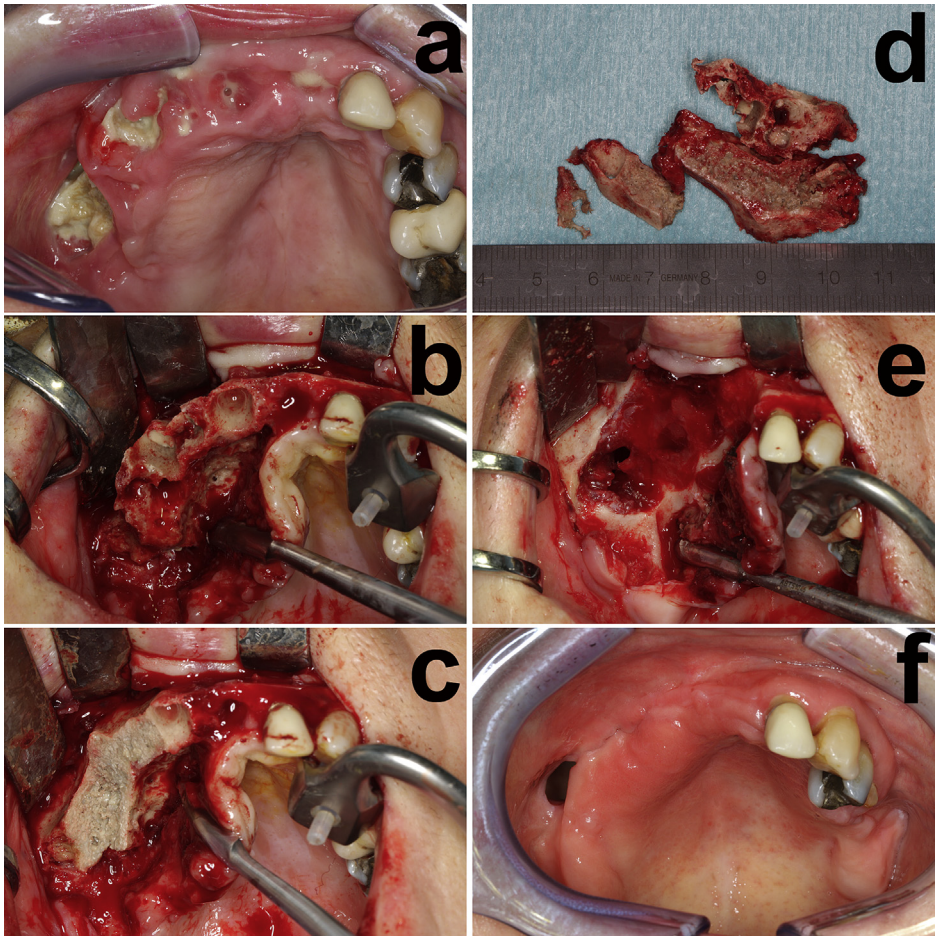

Fig. 5. Illustration of a 62-year old female patient suffering from metastatic breast cancer who received zoledronate intravenously (4 mg every 4 weeks) for more than 3 years and developed an extremely extended stage III MRONJ in her right maxilla with bone exposure suppuration which was also extremely painful on palpation (a). After antibiotic pre-treatment the patient was treated surgically. After exposure (b) the whole extent of the MRONJ lesion became visible which included parts of the hard palate and parts of the facial wall of the maxillary sinus. After removal of parts of the necrotic bone (c and d) it became obvious that the whole alveolar process of the right maxilla was necrotic and infected. The necrotic bone was completely removed using fluorescence-guided surgery (e) and a double-layered plastic wound closure was performed using the buccal fat pad and muco-periosteum. In the postoperative course the patient was free of pain but developed a wound healing disturbance and a oro-antral fistula. After complete healing there was no bone exposure but the oro-antral fistula persisted (f). As the patient was free of complaints she did want to go for another surgery to close the oro-antral fistula. So she was treated using an obturator prosthesis as described in detail elsewhere (Troeltzsch et al., 2015).

Table 1
Comparison of pre- and post-operative signs and symptoms of MRONJ in the patient cohort which was treated using fluorescence-guided surgery; n = number of patients (n = number of lesions).

	Pre-operatively Total n = 54 (65)	After first surgery Total n = 54 (65)	After second surgery in n = 4 (6); total 54 (65)
Bone exposure	53 (63)	5 (7)	1 (1)*
Pain/complaints	43 (51)	1 (1)	1 (1)**[a]
Impaired sensitivity N. V3	7 (7)	1 (1)	1 (1)**[b]
Sinusitis/oro-antral fistula	7 (8)	2 (2)	2 (2)**[b]
Pathological fracture	0	0	0

*change due to complete mucosal healing in 4 patients with 6 lesions who underwent second surgery.
**no change as none of the affected patients was treated surgically again.
[a] Due to worsening of underlying malignant disease.
[b] Due to patients wish and no need for second surgery.

confirmed significantly more mucosal healing for surgical treatment versus conservative protocols. Finally, a 2014 systematic review by Rupel et al. (2014), and another very recent systematic review meeting PRISMA guidelines (Liberati et al., 2009) which analysed data from 97 studies and 4,867 patients suggest that surgical treatment protocols are superior to conservative management (Fliefel et al., 2015).

The most important parts for a successful surgical treatment of MRONJ include pre- and postoperative antibiotic treatment, complete removal of the necrotic and often infected bone parts, smoothening of sharp bony edges and a complete and reliable plastic wound closure. The aim of the preoperative antibiotic treatment is to stop disease progression and to reduce infection in order to provide optimal conditions for the surgical treatment. The

complete removal of necrotic bone is essential to provide the conditions for bone and soft tissue healing and in order to avoid reinfection of necrotic bone parts. Fluorescence-guidance might be a tool to optimize the completeness of removal of necrotic bone parts. Smoothening of sharp bony edges is of special importance because of the remodeling suppression caused by antiresorptive drugs and seems therefore even more important when the antiresorptive activity is high (e.g. after multiple years of intravenous bisphosphonate intake or shortly after the last application of antiresorptive drugs with short half-life e.g. denosumab). The aim of the plastic wound closure is to ensure that the delayed and endangered healing of the jaw bone treated with antiresorptive drugs can take place in an undisturbed manner. In the experience of the authors of this article safe and reliable mucoperiostal flaps closed with multiple back stitches seems sufficient. However, it is recommended to perform double layered wound closure whenever possible. In this respect for example the use of the buccal fat pad in cases of MRONJ of the molar and premolar region of the maxilla and the use of the mylohyoid flap in the mandibular molar region might have advantages. The postoperative antibiotic treatment should protect the wound healing period and avoid reinfection of the bone. A prolonged antibiotic treatment seems to have advantages.

According to several guidelines including the AAOMS position paper and the ASBMR expert panel recommendation early stages of MRONJ should be treated conservatively and surgical treatment should only be applied to stages 2 and 3 (Khan et al., 2015; Ruggiero et al., 2014; Williams and O'Ryan, 2015). The authors of this paper disagree with these opinions especially in patients receiving intravenous administrations of bisphosphonates in the oncological setting (Otto et al., 2015). In fact treatment of all stage 0 and 1 lesions resulted in complete mucosal healing with minimal morbidity and a predictable and reasonable time frame. Furthermore, after complete mucosal healing the respective patients had no restrictions regarding their further oncological or osteological treatment including further antiresorptive treatment. Actually, surgical treatment of early MRONJ lesions offers a lot of advantages including the usually smaller extent of the lesions leading to less extended surgical removal of bone and minor functional impairments. Besides that lack of infection usually offers better conditions for surgical treatment. Therefore, the authors of this paper call for a re-evaluation of concepts and aim for a change of paradigms. Instead of long lasting, unpredictable conservative treatment approaches usually resulting in improvements of symptoms but rarely leading to complete mucosal healing should be replaced by early surgical interventions aiming in complete mucosal healing in a predictable timeframe and resulting in optimized functional outcomes as respective surgeries which frequently occur after unsuccessful conservative treatment approaches can be avoided. Indeed, it is worth mentioning that after changing our treatment concept to early surgical intervention we did not experience MRONJ cases, in which we had to perform continuity resections of the mandible and no microvascular reconstructions were necessary any more, which we experienced during the timeframe where we applied a more conservative treatment approach in early stages. So in fact so called conservative treatment protocols might lead to the necessity of more aggressive and large resections including all functional impairments over the long run (Williams and O'Ryan, 2015) The authors of this paper do not doubt that ablative surgery including continuity resections of the mandible and microvascular reconstructions are necessary in selected cases of MRONJ whereas a lot more cases of osteoradionecrosis require this radical treatment. We think that the progression of MRONJ cases presenting in early stages can be avoided when treated adequately. However, conservative treatment approaches and the role of drug holidays might well be different in MRONJ cases under denosumab especially in cases without prior bisphosphonate treatment because of the much shorter half-life of denosumab (26 days) when compared to bisphosphonates in bone (Otto, 2015).

Regarding the specific technique of fluorescence-guided bone resection it needs to be mentioned that it is not yet certain what exactly causes the intraoperative fluorescence. Recent reports suggest that there is an auto-fluorescence without tetracycline bone labeling, leading to similar bone fluorescence of tetracycline-exposed tissue (Vescovi et al., 2015; Ristow and Pautke, 2014). Indeed, it is well known that not only tetracycline but also components of the extracellular matrix e.g. calcified tissues (bone or teeth) have fluorescence properties (Pautke et al., 2010, 2011). A combination of these components might contribute to the fluorescence effects that can be used in the treatment of MRONJ. Therefore, further basic and clinical research is needed in order to investigate the fluorescence properties and their differences. Once the causes for fluorescence-guided surgical approaches might be suitable not only for MRONJ but also for osteoradionecrosis and osteomyelitis (Pautke et al., 2010).

Limitations of the present study include the inhomogeneous recall intervals of some of the patients which were mainly due to their underlying diseases and respective oncological treatment protocols. Furthermore there were only very few cases of MRONJ due to Denosumab intake. Given the much shorter half-life of Denosumab when compared to nitrogen-containing bisphosphonates there might be a different and more important role of conservative treatment protocols especially when there is no pretreatment with bisphosphonates and no further necessity of antiresorptive treatment. However, up to now there is no study which directly compares the outcome of conservative and surgical treatment and there is also no study comparing conventional surgical treatment versus fluorescence-guided surgery.

The available data might not yet be robust enough to inform guidelines on the treatment of MRONJ, especially as there is hardly any data on how to manage patients exposed to denosumab where conservative treatment might theoretically play a different role due to its much shorter half-life. There is an urgent need of prospective randomized trials comparing surgical and non-surgical treatment of MRONJ and including patient-centered outcome measures like quality of life before, during and after treatment. Ultimately, the clinical decision making will always be based on individual risk assessment, especially as most patients with MRONJ have multiple comorbidities, which require knowledge about the predictable efficacy and limitations of the all treatment options.

5. Conclusion

We conclude that fluorescence-guided bone resection is a reliable surgical treatment option for patients suffering from medication-related osteonecrosis of the jaw.

Conflicts of Interest

We declare that we have no conflicts of interest.

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References

Assaf AT, Zrnc TA, Riecke B, Wikner J, Zustin J, Friedrich RE, et al: Intraoperative efficiency of fluorescence imaging by Visually Enhanced Lesion Scope (VELscope) in patients with bisphosphonate related osteonecrosis of the jaw (BRONJ). J Craniomaxillofac Surg 42(5): e157–e164, 2014 Jul. http://dx.doi.org/10.1016/j.jcms.2013.07.014, [Epub 2013 Sep 4]. PMID: 24011463

Bedogni A, Saia G, Bettini G, Tronchet A, Totola A, Bedogni G, et al: Long-term outcomes of surgical resection of the jaws in cancer patients with bisphosphonate-related osteonecrosis. Oral Oncol 47: 420–424, 2011

Carlson ER, Basile JD: The role of surgical resection in the management of bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg 67: 85–95, 2009

Dore F, Filippi L, Biasotto M, Chiandussi S, Cavalli F, Di Lenarda R: Bone scintigraphy and SPECT/CT of bisphosphonate-induced osteonecrosis of the jaw. J Nucl Med 50: 30–35, 2009

Fabbricini R, Catalano L, Pace L, Del Vecchio S, Fonti R, Salvatore M, et al: Bone scintigraphy and SPECT/CT in bisphosphonate-induced osteonecrosis of the jaw. J Nucl Med 50: 1385, 2009 author reply 1385

Fleisher KE, Doty S, Kottal S, Phelan J, Norman RG, Glickman RS: Tetracycline-guided debridement and cone beam computed tomography for the treatment of bisphosphonate-related osteonecrosis of the jaw: a technical note. J Oral Maxillofac Surg 66: 2646–2653, 2008

Fliefel R, Troltzsch M, Kuhnisch J, Ehrenfeld M, Otto S: Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. Int J Oral Maxillofac Surg 44: 568–585, 2015

Graziani F, Vescovi P, Campisi G, Favia G, Gabriele M, Gaeta GM, et al: Resective surgical approach shows a high performance in the management of advanced cases of bisphosphonate-related osteonecrosis of the jaws: a retrospective survey of 347 cases. J Oral Maxillofac Surg 70: 2501–2507, 2012

Guggenberger R, Fischer DR, Metzler P, Andreisek G, Nanz D, Jacobsen C, et al: Bisphosphonate-induced osteonecrosis of the jaw: comparison of disease extent on contrast-enhanced MR imaging, [18F] fluoride PET/CT, and conebeam CT imaging. AJNR Am J Neuroradiol 34: 1242–1247, 2013

Hoff AO, Toth BB, Altundag K, Johnson MM, Warneke CL, Hu M, et al: Frequency and risk factors associated with osteonecrosis of the jaw in cancer patients treated with intravenous bisphosphonates. J Bone Miner Res 23: 826–836, 2008

Hutchinson M, O'Ryan F, Chavez V, Lathon PV, Sanchez G, Hatcher DC, et al: Radiographic findings in bisphosphonate-treated patients with stage 0 disease in the absence of bone exposure. J Oral Maxillofac Surg 68: 2232–2240, 2010

Jacobsen C, Metzler P, Obwegeser JA, Zemann W, Graetz KW: Osteopathology of the jaw associated with bone resorption inhibitors: what have we learned in the last 8 years? Swiss Med Wkly 142: w13605, 2012

Khan AA, Morrison A, Hanley DA, Felsenberg D, McCauley LK, O'Ryan F, et al: Diagnosis and management of osteonecrosis of the jaw: a systematic review and international consensus. J Bone Miner Res 30: 3–23, 2015

Khosla S, Burr D, Cauley J, Dempster DW, Ebeling PR, Felsenberg D, et al: Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American society for bone and mineral research. J Bone Miner Res 22: 1479–1491, 2007

Kyrgidis A, Triaridis S, Kontos K, Patrikidou A, Andreadis C, Constantinidis J, et al: Quality of life in breast cancer patients with bisphosphonate-related osteonecrosis of the jaws and patients with head and neck cancer: a comparative study using the EORTC QLQ-C30 and QLQ-HN35 questionnaires. Anticancer Res 32: 3527–3534, 2012

Liberati A, Altman DG, Tetzlaff J, Mulrow C, Gotzsche PC, Ioannidis JP, et al: The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. PLoS Med 6: e1000100, 2009

Marx RE, Sawatari Y, Fortin M, Broumand V: Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. J Oral Maxillofac Surg 63: 1567–1575, 2005

Montebugnoli L, Felicetti L, Gissi DB, Pizzigallo A, Pelliccioni GA, Marchetti C: Biphosphonate-associated osteonecrosis can be controlled by nonsurgical management. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 104: 473–477, 2007

Mucke T, Koschinski J, Deppe H, Wagenpfeil S, Pautke C, Mitchell DA, et al: Outcome of treatment and parameters influencing recurrence in patients with bisphosphonate-related osteonecrosis of the jaws. J Cancer Res Clin Oncol 137: 907–913, 2011

Nicolatou-Galitis O, Papadopoulou E, Sarri T, Boziari P, Karayianni A, Kyrtsonis MC, et al: Osteonecrosis of the jaw in oncology patients treated with bisphosphonates: prospective experience of a dental oncology referral center. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 112: 195–202, 2011

O'Ryan FS, Khoury S, Liao W, Han MM, Hui RL, Baer D, et al: Intravenous bisphosphonate-related osteonecrosis of the jaw: bone scintigraphy as an early indicator. J Oral Maxillofac Surg 67: 1363–1372, 2009

Otto S: Medication-related osteonecrosis of the jaws: bisphosphonates, denosumab, and new agents. Heidelberg, New York, Dordrecht, London: Springer. http://dx.doi.org/10.1007/978-3-662-43733-9, 2015

Otto S, Baumann S, Ehrenfeld M, Pautke C: Successful surgical management of osteonecrosis of the jaw due to RANK-ligand inhibitor treatment using fluorescence guided bone resection. J Craniomaxillofac Surg 41(7): 694–698, 2013 Oct. http://dx.doi.org/10.1016/j.jcms.2013.05.038, [Epub 2013 Jul 5]. PMID: 23830772

Otto S, Marx RE, Troltzsch M, Ristow O, Ziebart T, Al-Nawas B, et al: Comments on "diagnosis and management of osteonecrosis of the jaw: a systematic review and international consensus". J Bone Miner Res 30: 1113–1115, 2015

Otto S, Schreyer C, Hafner S, Mast G, Ehrenfeld M, Sturzenbaum S, et al: Bisphosphonate-related osteonecrosis of the jaws - characteristics, risk factors, clinical features, localization and impact on oncological treatment. J Craniomaxillofac Surg 40: 303–309, 2012

Pautke C, Bauer F, Bissinger O, Tischer T, Kreutzer K, Steiner T, et al: Tetracycline bone fluorescence: a valuable marker for osteonecrosis characterization and therapy. J Oral Maxillofac Surg 68: 125–129, 2010

Pautke C, Bauer F, Otto S, Tischer T, Steiner T, Weitz J, et al: Fluorescence-guided bone resection in bisphosphonate-related osteonecrosis of the jaws: first clinical results of a prospective pilot study. J Oral Maxillofac Surg 69: 84–91, 2011

Pautke C, Bauer F, Tischer T, Kreutzer K, Weitz J, Kesting M, et al: Fluorescence-guided bone resection in bisphosphonate-associated osteonecrosis of the jaws. J Oral Maxillofac Surg 67: 471–476, 2009

Pautke C, Kreutzer K, Weitz J, Knodler M, Munzel D, Wexel G, et al: Bisphosphonate related osteonecrosis of the jaw: a minipig large animal model. Bone 51: 592–599, 2012

Pautke C, Tischer T, Neff A, Horch HH, Kolk A: In vivo tetracycline labeling of bone: an intraoperative aid in the surgical therapy of osteoradionecrosis of the mandible. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 102: e10–e13, 2006

Ristow O, Otto S, Troeltzsch M, Hohlweg-Majert B, Pautke C: Treatment perspectives for medication-related osteonecrosis of the jaw (MRONJ). J Craniomaxillofac Surg 43: 290–293, 2015

Ristow O, Pautke C: Auto-fluorescence of the bone and its use for delineation of bone necrosis. Int J Oral Maxillofac Surg 43: 1391–1393, 2014

Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B: American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws–2009 update. J Oral Maxillofac Surg 67: 2–12, 2009

Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo TL, Merhotra B, et al, American Association of Oral and Maxillofacial Surgeons: position paper on medication-related osteonecrosis of the Jaw—2014 update. J Oral Maxillofac Surg 72(10): 1938–1956, 2014 Oct. http://dx.doi.org/10.1016/j.joms.2014.04.031 ,[Epub 2014 May 5]

Rupel K, Ottaviani G, Gobbo M, Contardo L, Tirelli G, Vescovi P, et al: A systematic review of therapeutical approaches in bisphosphonates-related osteonecrosis of the jaw (BRONJ). Oral Oncol 50: 1049–1057, 2014

Schubert M, Klatte I, Linek W, Muller B, Doring K, Eckelt U, et al: The saxon bisphosphonate register - therapy and prevention of bisphosphonate-related osteonecrosis of the jaws. Oral Oncol 48: 349–354, 2012

Stockmann P, Vairaktaris E, Wehrhan F, Seiss M, Schwarz S, Spriewald B, et al: Osteotomy and primary wound closure in bisphosphonate-associated osteonecrosis of the jaw: a prospective clinical study with 12 months follow-up. Support Care Cancer 18: 449–460, 2010

Then C, Horauf N, Otto S, Pautke C, von Tresckow E, Rohnisch T, et al: Incidence and risk factors of bisphosphonate-related osteonecrosis of the jaw in multiple myeloma patients having undergone autologous stem cell transplantation. Onkologie 35: 658–664, 2012

Troeltzsch M, Probst F, Troeltzsch M, Ehrenfeld M, Otto S: Conservative management of medication-related osteonecrosis of the maxilla with an obturator prosthesis. J Prosthet Dent 113: 236–241, 2015

Vescovi P, Giovannacci I, Otto S, Manfredi M, Merigo E, Fornaini C, et al: Medication-related osteonecrosis of the jaw: an autofluorescence-guided surgical approach performed with Er:YAG laser. Photomed Laser Surg 33: 437–442, 2015

Vescovi P, Manfredi M, Merigo E, Meleti M: Early surgical approach preferable to medical therapy for bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg 66: 831–832, 2008

Voss PJ, Joshi Oshero J, Kovalova-Muller A, Veigel Merino EA, Sauerbier S, Al-Jamali J, et al: Surgical treatment of bisphosphonate-associated osteonecrosis of the jaw: technical report and follow up of 21 patients. J Craniomaxillofac Surg 40: 719–725, 2012

Watters AL, Hansen HJ, Williams T, Chou JF, Riedel E, Halpern J, et al: Intravenous bisphosphonate-related osteonecrosis of the jaw: long-term follow-up of 109 patients. Oral Surg Oral Med Oral Pathol Oral Radiol 115: 192–200, 2013

Williams WB, O'Ryan F: Management of medication-related osteonecrosis of the jaw. Oral Maxillofac Surg Clin North Am 27: 517–525, 2015

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Abstract: We hypothesized that local infection plays a critical role in the pathogenesis of medication-related osteonecrosis of the jaw (MRONJ). Recent developments in molecular methods have revolutionized new approaches for the rapid detection of microorganisms including those difficult to culture. The aim of our study is to identify the bacterial profiles in MRONJ by microbiological culture and polymerase chain reactions (PCR). A retrospective analysis was performed on MRONJ patients from 2008 to 2014. The bacterial profile from MRONJ bone samples was determined using microbiological culture and PCR. Ninety five patients fulfilled the inclusion criteria with mean age of 69.85 ± 8.71 years. A female predilection was detected. The mandible was more commonly affected than maxilla. Tooth extraction was the frequent triggering factor. Breast cancer was the primary cause for administration and intravenous bisphosphonates were the most commonly administrated antiresorptive drugs. The majority of patients were classified as stage 2. Posterior teeth were most commonly affected. Based on bone culture results, the most common microorganism were both actinomyces and mixed flora. PCR confirmed the presence of actinomyces in 55 patients. Our data suggest that PCR might be an innovative method for detection of microorganisms difficult to culture using traditional microbiological techniques.

Role of microbiological culture and polymerase chain reaction (PCR) of Actinomyces in medication-related osteonecrosis of the jaw (MRONJ)

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INTRODUCTION

Medication-related osteonecrosis of the jaw (MRONJ) is a potentially devastating complication of antiresorptive drugs used globally to treat bone disorders as osteoporosis, skeletal complications associated with osseous metastasis and multiple myeloma (*Licata AA* 2005, *Peer A and Khamaisi M* 2015). Nowadays, the pathophysiology of MRONJ is not clearly understood. Numerous theories have been proposed, neither of which can provide an adequate explanation of the disease. MRONJ was perceived as a type of avascular necrosis due altered bone turnover or direct toxicity to the soft tissue, infection, inflammation, inhibition of angiogenesis or suppression of innate or acquired immunity have been identified as possible explanations of the disease process (*Mitsimponas KT et al.* 2014).

Bacterial infection to the maxillofacial region has been suggested as key factor for the pathogenesis and progression of MRONJ (*Otto S et al.* 2010, *Otto S et al.* 2010). The oral cavity comprises of more than 750 bacterial species existing as mixed biofilm communities (*Pushalkar S et al.* 2014). The mandible and maxilla are covered by thin layer of mucosa in close proximity to the external environment. After invasive dental procedures, oral trauma or soft tissue infection, microbial biofilms in the mouth and saliva gain access to the exposed jaw bone and play a significant role in the necrosis of the bone, inhibition of oral wound healing and facilitating bacterial colonization on bone surface (*Sedghizadeh PP et al.* 2012, *Li CL et al.* 2015). Actinomyces were regularly found in MRONJ suggesting a latent role of infection in the pathogenesis (*Hansen T et al.* 2006, *Hansen T et al.* 2007, *Lazarovici TS et al.* 2009). Actinomyces are filamentous gram-positive anaerobic bacteria that usually can be found in calculus, periodontal pockets, carious lesions and oral mucosal surfaces, in addition to the upper respiratory, gastrointestinal tracts and vagina. They are common saprophyte bacteria of low virulence in nature causing no disease as long as they stay on the surface of the mucosa but in certain conditions where the integrity of the mucosal barrier is compromised, the bacteria may be pathogenic and gain access to the oral tissues or jawbones

initiating a prolonged chronic inflammatory process, creating a tumor-like mass, tissue destruction, osteolysis and multiple sinus tracts (*Hall V* 2008, *Kaplan I et al.* 2009, *Norouzi F et al.* 2013).

MRONJ lesions are usually colonized by oral bacteria and the use of systemic antibiotics failed to restrict the bacterial colonization and effective healing of the lesion. It is important to identify the bacterial species colonizing jaw bone associated with the disease to delineate the pathogenesis. Moreover, it is not well understood whether the bacteria involved in MRONJ is similar or different to other biofilm associated bone infections in the oral cavity (*Ji X et al.* 2012). Recently, bone abnormalities were studied by various modalities but none proved to be reliable in describing the infectious nature of the disease. Recent advances using biomolecular profiling to describe MRONJ flora have decreased this gap (*Hinson AM et al.* 2014).

Here, we identify the bacterial profiles that colonize MRONJ bone samples determined by culture approaches and polymerase chain reactions (PCR) with clinical features of patients. This line of investigation could provide rationale in the future for MRONJ therapeutics and targeted antimicrobial therapy.

PATIENTS AND METHODS

This is a retrospective study of MRONJ patients treated at the Department of Oral and Maxillofacial Surgery, Ludwig-Maximillians-University Clinic, Munich from January 2008 to December 2014. Inclusion criteria were based on the American association of oral and maxillofacial surgery (AAOMS) Position paper (*Ruggiero SL et al.* 2014). Patients missing clinical, radiographic or follow-up data were excluded or if they had a history of head and neck radiation. Appropriate Institutional Review Board approval was obtained.

Clinical data relevant to the study were extracted and entered into an excel datasheet with a detailed history concerning: age, gender, location and teeth involved in the lesion, primary cause of the disease, comorbidities, clinical presentation, MRONJ clinical staging, type of antiresorptive drug, route of administration and pathological/microbiological findings of bone

samples. Bone samples were obtained from bone resection surgeries and were sent for microbiological investigations and PCR. Due to high likelihood of false positive culture from environmental exposure, we considered only at least strongly positive culture result (+2) as positive culture. One bone sample from each MRONJ patient was cut into fragments and prepared for microbiological analysis as described below.

Bone samples have been introduced in classical bacterial diagnostics. For this, aerobic cultures were prepared on Columbia blood-agar, MacConckey-agar and Columbia-CAN-agar, anaerobic cultures on Schaedler-agar and Schaedler-KV-agar (all agar plates from BD, Heidelberg, Germany). Besides, the swabs were cultivated in thioglycolate broth. All aerobe cultures have been read after 24h, 48h and 72h, the anaerobic cultures after 2d, 5d and 7d. The bacterial counts have been enumerated semi-quantitative and bacterial colonies were objected to MALDI-TOF MS for further species identification.

Samples were evaluated by the use of Microflex LT mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany) in linear positive-ion mode across the *m/z* range of 2,000 to 20,000 Da. Each spot was measured by using 240 laser shots at 60 Hz in groups of 40 shots per sampling area of the spot. Spectra were analyzed by using MALDI Biotyper software (v 3.1 – Build 65). Sample preparation included either the "direct transfer method", the "Extended Direct Transfer method (EDT)" or the "ethanol/formic acid extract method" as previously described (*Schulthess B et al.* 2014). Resulting spectra were compared against reference spectra using Bruker MALDI-TOF Biotyper software to obtain identification with a confidence score. For most isolates, the MSP (Main Spectral Projection) reference spectra were those contained in the Bruker database of 2013 (database version V 3.3.1.2) containing 364 genera, 2185 species and 4613 individual MSP. Results with score values >2 were considered as correct species identification, results displaying values of 1.5≤ and ≤2 were accepted as correct genus identification.

Identification of bacteria by sequencing of 16S rDNA has been performed as described previously with some modifications (*Wragg P et al.* 2014). In brief, crude bacterial lysates were prepared directly from culture plates by suspending bacteria from a clonal culture in 100 µl of RT-PCR grade water (approximately McFarland Standard 2.0) and placed in a hot block at 100 °C for 10 min. A ~800 bp-fragment of 16S rDNA was amplified using the universal primer pair FD1 5′-AGAGTTTGATCCTGGCTCAG-3′ and 800r 5′-GAGTACCAGGGTATCTAATCC-3′. Resulting PCR amplicons were sequenced using the same primers and standard sequencing methods. Data from both strands was aligned in SeqMan (DNASTAR Lasergene 8 Suite) to generate a contig of around 800 bp. The consensus sequences were then used to compare with online databases (NCBI BLAST—http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Ribosomal Database Project (http://rdp.cme.msu.edu/). Identification criteria of 99% sequence identity for identification to species level were applied (*Drancourt M et al.* 2000) where matches had to be to the species type strain. The identities of type strains, as well as accession numbers in NCBI for equivalent 16S rDNA sequences, are available at http://www.bacterio.cict.fr/ for all validly published bacterial species.

Statistical analysis

Descriptive statistics were computed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Results are expressed as mean values including standard error of the mean and range. Means were compared by statistical testing (Student's t-test), where $P < 0.05$ was considered to be significant.

RESULTS

A total of 150 patients were diagnosed with MRONJ from 2008 to 2014. However, 95 patients satisfied the inclusion criteria and form the basis of this study. Flow chart of the number of patients included in the study are illustrated in (Fig.1). The mean age of the patients was 69.9 ± 8.7 years; with a male to female ratio of 1:1.4 (39 males and 56 females).

Breast cancer was the primary cause for the administration of antiresorptive drugs (n=35; 36.8%), followed by prostate cancer (n=24; 25.3%) and osteoporosis (n=13; 13.7%) in addition to multiple myeloma (n=10; 10.5%), lung cancer (n=4; 4.2%) and finally other cancers (n=9; 9.5%). The relevant comorbidities identified included: diabetes mellitus (n=17; 17.9%), cardiovascular diseases (n=29; 30.5%), chemotherapy (n=57; 60%), irradiation other than head and neck (n=51; 53.7%), steroid intake (n=28; 29.5%), anti-angiogenic drugs (n=2; 2.1%) and smoking (n=28; 29.5%). The most commonly administrated antiresorptive drugs (ARD) were bisphosphonates (BPs) in 85 patients (89.5%) of which, zoledronate in 58 (61.1%), pamidronate in 3 (3.2%), ibandronate in 2 (2.1%), combination of BPs in 22 (23.1%). Only ten patients received denosumab (10.5%). Among the ARD groups, 79 patients (83.2%) had intravenous ARD, 6 patients (6.3%) with oral and 10 patients (10.5%) had subcutaneous injection. The baseline characteristics of the patients included in the study are listed in (Table 1).

Initial presentation of the lesion was only one case referred to stage 0 (1.1%) with no bone exposure but non-specific signs and symptoms of MRONJ. Fifteen patients (15.8%) were categorized as stage 1 where bone was exposed in the absence of pain and clinical signs of infection. The majority of cases (n=59; 62.1%),) were classified as stage 2 based on exposed necrotic bone in the maxillofacial region accompanied by pain or signs of infection. Twenty patients (21.1%). were presented with stage 3 lesions with complications such as pathological fracture, extraoral fistula formation, extension of the lesion to the inferior border of the mandible or to the floor of the maxillary sinus. Most of MRONJ lesions were located in the mandible (n=55; 57.9%), 25 patients (26.3%) had maxillary lesions and 15 patients (15.8%) had involvement of the maxilla and mandible. Characteristics of MRONJ lesions are presented in (Table 2). The posterior teeth specially the first and second molars were the most affected teeth by MRONJ than the anterior teeth. The frequency of MRONJ in teeth of each quadrant is represented in (Fig.2).

Regarding the onset of MRONJ, the most frequent signs and symptoms were: pain in 81 patients (85.3%), exposed bone in 70 patients (73.7%), disturbance in wound healing in 55 patients (57.9%), inflammation in 54 patients (56.8%), pus in 39 patients (41.1%), pathological fracture in 9 patients (9.5%), swelling in 55 patients (57.9%), fistula in 35 patients (36.8%) and sinus involvement in 13 patients (13.7%). The lesions were stratified into lesions with a known triggering event or spontaneous development of MRONJ. The most common events prior to the development of MRONJ lesions were extraction in 56 patients (58.9%), dentoalveolar surgery in 15 patients (15.8%), denture sores in 4 patients (4.2%), periodontal treatment in 7 patients (7.4%) and lesions developed spontaneously in 13 patients (13.7%). Histopathological examination of the bone specimens revealed typical picture of MRONJ lesions where nearly all the patients showed an active inflammatory process with necrotic bone (n=94, 98.9%), inflammatory cell infiltrate (n=87, 91.6%) and bacterial colonization (n=67, 70.5%). The characteristics of MRONJ lesions are illustrated in Table 2.

Ninety five patients had undergone microbiological culture tests. However, only 55 patients had undergone PCR for actinomyces. Based on bone culture results, the most common microorganism were both actinomyces and mixed oral flora (n=23, 24.2%) each then enterobacter group (n=19, 20%), streptococci (n=18, 18.9%), miscellaneous microorganisms (n=13, 13.6%), candida (n=9, 9.4%) and finally enterococcus (n=5, 5.2%) (Fig.3). As actinomyces were the most commonly found microorganisms, we therefore performed PCR to confirm the presence of actinomyces. Of the 55 patients, 53 (96.4%) were PCR and culture positive and 35 (63.6%) were positive only for PCR but negative for actinomyces culture. The results are shown in Table.3.

DISCUSSION

The main objective of this study was to identify microorganisms manifested in MRONJ with special attention to actinomyces using microbiological cultures and PCR which might be useful in assisting surgeons in making proper decisions on the treatment modality of the

disease based on the hypothesis that infection maybe the most important factor negatively influencing the onset and progression of MRONJ.

MRONJ can reduce the patient's quality of life and may produce significant morbidity due to impairment of chewing, swallowing and speaking as well as deterioration of facial aesthetics. Thus, it is of tremendous importance to treat those patients to adequately eliminate pain, control infection of soft and hard tissue and eradicate bone exposure (*Maurer P et al.* 2011). From the results of our study, it was proved that actinomyces were highly prevalent in MRONJ patients by microbiological culture which was consistent with an earlier study on MRONJ bone samples (*Hansen T et al.* 2007). A previous study on a pathological specimen of MRONJ lesion showed that the lesions were composed of areas with active inflammatory cells with acellular necrotic debris and bone resorption (*Favia G et al.* 2009). The histopathological findings of the bone samples in our study were similar.

The terminology MRONJ had been well recognised worldwide nowadays due to the increase in the prevalence of the disease. The pathogenesis of the disease raised many questions regarding the potential mechanisms underlying the pathophysiology (*Allen MR and Burr DB* 2009). Several mechanisms had also been proposed as: i) oversuppression of bone turnover, ii) a response to infection, iii) immunomodulation, iv) ischemia due to the antiangiogenetic effects of BPs, v) soft tissue toxicity. Arguably, all theories could play a role in the pathogenesis of BRONJ. However, none of them was able to explain why the jawbone is the exclusive target (*Otto S et al.* 2010, *Otto S et al.* 2010). However, microbial infection in the pathogenesis of MRONJ is debatable and is not fully elucidated with few publications referring to the importance of infection as a prime component in the multifactorial disease (*Otto S et al.* 2010, *Hinson AM et al.* 2014, *Katsarelis H et al.* 2015). In our study, we have confirmed the presence of actinomyces in the bone samples but it is not clearly known whether osteonecrosis occurs first and then infection of the necrotic lesion or infected lesion undergoes osteonecrosis (*Hoefert S* 2015, *Kim KM et al.* 2015). There are some evidences

showing that infection is necessary for osteonecrosis with formation of a bacterial biofilm in the lesion (*Sedghizadeh PP et al.* 2009, *Aspenberg P et al.* 2010, *Otto S et al.* 2010) as the oral cavity is occupied by hundreds of bacterial species existing as mixed biofilm. When the patient immunity is decreased, those microorganisms show opportunistic infection as actinomyces which are dominant pathogenic microorganisms detected at MRONJ by histopathological studies (*Boff RC et al.* 2014).

From our results, we confirmed that PCR using 16S rRNA was useful in identifying actinomyces directly from bone samples. PCR targeting the 16S rRNA gene of the actinomyces is highly conserved within species of the same genus and is thus considered the new standard for classification and identification of bacteria as well as a reliable method for the distinction of species that are difficult to cultivate (*Lau SK et al.* 2004, *Elsayed S et al.* 2006). PCR is superior to microbiological cultures in diagnosis of oral actinomyces as being highly sensitive and rapidly detecting actinomyces either dead or alive. Another advantage is that it quantifies DNA rather than viable organisms. However, culturing methods cannot detect non-viable bacteria (*Kaya D et al.* 2013). Previous studies have used different molecular methodologies to identify and differentiate actinomyces from oral samples after anaerobic cultivation, including PCR-RFLP, chromosomal DNA fingerprinting, 16S rRNA gene sequencing and oligonucleotide–DNA hybridization using universal primers or oligonucleotide probes (*Sato T et al.* 1998, *Ruby JD et al.* 2002, *Tang G et al.* 2004).

Fifty-three (96.4%) of the 55 bone samples reacted positively with the universal primer pair designed for actinomyces suggesting their presence. These results show that PCR targeting the 16S rRNA region can be used to detect actinomyces in MRONJ bone samples.

Microbiological cultures were used as a traditional technique to identify actinomyces from bone samples. Anaerobic culturing was done in all 95 samples. However, these results were confirmed by PCR for 55 bone samples. The positive PCR results of the bone samples that were negative to culture were attributed to the high sensitivity of the PCR compared to culture

methods, the way of transporting the specimens to the laboratory, death of some actinomyces during culturing and the inhibition of growth of actinomyces by the presence of other organisms affecting their ability to grow in culture. However, DNA from dead organisms can still be detected by PCR as explained by another study (*Kaya D et al.* 2013).

From our results, MRONJ occurred in the mandible twice as likely to be affected as in the maxilla which was in agreement with previous studies (*Boonyapakorn T et al.* 2008, *Thumbigere-Math V et al.* 2009). Age older than 65 years was found to be a risk factor for MRONJ. Some studies recognized no statistically significant correlation between ageing and MRONJ (*Vahtsevanos K et al.* 2009) whereas others have included advanced age as a potential co-factor (*Bamias A et al.* 2005). Correlations between MRONJ and comorbidities as diabetes mellitus, cardiovascular disease, chemotherapy or steroid intake have been discussed. These comorbidities affect bone remodelling by microvascular ischemia and compromised wound healing as well as impaired osteoblastic differentiation and function and the additional immunosuppressive and antiangiogenic effects (*O'Ryan FS and Lo JC* 2012, *Fliefel R et al.* 2015). The great majority of MRONJ occur in females. The reason for the female dominance seems to be due to the higher number of breast cancer patients compared with prostate cancer patients and the greater prevalence of osteoporosis in females than in men (*Bamias A et al.* 2005). MRONJ has been reported in patients with malignancies, particularly in those with breast and prostate cancer. (*Lopes RN et al.* 2015) The profile of patients affected by this complication seems to show a similar pattern in our study. The majority of patients presented with MRONJ were at stages II which is comparable to findings in other studies (*O'Ryan FS et al.* 2009, *Otto S et al.* 2013). The classic clinical presentation of MRONJ is bone exposure with signs of infection, swelling and a purulent discharge (*Lopes RN et al.* 2015). Our study has corroborated that MRONJ is more frequent in subjects on intravenous bisphosphonates as reported elsewhere (*Khosla S et al.* 2007, *Otto S et al.* 2012).

The cumulative risk of developing MRONJ was significantly greater in patients receiving zoledronic acid.

Although no consensus has been reached regarding the mechanism of MRONJ, in the present study, MRONJ developed either spontaneously or due to dentoalveolar reasons as tooth extraction, periodontal disease and denture trauma. Previous studies had shown that dental treatment is a risk factor for developing MRONJ (*Hoff AO et al.* 2008). In contrast, some studies had proved that tooth extraction and dentoalveolar surgical procedures aimed at treating and curing local infections leading to decreased risk for the development of MRONJ (*Saia G et al.* 2010, *Mozzati M et al.* 2013, *Otto S et al.* 2015). local infections were treated and overcome by the removal of infected teeth and suspicious bony lesions, and by antibiotic treatment and mucosal coverage of the extraction wounds, protecting the extraction sockets from bacterial ingrowth after extraction (*Otto S et al.* 2015).

One limitation of this study was that there was no control group of untreated MRONJ patients. In addition, no non-MRONJ patients were characterized for bacterial species. The number of patients was reduced from 150 to 95 due to the incomplete records or absence of histopathological, microbiological or PCR diagnosis.

CONCLUSION

The pathogenesis of MRONJ had raised many questions regarding the potential mechanisms underlying the pathophysiology with special attention to the role of microbial infection. Actinomyces were the most frequent microorganisms in the disease. However, this does not necessarily lead to the pathogenic role. PCR was found to be the most reliable method for the detection of these microorganisms.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Allen MR and Burr DB. The pathogenesis of bisphosphonate-related osteonecrosis of the jaw: so many hypotheses, so few data. J Oral Maxillofac Surg 67: 61-70,**2009**.

Aspenberg P, Genant HK, Johansson T, Nino AJ, See K, Krohn K, Garcia-Hernandez PA, Recknor CP, Einhorn TA, Dalsky GP, Mitlak BH, Fierlinger A and Lakshmanan MC. Teriparatide for acceleration of fracture repair in humans: a prospective, randomized, double-blind study of 102 postmenopausal women with distal radial fractures. J Bone Miner Res 25: 404-414,**2010**.

Bamias A, Kastritis E, Bamia C, Moulopoulos LA, Melakopoulos I, Bozas G, Koutsoukou V, Gika D, Anagnostopoulos A, Papadimitriou C, Terpos E and Dimopoulos MA. Osteonecrosis of the jaw in cancer after treatment with bisphosphonates: incidence and risk factors. J Clin Oncol 23: 8580-8587,**2005**.

Boff RC, Salum FG, Figueiredo MA and Cherubini K. Important aspects regarding the role of microorganisms in bisphosphonate-related osteonecrosis of the jaws. Arch Oral Biol 59: 790-799,**2014**.

Boonyapakorn T, Schirmer I, Reichart PA, Sturm I and Massenkeil G. Bisphosphonate-induced osteonecrosis of the jaws: prospective study of 80 patients with multiple myeloma and other malignancies. Oral Oncol 44: 857-869,**2008**.

Drancourt M, Bollet C, Carlioz A, Martelin R, Gayral JP and Raoult D. 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. J Clin Microbiol 38: 3623-3630,**2000**.

Elsayed S, George A and Zhang K. Intrauterine contraceptive device-associated pelvic actinomycosis caused by Actinomyces urogenitalis. Anaerobe 12: 67-70,**2006**.

Favia G, Pilolli GP and Maiorano E. Histologic and histomorphometric features of bisphosphonate-related osteonecrosis of the jaws: an analysis of 31 cases with confocal laser scanning microscopy. Bone 45: 406-413,**2009**.

Fliefel R, Tröltzsch M, Kühnisch J, Ehrenfeld M and Otto S. Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. International Journal of Oral and Maxillofacial Surgery 44: 568-585,**2015**.

Hall V. Actinomyces--gathering evidence of human colonization and infection. Anaerobe 14: 1-7,**2008**.

Hansen T, Kunkel M, Springer E, Walter C, Weber A, Siegel E and Kirkpatrick CJ. Actinomycosis of the jaws--histopathological study of 45 patients shows significant involvement in bisphosphonate-associated osteonecrosis and infected osteoradionecrosis. Virchows Arch 451: 1009-1017,**2007**.

Hansen T, Kunkel M, Weber A and James Kirkpatrick C. Osteonecrosis of the jaws in patients treated with bisphosphonates - histomorphologic analysis in comparison with infected osteoradionecrosis. J Oral Pathol Med 35: 155-160,**2006**.

Hinson AM, Smith CW, Siegel ER and Stack BC, Jr. Is bisphosphonate-related osteonecrosis of the jaw an infection? A histological and microbiological ten-year summary. Int J Dent 2014: 452737,**2014**.

Hoefert S. Microbiology and Antibiotics in the Context of Medication-Related Osteonecrosis of the Jaw. Medication-related Osteonecrosis of the Jaws: Bisphosphonates, Denosumab, and New Agents S. Otto. Heidelberg Springer 121-129 **2015.**

Hoff AO, Toth BB, Altundag K, Johnson MM, Warneke CL, Hu M, Nooka A, Sayegh G, Guarneri V, Desrouleaux K, Cui J, Adamus A, Gagel RF and Hortobagyi GN. Frequency and risk factors associated with osteonecrosis of the jaw in cancer patients treated with intravenous bisphosphonates. J Bone Miner Res 23: 826-836,**2008**.

Ji X, Pushalkar S, Li Y, Glickman R, Fleisher K and Saxena D. Antibiotic effects on bacterial profile in osteonecrosis of the jaw. Oral Diseases 18: 85-95,**2012**.

Kaplan I, Anavi K, Anavi Y, Calderon S, Schwartz-Arad D, Teicher S and Hirshberg A. The clinical spectrum of Actinomyces-associated lesions of the oral mucosa and jawbones: correlations with histomorphometric analysis. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology 108: 738-746,**2009**.

Katsarelis H, Shah NP, Dhariwal DK and Pazianas M. Infection and medication-related osteonecrosis of the jaw. J Dent Res 94: 534-539,**2015**.

Kaya D, Demirezen S, Hascelik G, Gulmez Kivanc D and Beksac MS. Comparison of PCR, culturing and Pap smear microscopy for accurate diagnosis of genital Actinomyces. J Med Microbiol 62: 727-733,**2013**.

Khosla S, Burr D, Cauley J, Dempster DW, Ebeling PR, Felsenberg D, Gagel RF, Gilsanz V, Guise T, Koka S, McCauley LK, McGowan J, McKee MD, Mohla S, Pendrys DG, Raisz LG, Ruggiero SL, Shafer DM, Shum L, Silverman SL, Van Poznak CH, Watts N, Woo SB, Shane E, American Society for B and Mineral R. Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research. J Bone Miner Res 22: 1479-1491,**2007**.

Kim KM, Rhee Y, Kwon YD, Kwon TG, Lee JK and Kim DY. Medication Related Osteonecrosis of the Jaw: 2015 Position Statement of the Korean Society for Bone and Mineral Research and the Korean Association of Oral and Maxillofacial Surgeons. J Bone Metab 22: 151-165,**2015**.

Lau SK, Woo PC, Fung AM, Chan KM, Woo GK and Yuen KY. Anaerobic, non-sporulating, Gram-positive bacilli bacteraemia characterized by 16S rRNA gene sequencing. J Med Microbiol 53: 1247-1253,**2004**.

Lazarovici TS, Yahalom R, Taicher S, Elad S, Hardan I and Yarom N. Bisphosphonate-related osteonecrosis of the jaws: a single-center study of 101 patients. J Oral Maxillofac Surg 67: 850-855,**2009**.

Li CL, Seneviratne CJ, Huo L, Lu WW and Zheng LW. Impact of Actinomyces naeslundii on bisphosphonate-related osteonecrosis of the jaws in ovariectomized rats with periodontitis. J Craniomaxillofac Surg 43: 1662-1669,**2015**.

Licata AA. Discovery, clinical development, and therapeutic uses of bisphosphonates. Ann Pharmacother 39: 668-677,**2005**.

Lopes RN, Rabelo GD, Rocha AC, Carvalho PA and Alves FA. Surgical Therapy for Bisphosphonate-Related Osteonecrosis of the Jaw: Six-Year Experience of a Single Institution. J Oral Maxillofac Surg 73: 1288-1295,**2015**.

Lopes RN, Rabelo GD, Rocha AC, Carvalho PAG and Alves FA. Surgical Therapy for Bisphosphonate-Related Osteonecrosis of the Jaw: Six-Year Experience of a Single Institution. Journal of Oral and Maxillofacial Surgery 73: 1288-1295,**2015**.

Maurer P, Sandulescu T, Kriwalsky MS, Rashad A, Hollstein S, Stricker I, Hoelzle F and Kunkel M. Bisphosphonate-related osteonecrosis of the maxilla and sinusitis maxillaris. International Journal of Oral and Maxillofacial Surgery 40: 285-291,**2011**.

Mitsimponas KT, Moebius P, Amann K, Stockmann P, Schlegel KA, Neukam FW and Wehrhan F. Osteo-radio-necrosis (ORN) and bisphosphonate-related osteonecrosis of the

jaws (BRONJ): the histopathological differences under the clinical similarities. Int J Clin Exp Pathol 7: 496-508,**2014**.
Mozzati M, Arata V and Gallesio G. Tooth extraction in osteoporotic patients taking oral bisphosphonates. Osteoporos Int 24: 1707-1712,**2013**.
Norouzi F, Aminshahidi M, Heidari B and Farshad S. Bacteremia Due to Actinomyces naeslundii in a T cell Lymphoma Child; a Case Report. Jundishapur J Microbiol 6: 306-308,**2013**.
O'Ryan FS, Khoury S, Liao W, Han MM, Hui RL, Baer D, Martin D, Liberty D and Lo JC. Intravenous bisphosphonate-related osteonecrosis of the jaw: bone scintigraphy as an early indicator. J Oral Maxillofac Surg 67: 1363-1372,**2009**.
O'Ryan FS and Lo JC. Bisphosphonate-related osteonecrosis of the jaw in patients with oral bisphosphonate exposure: clinical course and outcomes. J Oral Maxillofac Surg 70: 1844-1853,**2012**.
Otto S, Baumann S, Ehrenfeld M and Pautke C. Successful surgical management of osteonecrosis of the jaw due to RANK-ligand inhibitor treatment using fluorescence guided bone resection. J Craniomaxillofac Surg 41: 694-698,**2013**.
Otto S, Hafner S, Mast G, Tischer T, Volkmer E, Schieker M, Sturzenbaum SR, von Tresckow E, Kolk A, Ehrenfeld M and Pautke C. Bisphosphonate-related osteonecrosis of the jaw: is pH the missing part in the pathogenesis puzzle? J Oral Maxillofac Surg 68: 1158-1161,**2010**.
Otto S, Pautke C, Opelz C, Westphal I, Drosse I, Schwager J, Bauss F, Ehrenfeld M and Schieker M. Osteonecrosis of the Jaw: Effect of Bisphosphonate Type, Local Concentration, and Acidic Milieu on the Pathomechanism. Journal of Oral and Maxillofacial Surgery 68: 2837-2845,**2010**.
Otto S, Schreyer C, Hafner S, Mast G, Ehrenfeld M, Sturzenbaum S and Pautke C. Bisphosphonate-related osteonecrosis of the jaws - characteristics, risk factors, clinical features, localization and impact on oncological treatment. J Craniomaxillofac Surg 40: 303-309,**2012**.
Otto S, Troltzsch M, Jambrovic V, Panya S, Probst F, Ristow O, Ehrenfeld M and Pautke C. Tooth extraction in patients receiving oral or intravenous bisphosphonate administration: A trigger for BRONJ development? J Craniomaxillofac Surg 43: 847-854,**2015**.
Peer A and Khamaisi M. Diabetes as a Risk Factor for Medication-Related Osteonecrosis of the Jaw. Journal of Dental Research 94: 252-260,**2015**.
Pushalkar S, Li X, Kurago Z, Ramanathapuram LV, Matsumura S, Fleisher KE, Glickman R, Yan W, Li Y and Saxena D. Oral microbiota and host innate immune response in bisphosphonate-related osteonecrosis of the jaw. Int J Oral Sci,**2014**.
Ruby JD, Li Y, Luo Y and Caufield PW. Genetic characterization of the oral Actinomyces. Arch Oral Biol 47: 457-463,**2002**.
Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B and O'Ryan F. American Association of Oral and Maxillofacial Surgeons position paper on medication-related osteonecrosis of the jaw--2014 update. J Oral Maxillofac Surg 72: 1938-1956,**2014**.
Saia G, Blandamura S, Bettini G, Tronchet A, Totola A, Bedogni G, Ferronato G, Nocini PF and Bedogni A. Occurrence of bisphosphonate-related osteonecrosis of the jaw after surgical tooth extraction. J Oral Maxillofac Surg 68: 797-804,**2010**.
Sato T, Matsuyama J, Takahashi N, Sato M, Johnson J, Schachtele C and Hoshino E. Differentiation of oral Actinomyces species by 16S ribosomal DNA polymerase chain reaction-restriction fragment length polymorphism. Arch Oral Biol 43: 247-252,**1998**.
Schulthess B, Bloemberg GV, Zbinden R, Bottger EC and Hombach M. Evaluation of the Bruker MALDI Biotyper for identification of Gram-positive rods: development of a diagnostic algorithm for the clinical laboratory. J Clin Microbiol 52: 1089-1097,**2014**.

Sedghizadeh PP, Kumar SK, Gorur A, Schaudinn C, Shuler CF and Costerton JW. Microbial biofilms in osteomyelitis of the jaw and osteonecrosis of the jaw secondary to bisphosphonate therapy. J Am Dent Assoc 140: 1259-1265,**2009**.
Sedghizadeh PP, Yooseph S, Fadrosh DW, Zeigler-Allen L, Thiagarajan M, Salek H, Farahnik F and Williamson SJ. Metagenomic investigation of microbes and viruses in patients with jaw osteonecrosis associated with bisphosphonate therapy. Oral Surg Oral Med Oral Pathol Oral Radiol 114: 764-770,**2012**.
Tang G, Samaranayake LP and Yip HK. Genotypic diversity of oral Actinomyces naeslundii genospecies 1 and 2 in caries-active preschool children. Oral Microbiol Immunol 19: 371-378,**2004**.
Thumbigere-Math V, Sabino MC, Gopalakrishnan R, Huckabay S, Dudek AZ, Basu S, Hughes PJ, Michalowicz BS, Leach JW, Swenson KK, Swift JQ, Adkinson C and Basi DL. Bisphosphonate-related osteonecrosis of the jaw: clinical features, risk factors, management, and treatment outcomes of 26 patients. J Oral Maxillofac Surg 67: 1904-1913,**2009**.
Vahtsevanos K, Kyrgidis A, Verrou E, Katodritou E, Triaridis S, Andreadis CG, Boukovinas I, Koloutsos GE, Teleioudis Z, Kitikidou K, Paraskevopoulos P, Zervas K and Antoniades K. Longitudinal cohort study of risk factors in cancer patients of bisphosphonate-related osteonecrosis of the jaw. J Clin Oncol 27: 5356-5362,**2009**.
Wragg P, Randall L and Whatmore AM. Comparison of Biolog GEN III MicroStation semi-automated bacterial identification system with matrix-assisted laser desorption ionization-time of flight mass spectrometry and 16S ribosomal RNA gene sequencing for the identification of bacteria of veterinary interest. J Microbiol Methods 105: 16-21,**2014**.

TABLES LEGENDS

Table 1. Characteristics of patients diagnosed with MRONJ.

Table 2. Characteristics of MRONJ lesions

Table 3. PCR results of MRONJ bone samples

FIGURES LEGENDS

Figure 1. Flow chart of the number of patients included in the study.

Figure 2. Distribution of teeth involved in MRONJ at the different quadrants of maxilla and mandible where n is the number of teeth involved in each quadrant.

Figure 3. Pie charts showing distribution of microorganism in bone sample of MRONJ lesions from 2008 to 2014.

Role of microbiological culture and polymerase chain reaction (PCR) of Actinomyces in medication- related osteonecrosis of the jaw (MRONJ)

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Submission of a manuscript for publication in "Journal of Cranio-Maxillofacial Surgery"

Dear Professor Dr. Wiltfang,

On behalf of all the authors, I am pleased to submit an original research article entitled "**Role of microbiological culture and polymerase chain reaction (PCR) of Actinomyces in medication-related osteonecrosis of the jaw (MRONJ)**" for consideration for publication in JCMFS. In this manuscript, we hypothesized that local bacterial infections plays a critical role in the pathogenesis of medication-related osteonecrosis of the jaw (MRONJ). Recent developments in molecular methods have revolutionized new approaches for the rapid detection of microorganisms including those difficult to culture. Thus our study identified the bacterial profiles that colonize MRONJ bone samples by polymerase chain reaction (PCR) and microbiological culture with presenting clinical features of patients. Hereby, I confirm that this work is original and has not been published elsewhere nor is currently under consideration for publication elsewhere. All authors have approved the manuscript and agree with submission. The authors have no conflicts of interest to declare.
Please address all correspondence concerning this manuscript to me at (Sven.Otto@med.uni-muenchen.de). Thank you for your consideration to review this manuscript. We appreciate your time and look forward to your response

Sincerely,
Sven Otto.

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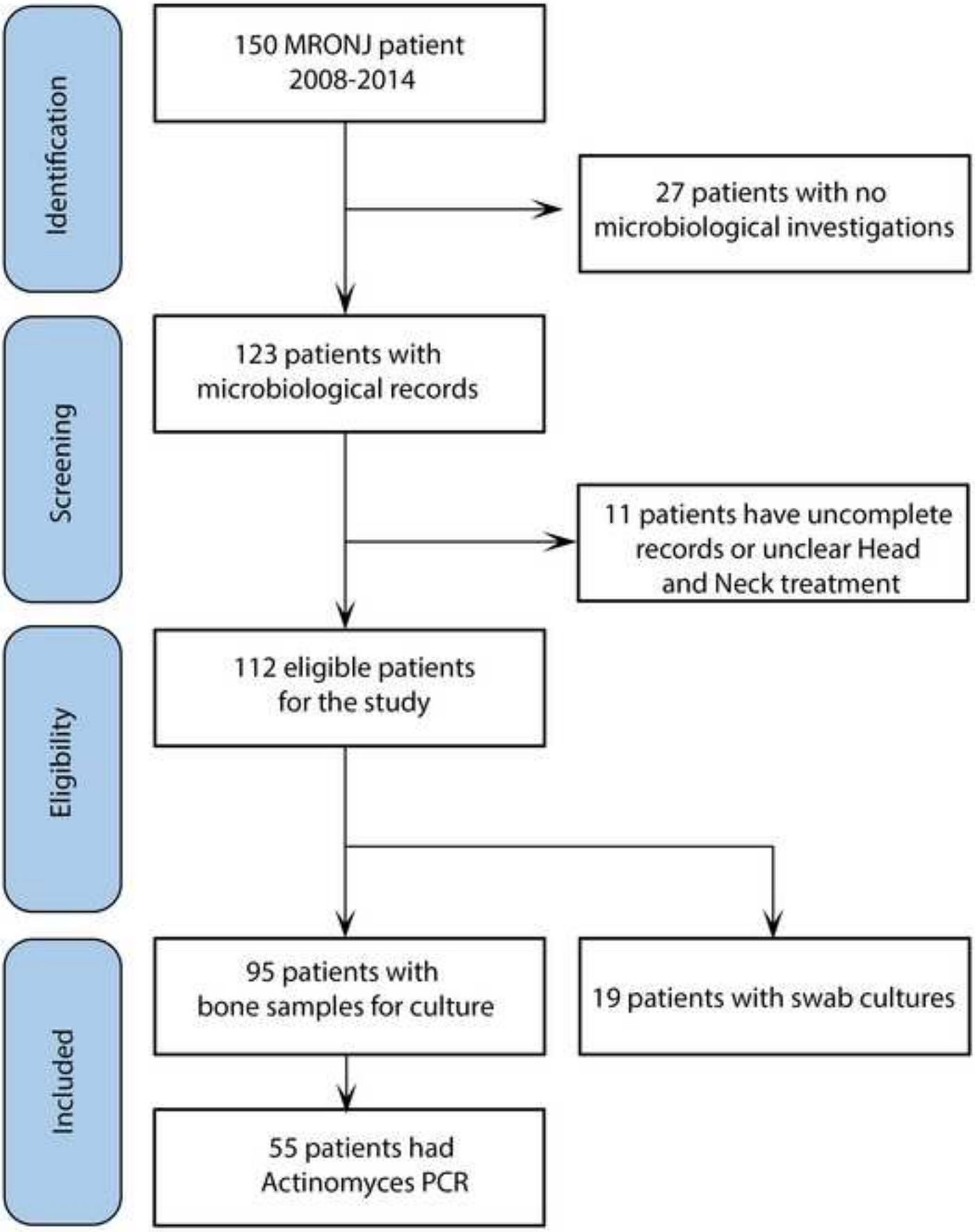

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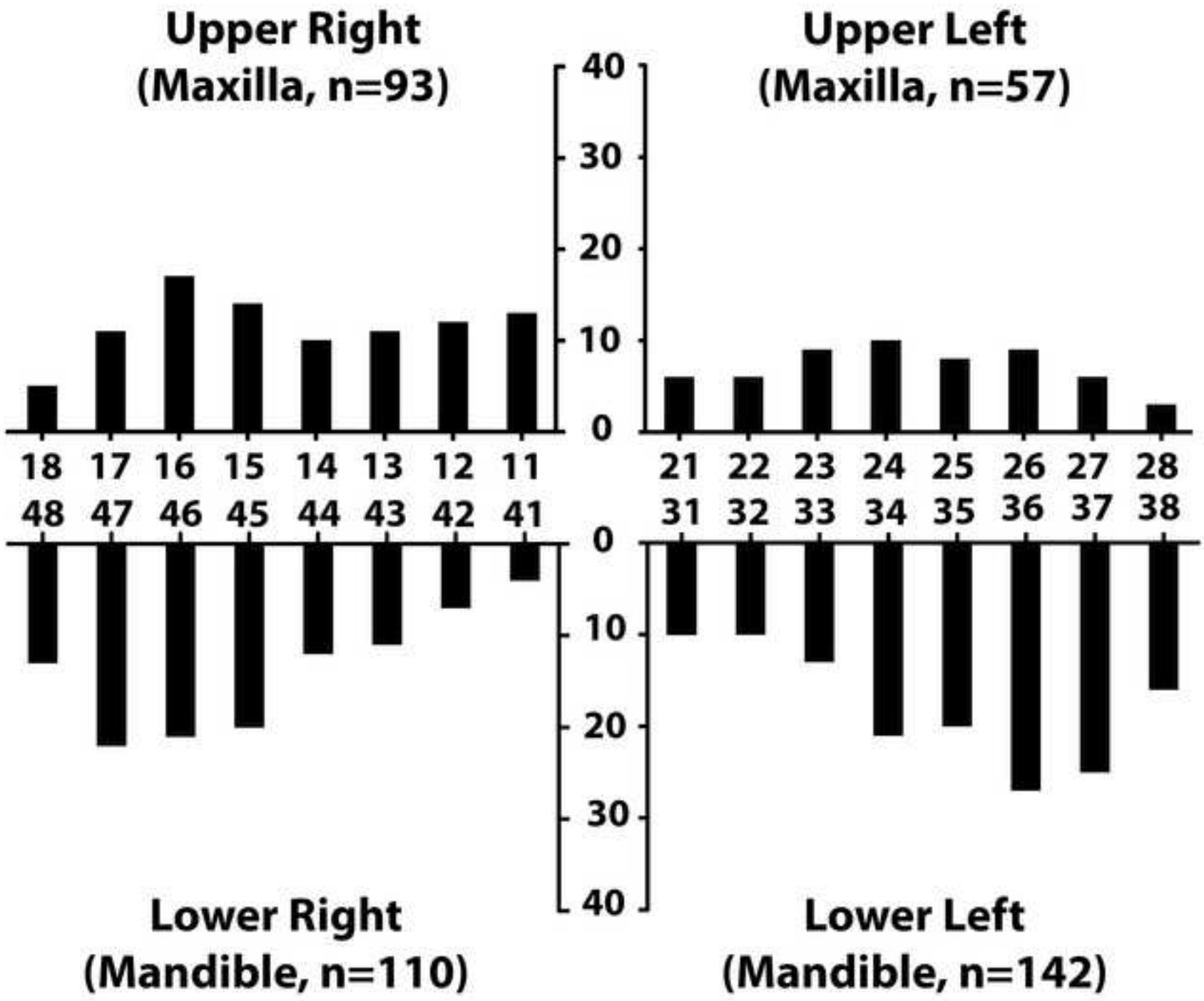

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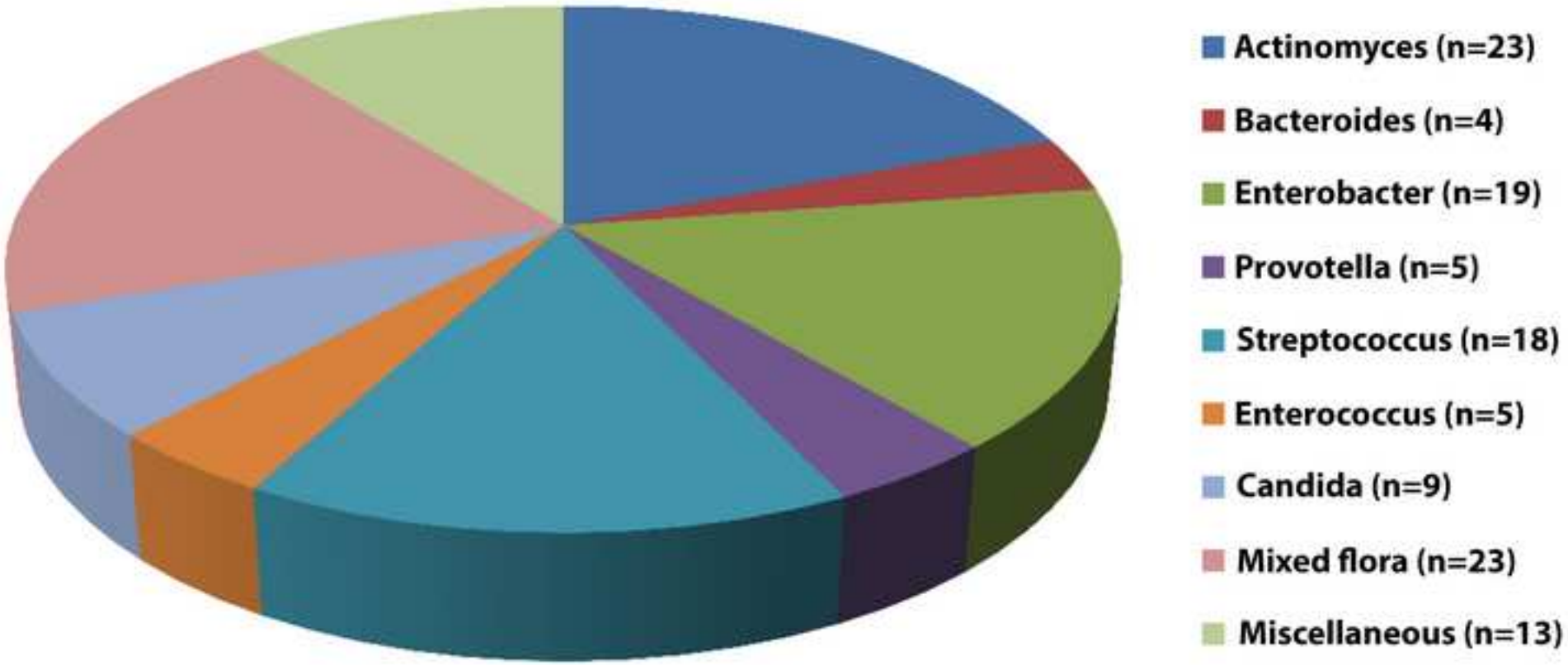

Highlights

1. We hypothesized that local bacterial infections plays a critical role in the pathogenesis of medication-related osteonecrosis of the jaw (MRONJ)
2. Bacterial profile of MRONJ bone samples was determined using microbiological culture and PCR.
3. Actinomyces were the most frequent microorganisms in the disease. However, this does not necessarily lead to the pathogenic role. PCR was found to be the most reliable method for the detection of these microorganisms.

Table 1: Characteristics of patients diagnosed with MRONJ.

Variable	Category	Number of patients (%) (n=95)
Age (years)	Mean	69.9 ± 8.7 years
Gender		
	Male	39 (41.1)
	Female	56 (58.9)
Primary cause		
	Breast cancer	35 (36.8)
	Prostate cancer	24 (25.3)
	Multiple myeloma	10 (10.5)
	Osteoporosis	13 (13.7)
	Lung cancer	4 (4.2)
	Other (Colon, Systemic Mastocytosis, Renal, Bladder, Thyroid, Endometrium)	9 (9.5)
Comorbidities		
	Diabetes Mellitus	17 (17.9)
	Cardiovascular disease	29 (30.5)
	Chemotherapy	57 (60)
	Irradiation (body)	51 (53.7)
	Steroid intake	28 (29.5)
	Antiangiogenic drugs	2 (2.1)
	Smoking	28 (29.5)
Antiresorptive drug (ARD)		
Bisphosphonate:		85 (89.5)
	Zoledronate	58 (61.1)
	Pamidronate	3 (3.2)
	Ibandronate	2 (2.1)
	Combination	22 (23.1)
Denosumab		10 (10.5)
Route of administration		
	Intravenous	79 (83.2)
	Oral	6 (6.3)
	Subcutaneous	10 (10.5)

Table 2: Characteristics of MRONJ lesions

Characteristics	Number of patients (%)
Staging of MRONJ	
Stage 0	1 (1.1)
Stage 1	15 (15.8)
Stage 2	59 (62.1)
Stage 3	20 (21.1)
Clinical presentation	
Pain	81 (85.3)
Exposed bone	70 (73.7)
Disturbance in wound healing	55 (57.9)
Inflammation	54 (56.8)
Pus	39 (41.1)
Pathological fracture	9 (9.5)
Swelling	55 (57.9)
Fistula	35 (36.8)
Sinus involvement	13 (13.7)
Histopathological Features	
Necrotic bone	94(98.9)
Inflammatory infiltrate	87(91.6)
Bacterial colonization	67(70.5)
Location	
Mandible	55 (57.9)
Maxilla	25 (26.3)
Both	15 (15.8)
Triggering events	
Extractions	56 (58.9)
Dentoalveolar surgery	15 (15.8)
Denture sore	4 (4.2)
Periodontal treatment	7 (7.4)
Spontaneous	13 (13.7)

Table 3: PCR results of MRONJ bone samples

Culture (n=55)	PCR (n, %) Positive	Negative
Positive	18(32.7)	0(0)
Negative	35(63.6)	2(3.6)
Total	53(96.4)	2(3.6)

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Comments on "Diagnosis and Management of Osteonecrosis of the Jaw: A Systematic Review and International Consensus"

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We read with great interest the recent article "Diagnosis and Management of Osteonecrosis of the Jaw: A Systematic Review and International Consensus" by Khan and colleagues.[1]

Medication-related osteonecrosis of the jaw (MRONJ) is a potentially severe adverse side effect of antiresorptive agents, and although a significant body of literature has been produced, there remains little evidence-based guidance for clinicians with respect to most aspects of this disease. Therefore, we applaud the attempt of Khan and colleagues to provide a much-needed systematic review.

However, it is important that any review on this topic is addressed on the basis of the best available evidence and a balanced analysis of the literature. More importantly, systematic reviews require rigorous research methods and a clear and transparent presentation of results in order to limit bias and maximize readability.[2–4] In the work of Khan and colleagues,[1] we have identified several issues that we suggest carry a risk of affecting the validity of their results.

Assessing the risk of bias is a crucial part of systematic reviews.[5,6] Khan and colleagues presented the criteria they used to assign level of evidence and grade recommendations, but unfortunately provided little information regarding qualitative assessment of reviewed studies, related risk of bias, as well as the process of article selection. Overall, it is hard to understand how and why articles were selected or excluded.

The presentation of data on incidence and prevalence makes the interpretation of the results difficult. It is well established that incidence data without definition of a time period can be meaningless;[7] nevertheless, results upon incidence of MRONJ are in several instances presented without mentioning the relevant time frame. There are also inconsistencies between different sections of the article: For example, in the abstract, it is stated that "in the osteoporosis patient population MRONJ incidence is estimated at 0.001 to 0.01%," whereas different figures are reported in the results (0.15% to $<$0.001% person-years of exposure). Furthermore, the authors state that the prevalence of MRONJ in the oncological setting ranges from "0 to 0.186%" whereas the work of Walter and colleagues, which they cite, reports a prevalence of 18.6%.[8]

Khan and colleagues report that the incidence of MRONJ in the osteoporosis population would only be "marginally higher than the incidence in the general population," which in the abstract is reported to be $<$0.001%.[1] This statement is quite

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*On behalf of the author team of the German S3 Guideline "Bisphosphonate-Associated Necrosis of the Jaw and Other Drug-Associated Necrosis of the Jaw."
**On behalf of the Italian Society of Maxillofacial Surgery.
***On behalf of the Italian Society of Oral Pathology and Medicine.
****On behalf of Cancer Network of North-Western Italy.

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confusing because it remains unclear what they mean by "incidence of jaw necrosis in the general population." Possibly the authors refer to other disorders that may cause jaw necrosis in the absence of antiresorptive therapy. We wonder whether it is appropriate to associate different populations with different disorders when incidence/prevalence is discussed in a systematic review. Also, we could not find any clear reference in the text supporting the reported $<0.001\%$ incidence; it remains uncertain where this figure comes from.

We also found it singular and rather unusual for a systematic review to provide a detailed description of an unrelated, poorly characterized, and not widely accepted disease entity, namely oral ulceration and bone sequestration (OUBS).[1] Its relevance to the intended systematic review on MRONJ remains unclear. The articles cited in the main part of the paper (Introduction)[9–11] do not provide convincing evidence regarding the impact this questionable disease may have upon patients, and certainly they cannot suggest that a significant portion of cases of MRONJ could, in fact, represent misdiagnosed OUBS.

The definition of MRONJ continues to cause significant controversy. Khan and colleagues seem to disregard the suggestions of different independent research groups who have called for a change in the traditional definition[12] so as to include the nonexposed variant of MRONJ,[13–23] which can represent up to 25% of all cases.[13] We wonder whether the authors concluded that these articles were in some way flawed and, therefore, had to be excluded from the systematic review. It is also rather surprising that they decided not to embrace the revised 2014 AAOMS consensus, which agrees that individuals presenting with bone that can be probed via sinus tracts do fit MRONJ definition.[24]

With respect to MRONJ treatment, readers would expect a systematic review to provide a balanced and fair comparison of the outcomes of different interventions, both surgical and nonsurgical. However, Khan and colleagues suggest that "conservative therapy is the mainstay of care" with no robust convincing evidence in support of this statement. Although we agree that there is a lack of consensus, as well as very little information on the outcomes of denosumab-related ONJ, we think that this review does not provide a fair and comprehensive summary of current knowledge and available evidence.

For example, when mucosal healing is considered the primary outcome,[25–28] a number of articles have reported that less than one-third of patients managed with long-term conservative treatment, especially in the oncological setting, would show evidence of mucosal healing (23% and 14.9% of Hoff and colleagues[29] and Nicolatou-Galitis and colleagues[30] case series, respectively). This means that the majority of MRONJ patients managed conservatively would present persistent jawbone exposure, which not only can affect their quality of life[31] but may also limit the oncological treatment options, including further antiresorptive treatment.[32,33] Although conservative treatment might be adequate to slow down disease progression and control pain and infections, there is increasing evidence supporting surgical treatment protocols. Case series from different research groups report percentages of mucosal healing that are consistently around and above 80%, with outcome endpoints ranging from 3 months to 7 years post-treatment. Examples include Carlson and colleagues (92%), [26] Stockmann and colleagues (89%), [34] Bedogni and colleagues (90%), [35] Schubert and colleagues (89%), [36] and Jacobsen and colleagues (78%). [37] Comparative studies also seem to confirm these results.[38,39] Finally, both the systematic review by Rupel and colleagues[40] and another recent systematic review meeting PRISMA guidelines[6] suggest that surgical therapy can be superior to conservative management.[41]

We feel that these are important aspects completing the review of Khan and colleagues.[1]

References

1. Khan AA, Morrison A, Hanley DA, et al. Diagnosis and management of osteonecrosis of the jaw: a systematic review and international consensus. J Bone Miner Res. 2015;30(1):3–23.
2. Sharif MO, Janjua-Sharif FN, Ali H, Ahmed F. Systematic reviews explained: AMSTAR-how to tell the good from the bad and the ugly. Oral Health Dent Manag. 2013;12(1):9–16.
3. Yuan Y, Hunt RH. Systematic reviews: the good, the bad, and the ugly. Am J Gastroenterol. 2009;104(5):1086–92.
4. Holmdahl L. A misleading meta-analysis of seprafilm. World J Surg. 2008;32(8):1888–9; author reply 1890–1.
5. Humphrey PR. Systematic reviews of diagnostic test evaluations: what's behind the scenes? ACP J Club. 2004;141(3):A14.
6. Liberati A, Altman DG, Tetzlaff J, et al. The PRISMA statement for reporting systematic reviews and meta-analyses of studies that evaluate health care interventions: explanation and elaboration. PLoS Med. 2009;6(7):e1000100.
7. Noordzij M, Dekker FW, Zoccali C, Jager KJ. Measures of disease frequency: prevalence and incidence. Nephron Clin Pract. 2010;115 (1):17–20.
8. Walter C, Al-Nawas B, Grotz KA, et al. Prevalence and risk factors of bisphosphonate-associated osteonecrosis of the jaw in prostate cancer patients with advanced disease treated with zoledronate. Eur Urol. 2008;54(5):1066–72.
9. Peters E, Lovas GL, Wysocki GP. Lingual mandibular sequestration and ulceration. Oral Surg Oral Med Oral Pathol. 1993;75(6): 739–43.
10. Scully C. Oral ulceration: a new and unusual complication. Br Dent J. 2002;192(3):139–40.
11. Sonnier KE, Horning GM. Spontaneous bony exposure: a report of 4 cases of idiopathic exposure and sequestration of alveolar bone. J Periodontol. 1997;68(8):758–62.
12. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg. 2007;65(3):369–76.
13. Fedele S, Bedogni G, Scoletta M, et al. Up to a quarter of patients with osteonecrosis of the jaw associated with antiresorptive agents remain undiagnosed. Br J Oral Maxillofac Surg. 2015;53(1): 13–7.
14. Fedele S, Porter SR, D'Aiuto F, et al. Nonexposed variant of bisphosphonate-associated osteonecrosis of the jaw: a case series. Am J Med. 2010;123(11):1060–4.
15. Hutchinson M, O'Ryan F, Chavez V, et al. Radiographic findings in bisphosphonate-treated patients with stage 0 disease in the absence of bone exposure. J Oral Maxillofac Surg. 2010;68(9): 2232–40.
16. Junquera L, Gallego L. Nonexposed bisphosphonate-related osteonecrosis of the jaws: another clinical variant? J Oral Maxillofac Surg. 2008;66(7):1516–7.
17. Mawardi H, Treister N, Richardson P, et al. Sinus tracts—an early sign of bisphosphonate-associated osteonecrosis of the jaws? J Oral Maxillofac Surg. 2009;67(3):593–601.
18. Patel S, Choyee S, Uyanne J, et al. Non-exposed bisphosphonate-related osteonecrosis of the jaw: a critical assessment of current definition, staging, and treatment guidelines. Oral Dis. 2012;18(7): 625–32.
19. Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B. American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaws—2009 update. J Oral Maxillofac Surg. 2009;67(5 Suppl):2–12.

20. Yarom N, Fedele S, Lazarovici TS, Elad S. Is exposure of the jawbone mandatory for establishing the diagnosis of bisphosphonate-related osteonecrosis of the jaw? J Oral Maxillofac Surg. 2010;68(3):705.
21. Schiodt M, Reibel J, Oturai P, Kofod T. Comparison of nonexposed and exposed bisphosphonate-induced osteonecrosis of the jaws: a retrospective analysis from the Copenhagen cohort and a proposal for an updated classification system. Oral Surg Oral Med Oral Pathol Oral Radiol. 2014;117(2):204–13.
22. Colella G, Campisi G, Fusco V. American Association of Oral and Maxillofacial Surgeons position paper: bisphosphonate-related osteonecrosis of the jaws—2009 update: the need to refine the BRONJ definition. J Oral Maxillofac Surg. 2009;67(12):2698–9.
23. Bedogni A, Fusco V, Agrillo A, Campisi G. Learning from experience. Proposal of a refined definition and staging system for bisphosphonate-related osteonecrosis of the jaw (BRONJ). Oral Dis. 2012;18(6):621–3.
24. Ruggiero SL, Dodson TB, Fantasia J, et al. American Association of Oral and Maxillofacial Surgeons position paper on medication-related osteonecrosis of the jaw—2014 update. J Oral Maxillofac Surg. 2014;72(10):1938–56.
25. Assaf AT, Zrnc TA, Riecke B, et al. Intraoperative efficiency of fluorescence imaging by Visually Enhanced Lesion Scope (VELscope) in patients with bisphosphonate related osteonecrosis of the jaw (BRONJ). J Craniomaxillofac Surg. 2014;42(5):e157–64.
26. Carlson ER, Basile JD. The role of surgical resection in the management of bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg. 2009;67(5 Suppl):85–95.
27. Pautke C, Bauer F, Otto S, et al. Fluorescence-guided bone resection in bisphosphonate-related osteonecrosis of the jaws: first clinical results of a prospective pilot study. J Oral Maxillofac Surg. 2011; 69(1):84–91.
28. Vescovi P, Manfredi M, Merigo E, Meleti M. Early surgical approach preferable to medical therapy for bisphosphonate-related osteonecrosis of the jaws. J Oral Maxillofac Surg. 2008;66(4):831–2.
29. Hoff AO, Toth BB, Altundag K, et al. Frequency and risk factors associated with osteonecrosis of the jaw in cancer patients treated with intravenous bisphosphonates. J Bone Miner Res. 2008;23(6): 826–36.
30. Nicolatou-Galitis O, Papadopoulou E, Sarri T, et al. Osteonecrosis of the jaw in oncology patients treated with bisphosphonates: prospective experience of a dental oncology referral center. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2011;112(2):195–202.
31. Kyrgidis A, Triaridis S, Kontos K, et al. Quality of life in breast cancer patients with bisphosphonate-related osteonecrosis of the jaws and patients with head and neck cancer: a comparative study using the EORTC QLQ-C30 and QLQ-HN35 questionnaires. Anticancer Res. 2012;32(8):3527–34.
32. Then C, Horauf N, Otto S, et al. Incidence and risk factors of bisphosphonate-related osteonecrosis of the jaw in multiple myeloma patients having undergone autologous stem cell transplantation. Onkologie. 2012;35(11):658–64.
33. Otto S, Schreyer C, Hafner S, et al. Bisphosphonate-related osteonecrosis of the jaws—characteristics, risk factors, clinical features, localization and impact on oncological treatment. J Craniomaxillofac Surg. 2012;40(4):303–9.
34. Stockmann P, Vairaktaris E, Wehrhan F, et al. Osteotomy and primary wound closure in bisphosphonate-associated osteonecrosis of the jaw: a prospective clinical study with 12 months follow-up. Support Care Cancer. 2010;18(4):449–60.
35. Bedogni A, Saia G, Bettini G, et al. Long-term outcomes of surgical resection of the jaws in cancer patients with bisphosphonate-related osteonecrosis. Oral Oncol. 2011;47(5):420–4.
36. Schubert M, Klatte I, Linek W, et al. The saxon bisphosphonate register—therapy and prevention of bisphosphonate-related osteonecrosis of the jaws. Oral Oncol. 2012;48(4):349–54.
37. Jacobsen C, Metzler P, Obwegeser JA, Zemann W, Graetz KW. Osteopathology of the jaw associated with bone resorption inhibitors: what have we learned in the last 8 years? Swiss Med Wkly. 2012;142:w13605.
38. Mucke T, Koschinski J, Deppe H, et al. Outcome of treatment and parameters influencing recurrence in patients with bisphosphonate-related osteonecrosis of the jaws. J Cancer Res Clin Oncol. 2011; 137(5):907–13.
39. Graziani F, Vescovi P, Campisi G, et al. Resective surgical approach shows a high performance in the management of advanced cases of bisphosphonate-related osteonecrosis of the jaws: a retrospective survey of 347 cases. J Oral Maxillofac Surg. 2012;70(11):2501–7.
40. Rupel K, Ottaviani G, Gobbo M, et al. A systematic review of therapeutical approaches in bisphosphonates-related osteonecrosis of the jaw (BRONJ). Oral Oncol. 2014;50(11):1049–57.
41. Fliefel R, Tröltzsch M, Kühnisch J, Ehrenfeld M, Otto S. Treatment strategies and outcomes of bisphosphonate related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. Int J Oral Maxillofac Surg. Epub 2015 Feb 25.

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Systematic review of oral ulceration with bone sequestration

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ABSTRACT

Background: This article represents the first systematic review entirely dedicated toward a disease called oral ulceration with bone sequestration (OUBS). We performed this review in order to further define and outline this disease. A secondary interest was to recognize the prevalence and importance of OUBS in relation to other oral disorders accompanied by ulceration and bone exposure.
Material and methods: The systematic review was registered with PROSPERO (registration number CRD42015024294) and performed in cooperation with Harvard's Countway Library. Searches were built using MeSH terms and proximity operators previously mentioned in OUBS descriptions. Database searches were performed through EMBASE, Medline, and PubMed, followed by a handsearch of bibliographies for relevant articles. Articles were assessed against eligibility and inclusion criteria centering on bone exposure without known etiologic cause. We sought to gather information on patient age, sex, anatomical location, clinical presentation, and comorbidities. PRISMA guidelines were followed.
Results: The searches identified 766 records total. Despite considerable inspection, we found only 8 articles qualifying for our review. In the 8 articles, there were a total of 24 patients fulfilling the criteria of OUBS. Although some abstracts mentioned idiopathic nature, most authors presented clinical cases with probable causes to ulceration and sequestration. The mean age of these patients was 43.21 ± 11.94 years. The male to female ratio was 3:1. The predominant area of occurrence was the mandible (n = 23, 95.8%).
Conclusion: The representation of OUBS in the literature remains scarce. More data must be generated and gathered on the concept of OUBS so as to determine the true incidence and importance of this disease. Despite rare occurrences of conditions characterizing OUBS, the recent discussion of this topic in the scientific community calls for more knowledge to be brought forth, with great benefit to patients suffering from ulcerative diseases and osteonecrosis.

1. Introduction

Oral ulceration with bone sequestration (OUBS) has been used variably by authors and practitioners to characterize oral ulceration and bone sequestration without an etiologic cause (Khan et al., 2015). The most common locations reported for ulceration and subsequent bone exposure occur at the mylohyoid ridge, mandibular tori, palatal tori, and mandibular exostosis (Farah and Savage, 2003). No precise definition for OUBS has been provided or accepted, and there has been recent debate in the literature pertaining to the disorder's conceptualization (Khan et al., 2015; Otto et al., 2015a). It is a rare condition which contains cases previously recognized by the term 'lingual mandibular sequestration and ulceration' (Peters et al., 1993). Almazrooa and Woo (2009) define OUBS as, "spontaneous sequestration of the lingual mandibular bone, usually in the area of the mylohyoid ridge, in patients with no significant underlying systemic condition." Previously, to the best of our knowledge, only one review has been attempted on OUBS (Khan et al., 2015).

The objective of this current systematic review was to distinguish between various ulcerative disorders of the oral cavity and that of OUBS. Although oral ulceration is common, it is normally self-limiting and the progression to bone exposure and

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sequestration is extremely rare (Farah and Savage, 2003). Medical practitioners still must be aware of the local, systemic, and anatomical predispositions which lead to increasingly destructive ulcerative disorders. Our secondary interest concerned assessing the prevalence and importance of OUBS in relation to other oral maladies associated with bone exposure. The determination of a general prevalence value will be significant since OUBS has recently been stressed as a potential differential diagnosis pertaining to ONJ (Khan et al., 2015). We sought to collect articles and case reports mentioning idiopathic bone exposure or sequestration in order to begin constructing a framework for characterizing this concept.

2. Materials and methods

This systematic review was performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2009). Searches were conducted on 13th July 2015 after being registered with PROSPERO (registration #CRD42015024294). No date limits were applied for the beginning of the search in order to obtain all relevant articles. Research experts at Harvard's Countway Library were consulted for expertise in article identification. Databases were screened based on the MeSH and Emtree terms developed in conjunction with Harvard's Countway Library. The search was ended on 13th July 2015.

We searched Embase, MEDLINE, and PubMed for articles that mentioned key words for oral ulceration with exposed, necrotic, idiopathic, unexplained, spontaneous, or sequestered bone. Controlled vocabulary terms (both MeSH and Emtree) were used when available and appropriate. Searches were linked by OR operators and intersections of concepts linked consecutively with AND statements. Proximity operators (NEAR/n) allowed for detection of words possibly combined in unpredictable ways. Appendix 1 contains search algorithms used. Each search contains field tags which tell interfaces which indexes to search. A final handsearch through bibliographies of relevant articles was conducted in order to accumulate as much information on OUBS as possible.

Inclusion criteria were met if: 1) the abstract made any discussion on oral ulceration and bone sequestration without providing an etiologic cause; 2) an 'idiopathic' or an 'unknown' etiology was stated in the abstract regardless of pathologic mechanisms discussed; 3) full article in English language was available. Records were excluded if one of the following criteria pertained: 1) language other than English; 2) pertaining diagnostic methods unrelated to OUBS; 3) pertaining to animals and unrelated to OUBS; 4) pertaining to therapy unrelated to OUBS; 5) pertaining to topics irrelevant to OUBS; 6) etiologic cause of oral ulceration or bone sequestration was given in abstract (i.e. antiresorptive drugs, osteomyelitis, osteoradionecrosis, trauma, neoplasia, etc). This systematic review followed the PRISMA guidelines (6).

3. Results

The searches returned a total of 766 records. After duplicates were removed, 726 records remained (Fig. 1). All 726 articles were

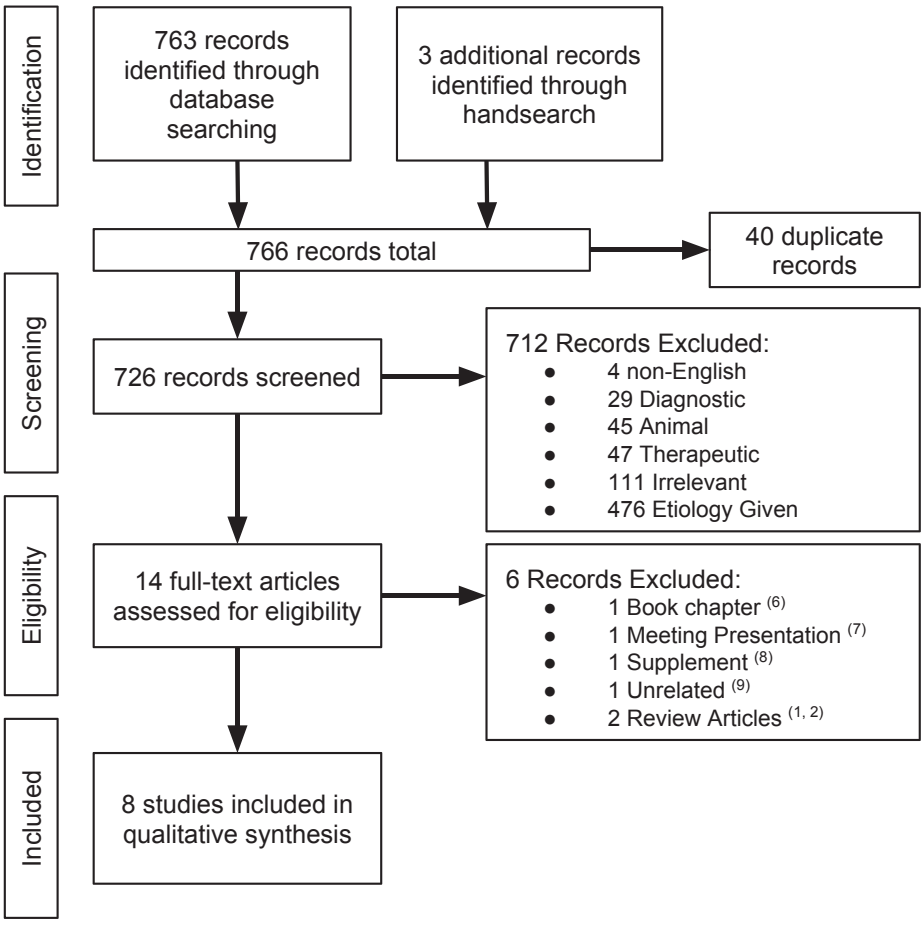

Fig. 1. Flow chart for record selection.

screened for titles and abstracts and 712 records were excluded based on the exclusion criteria. Of the records excluded, 4 were not in English, 29 discussed diagnostic material unrelated to OUBS, 45 pertained to animals, 47 discussed therapy unrelated to OUBS, 111 were irrelevant to the topic, and 476 discussed or specified an etiology. These etiologic causes occurred individually and in combination within categories associated with infection: bacteria and osteomyelitis (118 records), fungal (9 records), viral (7 records), myiasis (2 records); treatment: systemic medications such as bisphosphonates (155 records), denosumab (4 records), and other drugs including antiangiogenic agents (11 records), drugs such as cocaine and Krokadil (5 records), osteoradionecrosis (18 records); trauma (8 records); and alternative causes such as neoplasia (63 records), congenital or genetic defects (39 records), autoimmune or autoinflammatory (23 records), avascular necrosis (5 records), and other various bone disorders (9 records). A post-analysis term search for 'idiopathic' was performed and identified 20 abstracts in which it was used as a descriptor, however none of these articles made it to the final inclusion stage. This selection process left 14 records eligible for full-text review.

Of these 14 articles, 8 were synthesized in the qualitative analysis (Table 1 and Supplement 1 & 2). Six papers were excluded: Huebsch (1965) was not available electronically, was published in 1965, and did not specify idiopathic causes. Two records by Khan (2014); Khan et al. (2013) were excluded because one was a conference presentation and the other a supplemental document. Boffano et al. (2012) was excluded because although mentioning idiopathic causes of mandibular fractures in the abstract, the paper itself made no mention of idiopathic pathologies. Two papers pertained to OUBS and were valuable in its conceptualization (Khan et al., 2015; Farah and Savage, 2003), however, neither article presented new clinical cases on OUBS so they were not included in the analysis. The 8 remaining articles presented 24 different patient cases in total.

Papers were read in full to extract information for the completion of Table 1. If an article failed to present information or characteristics of the clinical presentation we identified and calculated it as absent in the table. No meta analysis was performed due to low number of cases and the possibility of ascertainment bias in these patient's access to care. Racial categories were excluded due to outdated terms (i.e. Mongoloid, East Indian) used in older articles. Readers can also consider implications of notoriety bias with ONJ, discussed by De Boissieu et al. (2014). However, as this bias regarded bisphosphonates in relation to chemotherapeutic agents and not idiopathic causes, its relevance to OUBS was minimal.

In the 8 articles, there were a total of 24 patients fulfilling the criteria of OUBS. Quantitative data was reported but is supported only through qualitative descriptions as the literature contained insufficient power ($n < 30$). The mean age of these patients was 43.21 ± 11.94 years. There was a male predilection in the ratio of 3:1 as there were 18 male patients (75%) and 6 female patients (25%). The most common location was the mandible ($n = 23$, 95.8%), with one case occurring in both the maxilla and mandible ($n = 1$, 4.2%), and no cases occurring solely in the maxilla ($n = 0$, 0.0%). Comorbidities that were detected in the patients included: 4 patients presenting with systemic disease (rheumatoid arthritis, glomerulosclerosis, type II diabetes, allergic disorder), 3 patients presented with infections (periodontitis, actinomyces infection), and 2 patients receiving relevant medications (prednisolone and bisphosphonates, and methotrexate). The most common predisposing factors were loss of lingual inclination in 9 patients (extractions, restoration, missing teeth), exostoses and tori in 8 patients, or 6 patients with other causes (1 with bruxism, 1 with foreign particulate, 1 with periodontal scaling, 2 with minor aphthous stomatitis, and 2 with a stress-induced tongue thrust habit), as well as no causes indicated in 6 cases. Clinically, all 24 patients presented with bone sequestration. Some clinically relevant information was missing and marked as absent, as such, 17 cases contained descriptions of ulceration ($n = 17$, 70.8%), 3 cases described no ulceration ($n = 3$, 12.5%), and 4 cases did not present information on ulceration ($n = 4$, 16.7%). Bone exposure was explicitly described in 11 cases ($n = 11$, 45.8%), and sinus tract involvement was specified in 2 patients ($n = 2$, 8.3%).

None of the articles included in the study contained reliable information on the prevalence or incidence of OUBS in the general population. Due to limited number of patients identified and without data regarding the underlying general population, no calculation of prevalence or incidence could be performed.

4. Discussion

This systematic review attempted to distinguish between OUBS and other oral ulcerative disorders, as well as to assess the prevalence and importance of OUBS. OUBS has recently been stressed with more attention as a differential diagnosis for osteonecrosis of the jaw (ONJ). There is a high prevalence of oral soft tissue lesions in the general population, although progression to bone sequestration is extremely rare (Shulman et al., 2004). Multiple risk factors can cause the accumulation and perpetuation of ulcers however, in which local and systemic factors can lead to sequestration via independent pathomechanisms. These cases present frequently to health clinics and it is essential that medical practitioners are aware of the underlying disorders in their provision of treatment. OUBS is a recent concept to the osteologic field and this report aims to clarify its conceptualization and understanding.

The rare occurrence of OUBS, its poor representation in the literature, differing definitions and descriptions by authors, lack of understanding or awareness by practitioners, and perhaps inadequate retrieval methods from the literary searches above limit the power of this review. Further limitations to the retrieval of relevant articles pertain to a publication bias in which disorders with undetermined causes would be less likely to be written up by practitioners, and less likely to be published by journals. We chose broad terms in our search which would underlie descriptions of OUBS. However, we found this terminology used in a wide array of abstracts. For instance, the term idiopathic was alone used as a descriptive term by authors in 20 abstracts, none of which made it to our qualitative analysis. Frequently records came up for medication related osteonecrosis of the jaw (MRONJ) cases in which authors used the terms idiopathic in a non-specific manner. Although we were searching for OUBS we found many more records concerning disorders such as bisphosphonate related osteonecrosis of the jaw (BRONJ), osteomyelitis, osteoradionecrosis, neoplasia, and other bone disorders (Lee et al., 2015; Mücke et al., 2015; Otto et al., 2015b).

We identified 8 articles pertaining to disorders which could be classified as OUBS. Although most authors describe an idiopathic disorder in the abstract, all but two articles contained detailed clinical presentations which described and discussed some distinct ulcerative etiology. Furthermore, many articles even discussed the role of these local and systemic factors pertaining to the patient case, as well as the pathological process which linked such an ulcerative event to bone sequestration.

We determined a predilection for middle age (mean 43.21 ± 11.94 years), with only 1 case occurring in a patient under 32 years old. This finding is supported with the knowledge that ulcerations are more likely to occur with age, occurring due to weakening mucosa and impaired immunity and increased probability of losing teeth (Dhanuthai et al., 2015). It was also much more likely that males suffered from ulceration and bone sequestration

Table 1
Qualitative analysis.

Article	Case #	Age	Sex	Anatomical location	Clinical presentation	Comorbidities
Carrard et al., 2009 [24]	1	38	Male	Bilateral exostoses in mandibular lingual molar area	Bilateral ulcerations (15 × 7 mm) with exposed necrotic bone sequestrum; symptomatic 1 month	Bilateral exostoses in lingual mandibular cortical bone; Bruxism
Flaitz, 2000 [29]	1	56	Female	Left lingual mucosa covering mylohyoid ridge near left molars	Ulcer (3 × 8 mm) with exposed necrotic bone sequestrum; symptomatic 2 months, worsening last 2 weeks	Rheumatoid arthritis managed with prednisone and methotrexate
Jackson and Malden (2007) [20]	1	41	Male	Right lingual mucosa covering mylohyoid ridge near molars	12 mm ulceration and exposed necrotic bone sequestrum; symptomatic 1 week	1 week post-extraction #48; #47 also missing
	2	57	Male	Right lingual mucosa covering mylohyoid ridge and molar region	15 mm ulceration and exposed necrotic bone sequestrum; symptomatic 1 week	1 month post-extraction #47; food debris present
	3	48	Male	Left lingual mucosa covering mylohyoid ridge near molar region	12 mm ulceration and exposed necrotic bone sequestrum; symptomatic 2 months	Glomerulosclerosis managed with prednisolone and alendronic acid, actinomyces-like infection
Kessler (2005) [30]	1	40	Male	Right lingual mucosa covering mylohyoid ridge near molar region	Ulceration and exposed necrotic bone sequestrum; symptomatic 3 weeks	None described by practitioner
Koshal et al. (2010) [16]	1	5	Male	Left mandibular buccal alveolus with deciduous canine and first molar (#73 & #74)	Demarcated, necrotic, non-healing alveolar bone division; symptomatic 2 months	None described by practitioner
Peters et al. (1993) [4]	1	53	Female	Lingual mucosa covering mylohyoid ridge	3 mm ulcer with 2 mm sequestrum; symptomatic 12 weeks	None described by practitioner
	2	55	Male	Lingual mucosa covering mylohyoid ridge	3 mm ulcer with 3 mm sequestrum; symptomatic 8 weeks	Type II Diabetes
	3	42	Female	Lingual mucosa covering mylohyoid ridge	3 mm sequestrum; symptomatic 3 weeks	Bilateral mandibular tori; missing first molar
	4	32	Male	Lingual mucosa covering mylohyoid ridge	3–4 mm ulcer with 4 mm sequestrum; symptomatic “few months”	Missing first and third molar
	5	50	Female	Lingual mucosa covering mylohyoid ridge	4 mm sequestrum	None described by practitioner
	6	34	Male	Lingual mucosa covering mylohyoid ridge	3 mm sequestrum; symptomatic 2 weeks	None described by practitioner
	7	33	Male	Lingual mucosa covering mylohyoid ridge	8 mm ulcer with 6 mm sequestrum; symptomatic 1 week	Restorations 1 week prior to onset
	8	47	Male	Lingual mucosa covering mylohyoid ridge	6 mm sequestrum	None described by practitioner
	9	57	Male	Lingual mucosa covering mylohyoid ridge	3 mm ulcer with 2 mm sequestrum; symptomatic 3 weeks	Missing second and third molar
	10	55	Female	Lingual mucosa covering mylohyoid ridge	4 mm ulcer with 10 mm sequestrum	Bilateral mandibular tori; Missing all posterior molars
	11	40	Male	Lingual mucosa covering mylohyoid ridge	8 mm ulcer with 3 mm sequestrum; symptomatic 2 weeks	Bilateral mandibular tori; Missing third molar; Hypertension controlled with medication; recent flu
Scully (2002a,b) [14]	1	45	Male	Inferior to left mandibular lingual oblique ridge	5 mm ulcer with 3 mm sequestrum; symptomatic 5 days	Scaling session few days prior to onset; minor aphthous stomatitis
	2	53	Male	Inferior to left mandibular lingual oblique ridge	5 mm ulcer with 4 mm sequestrum; symptomatic 10 days	History of minor aphthous stomatitis
Sonnier and Horning (1997) [15]	1	32	Male	Left lingual mucosa near tooth #34	Ulceration with 3 bony lesions on central tori; symptomatic 4 months	Bilateral mandibular tori; Stressful habit (tongue thrush) during periodontal residency
	2	53	Male	Bilateral mandibular lingual mucosa	10 mm ulcers bilaterally with necrotic bone sequestrum; symptomatic 2 weeks	Exostoses present along entire lingual mandible; Periodontitis (4–7 mm probing depth)
	3	38	Female	Right lingual mucosa on mandibular near teeth #45 and #46	No ulceration, 3 sinus tracts leading to multiple bony lesions (5 × 15 × 1 mm) on exostoses; symptomatic 2 weeks	Exostoses present near tooth #45 and #46; multiple restorations
	4	33	Male	Biopsy on facial gingiva near teeth #15 and #16; sequestrum near tooth #28; sequestrum near sinus tract of tooth #26	No ulceration, 1 sinus tract, 3 × 2 × 1 mm sequestrum; symptomatic 1 month	Allergic gingivitis\mucositis; Periodontitis (5–7 mm probing depths); Early multifocal osseous dysplasia; Vasculitis; Hyperplasia with intermittent exostosis

*Tooth numbering according to the FDI World Dental Federation notation.

(n = 18 males, 75%). Sex could play a role in ulceration for multiple reasons from increased alcohol consumption, tobacco use, increased masticatory loads, prominent mylohyoid ridge, but none of these can be discussed with adequate power.

After review it was noted that this area of research has three foundational papers, Peters et al. (1993), Scully (2002a,b), and Sonnier and Horning (1997). We identified only one article, Koshal and the presenting 5 year-old patient, which was not reliant on these authors for substantiation (Koshal et al., 2010). The significant role these papers continue to sustain should call for critical review. These articles were published in 1992, 1997, and 2002, respectively. The advancement in molecular pathology, cytopathology, and histopathology over the past decade is not part of the case reports and disease identification process. Furthermore, some descriptions

were performed retrospectively, lacking information on clinical presentation, anatomical location, and possible risk factors. Interestingly, these foundational articles stress ulcerative risk factors such as trauma, infection, systemic disorders, neoplasia, anatomical predispositions, and others. This retrieval process has indicated that many authors are using the term idiopathic (as well as spontaneous, unexplained, etc.), when actually discussing cases with these identifiable predisposing factors. Finally, these papers present only 17 patients total and with little published literature recently, authors have been overly reliant on these papers. Some authors have even cited letters to the editor such as Scully (2002a,b), Friel and Macintyre (2002), and Dhanrajani (2008) for validation of the OUBS concept.

Oral ulcerations are quite common in the general population. Although most ulcers resolve within a few days to weeks, the high prevalence causes frequent presentation to medical practitioners for evaluation and proper treatment. Ulcerative events occur due to a combination of predisposing factors. Anatomically, the mylohyoid ridge of the lingual mandible is the most common area associated with OUBS, secondary being mandibular tori followed by mandibular exostoses (Farah and Savage, 2003). These areas are prone to ulceration as the mucosa which covers these prominences is stretched thin and harbors minimal amounts of connective tissue (Chanavaz, 1995). The lack of connective tissue predisposes these areas to ulceration from sudden or chronic trauma, while the decreased blood supply allows for progressive ulceration via impaired abilities to heal and fight infections (Chanavaz, 1995).

As authors have previously mentioned, there is a pathological link between ulceration and the subsequent bacterial colonization which results an avascular insult (Farah and Savage, 2003; Carrad et al., 2009; Otto et al., 2015a). Similar to the predisposition of the mucosa, the cortical bone of the maxilla and mandible is far removed from the alveolar blood supply (Chanavaz, 1995). Tori and exostoses only further exaggerate this lack of perfusion, lengthening the ischemic insult and slowing healing process which will result in necrosis. Finally, the posterior mandible harbors a large number of bacteria and debris due to decreased flexibility of the tongue and increased masticatory loads which lead to trauma and chronic irritation (Farah and Savage, 2003). Indeed, many of the histologic reports in this review specified high bacterial infiltrates in the sequestered bone.

For the identification of ulcerative risk factors, Almazrooa and Woo (2009) have provide a basic outline for which we have expanded upon in Table 2. The staging and treatment of OUBS would be unproductive. As OUBS includes multiple disorders of various etiology, patients care would be more effective when specified at the underlying causes. Healthcare professionals must identify the comorbidities and varying combination of risk factors which each case presents. This treatment, specified toward the management of a specific risk factors, is more beneficial. For instance, lesions caused by recurrent aphthous stomatitis (RAS) will require an antibacterial mouthrinse (Tarakji et al., 2015), mandibular and palatal tori may require surgical removal (García-García et al., 2010), loss of lingual inclination will require prosthetic rehabilitation and dental realignment, osteomyelitis can be treated from antibiotic treatment to decortication (Agarwal et al., 2014), and the treatment of cases related to bisphosphonates or denosumab will vary from conservative measures to invasive surgery depending on the stage and severity.

As cytologic, histologic, and pathologic innovations continue and electronic records maintain full patient reports, previously classified 'idiopathic lesions' will continue to move towards various established etiologies. The idiopathic label is retreating and giving way to more scientific classifications. OUBS is such a label, and patients stand to benefit much more when their treatment is aimed at their explicit disorder and known disease pathogenesis. When

Table 2
Risk factors of ulceration and bone sequestration.

Category	Condition
Infection	
Bacterial	Actinomyces (Kaplan et al., 2009); Syphilis (Scott and Flint, 2005); Tuberculosis (Andrade and Mhatre, 2012)
Viral	EBV (Dojcinov et al., 2010); HCV (Carrozzo and Scally, 2014); HIV (Reznik, 2005); HSV (Arduino and Porter, 2008); VZV (Mendieta et al., 2005)
Fungal	Aspergillus (Sugata et al., 1994); Candidiasis (Garcia-Cuesta et al., 2014); Mucormycosis (Rahman et al., 2013)
Parasitic	Leishmaniasis (Nadler et al., 2014); Myiasis (Moshref et al., 2008)
Treatment	
Systemic Medications	Antiangiogenic Agents, Bisphosphonates, Denosumab, TKIs, mTOR inhibitors (Hamadeh et al., 2015); Calcium Antagonists (Cohen et al., 1999); Corticosteroids (Mouries et al., 2013); Immunosuppressants (López-Pintor et al., 2010)
Direct Toxicities	Dental Agents (arsenic paste (Chen and Sung, 2014) and formocresol (Ege et al., 2014)) Heavy Metals (Dunsche et al., 2003); Cocaine (Seyer et al., 2002)
Radiation	Osteoradionecrosis (Reuther et al., 2003)
Trauma	
Dental Trauma	Dentures (Martori et al., 2014); Toothbrushing (Endo et al., 2006); Impaction (Prodromidis et al., 2011)
Mastication Trauma	Predisposing Factors: Exostoses of Mandible (Kermer et al., 1996); Mandibular or Palatal Tori (Cortes et al., 2014); Prominent Mylohyoid Ridge; Missing Teeth; Loss of Lingual Inclination; Clenching/Grinding/Bruxism (Sirirungrojying and Kerdpond, 1999)
Other	Procedural Trauma/Intubation (Almazrooa et al., 2010); Vomiting (Russo et al., 2008); Foreign Body Entrapment (Ho et al., 2010); Self Inflicted Gingival Injury (Alonso Chevitarese et al., 2004)
Other	
Neoplasia	Adenocarcinoma (Orlandi et al., 2007); Sarcomas (Hagström et al., 2011); Leukemia (Vourexakis, 2015); Lymphoma (NK\T\B Cell) (Darling et al., 2012); Burkitt's Lymphoma (Ferry, 2006)
Immunogenic	Diabetes (Silva et al., 2015); Crohn Disease (Rowland et al., 2010); Ulcerative Colitis (Elahi et al., 2012); Arthritis (Kato et al., 2014); SLE (Khatibi et al., 2012); Wegener's Granulomatosis (Genuis and Pewarchuk, 2014); SAPHO (Scully et al., 2008); GVHD (Margaix-Muñoz et al., 2015); Behçet's Disease (Unizony et al., 2015); Lichen Planus (Gupta and Jawanda, 2015)
Bone Disorders	Systemic Sclerosis (Nagy et al., 1994); Osteopetrosis (Albuquerque et al., 2006); Cemento-Osseous Dysplasia (Rao et al., 2014); Florid Osseous Dysplasia (Rekabi et al., 2013); CNO (Winters and Tatum., 2014); CRMO (Monsour and Dalton, 2010)
Congenital\ Genetic Defects	Gorham's Disease (Reddy and Jatti, 2012); Paget's Disease (Polisetti et al., 2014); Acatalasia (Delgado and Calderón, 1979); Maxillomandibular Arteriovenous Malformations (Churojana et al., 2012); Congenital Insensitivity to Pain with Anhidrosis (Fruchtman et al., 2013); Chronic Granulomatous Disease (Dar-Odeh et al., 2010); Cherubism (Mehrotra et al., 2011)
Other	Avascular Necrosis (Nguyen and Heggie, 2014); Recurrent Aphthous Stomatitis (Akintoye and Greenberg, 2014); Anemia (K et al., 2014); Neutropenia (Shete et al., 2012); Pyoderma Gangrenosum (Paramkusam et al., 2010); Perry-Romberg Syndrome (Al-Aizari et al., 2015); Hyperparathyroidism (Praveen and Thriveni, 2012); Hypothyroidism or Hyperthyroidism (Chandna and Bathla, 2011); Age Extremities (Dhanuthai et al., 2015); Excessive Tobacco or Alcohol (Chandra and Govindraju, 2012); Malnutrition (Thomas and Mirowski, 2010)

no etiology is apparent, we should no longer default to an unexplained cause. Therefore moving forward, we propose that the ulcerative disorders currently identified by the label OUBS should be separately classified as distinct processes. OUBS can maintain its idiopathic conception but more material, knowledge, and cases must be generated, presented, and discussed.

5. Conclusion

Similar to the call by other authors, there needs to be further discussion on the topic of OUBS. Furthermore, more clinical data is necessary. Both institutions and practitioners should identify and publish cases of ulceration and bone sequestration taking care to note the formatted information of Table 1 and the etiologies provided in Table 2. The current findings of this systematic review demonstrate that the OUBS concept cannot be regarded as one distinct entity, but rather it incorporates multiple, ulcerative disorders occurring in various locations, and due to a variety of both local and systemic effects. Practitioners must acknowledge many etiologic causes of ulceration, the predisposition for certain anatomical areas and abnormalities, and the link between ulceration and the subsequent insult leading to bone sequestration.

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All authors state that they have no conflict of interest.

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Appendix 1. Search Algorithms

Search A

Embase, MEDLINE in Embase.com (Elsevier)
13 July 2015, 584 records

(((oral OR mouth OR lingual OR palatal OR palate OR mandibl* OR maxilla* OR buccal OR jaw) NEAR/9 (lesion* OR ulcer*)):ab,ti OR 'mouth ulcer'/exp)
AND
(((necro* OR sequest* OR exposed OR exposure) NEAR/4 (bone* OR mandib* OR maxill* OR jaw)):ab,ti OR osteonec*:ab,ti OR (osteo NEXT/1 necrot*):ab,ti OR osteomyelitis:ab,ti OR 'jaw osteonecrosis'/exp OR 'osteomyelitis'/exp OR 'osteonecrosis':de OR 'osteomyelitis':de)

Search B

PubMed
13 July 2015, 10 records

((oral[tiab] OR mouth[tiab] OR lingual[tiab] OR palatal[tiab] OR palate[tiab] OR mandibl*[tiab] OR maxilla*[tiab] OR buccal[tiab] OR jaw[tiab]) AND (lesion*[tiab] OR ulcer*[tiab]))
AND
((necro*[tiab] OR sequest*[tiab] OR exposed[tiab] OR exposure OR osteonec*[tiab] OR osteo necrot*[tiab] osteomyelitis[tiab]) AND (bone*[tiab] OR mandib*[tiab] OR maxill*[tiab] OR jaw[tiab]))
NOT
medline[sb]

Search C

EMBASE
13th July 2015, 169 records

idiopathic:ab,ti OR unexplained:ab,ti OR spontaneous:ab,ti OR ((unknown OR unexplained) NEAR/3 (cause* OR etiolog*)):ab,ti AND (((necro* OR sequest* OR exposed OR exposure) NEAR/4 (bone* OR mandib* OR maxill* OR jaw)):ab,ti OR osteonec*:ab,ti OR osteo AND necrot*:ab,ti OR osteomyelitis:ab,ti OR 'jaw osteonecrosis'/exp OR 'jaw osteonecrosis' OR 'osteomyelitis'/exp OR 'osteomyelitis' OR 'osteonecrosis':de OR 'osteomyelitis':de) AND (oral:ab,ti OR mouth:ab,ti OR lingual:ab,ti OR palatal:ab,ti OR palate:ab,ti OR mandibl*:ab,ti OR maxilla*:ab,ti OR buccal:ab,ti OR jaw:ab,ti)

Search D

Handsearch
13th–20th July 2015, 3 records

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jcms.2015.11.014.

References

Agarwal A, Kumar N, Tyagi A, De N: Primary chronic osteomyelitis in the mandible: a conservative approach. BMJ Case Rep. http://dx.doi.org/10.1136/bcr-2013-202448, 2014
Akintoye SO, Greenberg MS: Recurrent aphthous stomatitis. Dent Clin North Am 58(2): 281–297, 2014
Al-Aizari NA, Azzeghaiby SN, Al-Shamiri HM, Darwish S, Tarakji B: Oral manifestations of Parry-Romberg syndrome: a review of literature. Avicenna J Med 5(2): 25–28, 2015
Albuquerque MA, Melo ES, Jorge WA, Cavalcanti MG: Osteomyelitis of the mandible associated with autosomal dominant osteopetrosis: a case report. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 102(1): 94–98, 2006
Almazrooa SA, Woo SB: Bisphosphonate and nonbisphosphonate-associated osteonecrosis of the jaw: a review. J Am Dent Assoc 140(7): 864–875, 2009
Almazrooa SA, Chen K, Nascimben L, Woo SB, Treister N: Case report: osteonecrosis of the mandible after laryngoscopy and endotracheal tube placement. Anesth Analg 111(2): 437–441, 2010 Aug
Alonso Chevitarese AB, Della Valle D, Primo L: Self-inflicted gingival injury in a pediatric patient: a case report. J Dent Child (Chic) 71(3): 215–217, 2004 Sep–Dec
Andrade NN, Mhatre TS: Orofacial tuberculosis-A 16-year experience with 46 cases. J Oral Maxillofac Surg 70(1): e12–e22, 2012
Arduino PG, Porter SR: Herpes Simplex Virus Type 1 infection: overview on relevant clinico-pathological features. J Oral Pathol Med 37(2): 107–121, 2008 Feb
Boffano P, Roccia F, Gallesio C, Garzino-Demo P, Ramieri G, Berrone S: Surgical management of pathologic mandibular fractures. J Craniofac Surg 23(6): e560–e562, 2012
Carrard VC, Sieck GG, Chaves AM, Filho MS, Rados PV: Oral ulceration with bone sequestration – case report. RFO 14(2): 149–152, 2009
Carrozzo M, Scally K: Oral manifestations of hepatitis C virus infection. World J Gastroenterol 20(24): 7534–7543, 2014 Jun 28
Chanavaz M: Anatomy and histophysiology of the periosteum: quantification of the periosteal blood supply to the adjacent bone with 85Sr and gamma spectrometry. J Oral Implantol 21: 214–219, 1995
Chandna S1, Bathla M: Oral manifestations of thyroid disorders and its management. Indian J Endocrinol Metab 15(Suppl. 2): S113–S116, 2011
Chandra P, Govindraju P: Prevalence of oral mucosal lesions among tobacco users. Oral Health Prev Dent 10(2): 149–153, 2012
Chen G, Sung PT: Gingival and localized alveolar bone necrosis related to the use of arsenic trioxide paste–two case reports. J Formos Med Assoc 113(3): 187–190, 2014 Mar

Churojana A, Khumtong R, Songsaeng D, Chongkolwatana C, Suthipongchai S: Life-threatening arteriovenous malformation of the maxillomandibular region and treatment outcomes. Interv Neuroradiol 18(1): 49–59, 2012 Mar
Cohen DM, Bhattacharyya I, Lydiatt WM: Recalcitrant oral ulcers caused by calcium channel blockers: diagnosis and treatment considerations. J Am Dent Assoc 130(11): 1611–1618, 1999 Nov
Cortes AR, Jin Z, Morrison MD, Arita ES, Song J, Tamimi F: Mandibular tori are associated with mechanical stress and mandibular shape. J Oral Maxillofac Surg 72(11): 2115–2125, 2014 Nov
Dar-Odeh NS, Hayajneh WA, Abu-Hammad OA, Hammad HM, Al-Wahadneh AM, Bulos NK, et al: Orofacial findings in chronic granulomatous disease: report of twelve patients and review of the literature. BMC Res Notes 17(3): 37, 2010 Feb
Darling MR, Cuddy KK, Rizkalla K: Hodgkin lymphoma of the oral mucosa. Head Neck Pathol 6(4): 507–510, 2012 Dec
De Boissieu P, Kanagaratnam L, Abou Taam M, Roux M-P, Dramé M, Trenque T: Notoriety bias in a database of spontaneous reports: the example of osteonecrosis of the jaw under bisphosphonate therapy in the French national pharmacovigilance database. Pharmacoepidemiol Drug Saf 23(9): 989–992, 2014
Delgado W, Calderón R: Acatalasia in two Peruvian siblings. J Oral Pathol 8(6): 358–368, 1979 Dec
Dhanrajani PJ: Lingual mucosal ulceration with mandibular sequestration (Dent Update 2007;34: 573–577). Dent Update Nov 35(9): 642, 2008
Dhanuthai K, Rojanawatsirivej S, Somkotra T, Shin H-I, Hong S-P, Darling M, et al: Geriatric oral lesions: a multicentric study Geriatrics and Gerontology International 2015. Geriatr Gerontol Int. http://dx.doi.org/10.1111/ggi.12458, 2015 Feb 6
Dojcinov SD, Venkataraman G, Raffeld M, Pittaluga S, Jaffe ES: EBV positive mucocutaneous ulcer – a study of 26 cases associated with various sources of immunosuppression. Am J Surg Pathol 34: 405–417, 2010
Dunsche A, Kåstel I, Terheyden H, Springer ING, Christophers E, Brasch J: Oral lichenoid reactions associated with amalgam: improvement after amalgam removal. Br J Dermatol 148(1): 70–76, 2003
Ege B, Demirkol M, Mustafa R, Aras MH: A tunnel shape defect on maxillary bone after accidental injection of formocresol instead of anesthetic solution. J Craniofac Surg 25(5): e451–e452, 2014 Sep
Elahi M, Telkabadi M, Samadi V, Vakili H: Association of oral manifestations with ulcerative colitis. Gastroenterol Hepatol Bed Bench 5(3): 155–160, 2012
Endo H, Rees TD, Hallmon WW, Kono Y, Kato T: Self-inflicted gingival injuries caused by excessive oral hygiene practices. Tex Dent J 123(12): 1098–1104, 2006 Dec
Farah CS, Savage NW: Oral ulceration with bone sequestration. Aust Dent J 48: 61–64, 2003
Ferry JA: Burkitt's lymphoma: clinicopathologic features and differential diagnosis. Oncologist 11(4): 375–383, 2006
Flaitz CM: Oral and maxillofacial pathology case of the month. Lingual mandibular sequestration and ulceration. Tex Den J 117(12), 2000 (34, 40-41)
Friel P, Macintyre DR: Bone sequestration from lower third molar region. Br Dent J 193(7): 366, 2002
Fruchtman Y, Perry ZH, Levy J: Morbidity characteristics of patients with congenital insensitivity to pain with anhidrosis (CIPA). J Pediatr Endocrinol Metab 26(3–4): 325–332, 2013
Garcia-Cuesta C, Sarrion-Pérez MG, Bagán JV: Current treatment of oral candidiasis: a literature review. J Clin Exp Dent 6(5): e576–e582, 2014 Dec 1
García-García AS, Martínez-González JM, Gómez-Font R, Soto-Rivadeneira A, Oviedo-Roldán L: Current status of the torus palatinus and torus mandibularis. Med Oral Patol Oral Cir Bucal 1(15), 2010 Mar (2)
Genuis K, Pewarchuk J: Granulomatosis with polyangiitis (Wegener's) as a necrotizing gingivitis mimic: a case report. J Med Case Rep 8: 297, 2014 Sep 7
Gupta S, Jawanda MK: Oral Lichen Planus: an update on etiology, pathogenesis, clinical presentation, diagnosis and management. Indian J Dermatol 60(3): 222–229, 2015 May–Jun
Hagström J, Mesimäki K, Apajalahti S, Haglund C, Rönty M, Sarlomo-Rikala M: A rare case of oral epithelioid sarcoma of the gingiva. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 111(4), 2011 Apr
Hamadeh IS, Ngwa BA, Gong Y: Drug induced osteonecrosis of the jaw. Cancer Treat Rev 41(5): 455–464, 2015 May
Ho W, Lai PC, Walters JD: Chronic swelling from entrapment of acrylic resin in a surgical extraction site. Contemp Clin Dent 1(3): 193–195, 2010 Jul
Huebsch RF: Acute lesions of the oral cavity. Dental Clin North America: 577–589, 1965
Jackson I, Malden N: Lingual mucosal ulceration with mandibular sequestration. Dental Update 34(9), 2007 573-574, 576-577
K S, B S, Palaneeswari MS, Devi AJM: Significance of ferritin in recurrent oral ulceration. J Clin Diagn Res 8(3): 14–15, 2014 Mar
Kaplan I, Anavi K, Anavi Y, Calderon S, Schwartz-Arad D, Teicher S, et al: The clinical spectrum of actinomyces-associated lesions of the oral mucosa and jawbones: correlations with histomorphometric analysis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 108(5): 738–746, 2009
Kato K, Segami N, Fukuda H, Minato H: Rheumatoid nodule in the lower lip of a patient with rheumatoid arthritis: a novel case report and review of literature. J Oral Maxillofac Surg 72(8): 1532, 2014 Aug
Kermer C, Rasse M, Undt G, Lang S: Cartilaginous exostoses of the mandible. Int J Oral Maxillofac Surg 25(5): 373–375, 1996 Oct
Kessler HP: Oral and maxillofacial pathology case of the month. Lingual mandibular sequestration with ulceration. Tex Dent J 122(2), 2005 198-199, 206-207
Khan A: Osteonecrosis of the jaw: report from the international. ONJ Task Force Osteoporos Int 25(Suppl. 2): S148–S149, 2014
Khan A, Morrison A, Hanley D, Felsenberg D, McCauley L, O'Ryan F, et al: International consensus on diagnosis and management of osteonecrosis of the jaw. J Bone Miner Res (Suppl. 1), 2013
Khan AA, Morrison A, Hanley DA, Felsenberg D, McCauley LK, O'Ryan F, et al: International task force on osteonecrosis of the jaw. Diagnosis and management of osteonecrosis of the jaw: a systematic review and international consensus. J Bone Miner Res 30(1): 3–23, 2015 Jan
Khatibi M, Shakoorpour AH, Jahromi ZM, Ahmadzadeh A: The prevalence of oral mucosal lesions and related factors in 188 patients with systemic lupus erythematosus. Lupus 21(12): 1312–1315, 2012
Koshal S, Chaudhry SI, Johnson A, Porter SR: Idiopathic loss of deciduous teeth and associated alveolus. Oral Dis 16(6), 2010 (570-)
Lee M, Chin RY, Eslick GD, Sritharan N, Paramaesvaran S: Outcomes for microvascular free flap reconstruction for mandibular osteoradionecrosis: a systematic review. J Craniomaxillofac Surg, 2015 Mar 20 S1010–5182(15)00058-X
López-Pintor RM, Hernández G, de Arriba L, de Andrés A: Comparison of oral lesion prevalence in renal transplant patients under immunosuppressive therapy and healthy controls. Oral Dis 16(1): 89–95, 2010 Jan
Margaix-Muñoz M, Bagán JV, Jiménez Y, Sarrión MG, Poveda-Roda R: Graft-versus-host disease affecting oral cavity. A review. J Clin Exp Dent 7(1): e138–e145, 2015 Feb 1
Martori E, Ayuso-Montero R, Martinez-Gomis J, Viñas M, Peraire M: Risk factors for denture-related oral mucosal lesions in a geriatric population. J Prosthet Dent 111(4): 273–279, 2014 Apr
Mehrotra D, Kesarwani A, Nandlal: Cherubism: case report with review of literature. J Maxillofac Oral Surg 10(1): 64–70, 2011 Mar
Mendieta C, Miranda J, Brunet LI, Gargallo J, Bernini L: Alveolar bone necrosis and tooth exfoliation following herpes zoster infection: a review of the literature and case report. J Periodontol 76: 148–153, 2005
Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group: Preferred reporting items for systematic reviews and meta-analyses: the PRISMA Statement. PLoS Med 6(6): e1000097, 2009
Monsour PA, Dalton JB: Chronic recurrent multifocal osteomyelitis involving the mandible: case reports and review of the literature. Dentomaxillofac Radiol 39(3): 184–190, 2010 Mar
Moshref M, Ansari G, Lotfi A: Oral gingival myiasis:a case report. Int J Trop Med 3: 97–100, 2008
Mouries F, Fricain J, Catros S, Géniaux H, Haramburu F, Miremont-Salame G: Adverse drug reactions in odontology: a retrospective study in a teaching hospital. Drug Saf 36(9), 2013 (896-)
Mücke T, Koschinski J, Wolff KD, Kanatas A, Mitchell DA, Loeffelbein DJ, et al: Quality of life after different interventions in head and neck cancer patients. J Craniomaxillofac Surg 43(9): 1895–1898, 2015 Nov
Nadler C, Enk CD, Leon GT, Samuni Y, Maly A, Czerninski R: Diagnosis and management of oral leishmaniasis—case series and literature review. J Oral Maxillofac Surg 72(5): 927–934, 2014 May
Nagy G, Kovács J, Zeher M, Czirják L: Analysis of the oral manifestations of systemic sclerosis. Oral Surg Oral Med Oral Pathol 77(2): 141–146, 1994 Feb
Nguyen EV, Heggie AA: Avascular necrosis of the midface secondary to disseminated intravascular coagulation. Int J Oral Maxillofac Surg 43(12): 1441–1444, 2014 Dec
Orlandi A, Basso M, Di Salvatore M, Federico F, Cassano A, Barone C: Lung adenocarcinoma with mandibular metastatic lesion: case report. Med Oral Patol Oral Cir Bucal 12: e424–e427, 2007
Otto S, Marx RE, Tröltzsch M, Ristow O, Ziebart T, Al-Nawas B, et al: Comments on "Diagnosis and management of osteonecrosis of the jaw: a systematic review and international consensus". J Bone Miner Res 30(6): 1113–1115. http://dx.doi.org/10.1002/jbmr.2525, 2015a
Otto S, Troeltzsch M, Burian E, Mahaini S, Probst F, Pautke C, et al: Ibandronate treatment of diffuse sclerosing osteomylitis of the mandible: pain relief and insight into pathogenesis. J Craniomaxillofac Surg 43(9): 1837–1842, 2015b
Paramkusam G, Meduri V, Gangeshetty N: Pyoderma gangrenosum with oral involvement – case report and review of the literature. Int J Oral Sci 2(2): 111–116, 2010 Jun
Peters E, Lovas GL, Wysocki GP: Lingual mandibular sequestration and ulceration. Oral Surg Oral Med Oral Pathol 75(6): 739–743, 1993
Polisetti N, Neerupakam M, Prathi VS, Prakash J, Vaishnavi D, Beeraka SS, et al: Osteonecrosis secondary to Paget's disease: radiologic and pathologic features. J Clin Imaging Sci. http://dx.doi.org/10.4103/2156-7514.129262, 2014 Mar 21
Praveen AH, Thriveni R: Maxillary and mandibular hyperparathyroidism. Natl J Maxillofac Surg 3(1): 51–54, 2012 Jan
Prodromidis GI, Tosios KI, Koutlas IG: Cemento-osseous dysplasia-like lesion and complex odontoma associated with an impacted third molar. Head Neck Pathol 5(4): 401–404, 2011 Dec
Rahman A, Akter K, Hossain S, Rashid HU: Rhino-orbital mucourmycosis in a non-immunocompromised patient. BMJ Case Rep. http://dx.doi.org/10.1136/bcr-2012-007863, 2013 Feb 6
Rao GS, Kamalapur MG, Acharya S: Focal cemento-osseous dysplasia masquerading as benign cementoblastoma: a diagnostic dilemma. J Oral Maxillofac Pathol 18(1): 150, 2014 Jan
Reddy S, Jatti D: Gorham's disease: a report of a case with mandibular involvement in a 10-year follow-up study. Dentomaxillofac Radiol 41(6): 520–524, 2012 Sep

Rekabi AR, Ashouri R, Torabi M, Parirokh M, Abbott PV: Florid cemento-osseous dysplasia mimicking apical periodontitis: a case report. Aust Endod J 39(3): 176–179, 2013 Dec
Reuther T, Schuster T, Mende U, Kubler A: Osteoradionecrosis of the jaws as a side effect of radiotherapy of head and neck tumor patients: are part of a thirty year retrospective review. Int J Oral Maxillofac Surg 32(3): 289–295, 2003
Reznik DA: Oral manifestations of HIV disease. Top HIV Med 13(5): 143–148, 2005 Dec–2006 Jan
Rowland M, Fleming P, Bourke B: Looking in the mouth for Crohn's disease. Inflamm Bowel Dis 16: 332–337, 2010
Russo Lo, Campisi G, Di Fede O, Di Liberto C, Panzarella V, Lo Muzio L: Oral manifestations of eating disorders: a critical review. Oral Dis 14: 479–484, 2008
Scott CM, Flint SR: Oral syphilis – re-emergence of an old disease with oral manifestations. Int J Oral Maxillofac Surg 34: 58–63, 2005
Scully C: Oral ulceration: a new unusual complication. Br Dent J 192: 139–140, 2002a
Scully CA: Oral ulceration. Br Dent J 192(11): 607, 2002b
Scully C, Hodgson T, Lachmann H: Auto-inflammatory syndromes and oral health. Oral Dis 14(8): 690–699, 2008
Seyer BA, Grist W, Muller S: Aggressive destructive midfacial lesion from cocaine abuse. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 94(4): 465–470, 2002
Shete M, Thompson JW, Naidu SI, Stocks RM, Wang WC: Olaryngologic manifestations in children with chronic neutropenia. Int J Pediatr Otorhinolaryngol 76(3): 392–395, 2012 Mar
Shulman JD, Beach MM, Rivera-Hidalgo F: The prevalence of oral mucosal lesions in U.S. adults: data from the third national health and nutrition examination survey, 1988–1994. J Am Dent Assoc 135(9): 1279–1286, 2004 Sep
Silva MF, Barbosa KG, Pereira JV, Bento PM, Godoy GP, Gomes DQ: Prevalence of oral mucosal lesions among patients with diabetes mellitus types 1 and 2. An Bras Dermatol 90(1): 49–53, 2015 Jan–Feb
Sirirungrojying S, Kerdpond D: Relationship between oral tori and temporomandibular disorders. Int Dent: 101–104, 1999
Sonnier KE, Horning GM: Spontaneous bony exposure: a report of 4 cases of idiopathic exposure and sequestration of alveolar bone. J Periodontol 68: 758–762, 1997
Sugata T, Myoken Y, Kyo T-I, Fujihara M: Invasive oral aspergillosis in immunocompromised patients with leukemia. J Oral Maxillofac Surg 52(4): 382–386, 1994
Tarakji B, Gazal G, Al-Maweri SA, Azzeghaiby SN, Alaizari N: Guideline for the diagnosis and treatment of recurrent aphthous stomatitis for dental practitioners. J Int Oral Health 7(5): 74–80, 2015 May
Thomas DM, Mirowski GW: Nutrition and oral mucosal diseases. Clin Dermatol 28(4): 426–431, 2010 Jul–Aug
Unizony SH, Kim ND, Hoang MP: Case records of the mass general hospital. Case 7-2015: a 25-year-old man with oral ulcers, rash, and odynophagia. N Engl J Med 372(9): 864–872, 2015 Feb 26
Vourexakis Z: Oral lesions presenting as an early sign of acute leukaemia. BMJ Case Rep. http://dx.doi.org/10.1136/bcr-2014-205100, 2015 Jan 29
Winters R, Tatum 3rd SA: Chronic nonbacterial osteomyelitis. Curr Opin Otolaryngol Head Neck Surg 22(4): 332–335, 2014 Aug

Stem Cells and Development

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GENE THERAPY FOR BONE DEFECTS IN ORAL AND MAXILLOFACIAL SURGERY: A SYSTEMATIC REVIEW AND META-ANALYSIS

Journal:	*Stem Cells and Development*
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Keyword:	Comprehensive Review, Genetic Engineering, Growth Factors, MSC, Osteogenesis
Abstract:	Craniofacial bone defects are challenging problems for maxillofacial surgeons over the years. With the development of cell and molecular biology, gene therapy is a breaking new technology with the aim of regenerating tissues by acting as a delivery system for therapeutic genes in the craniofacial region rather than treating genetic disorders. A systematic review was conducted summarizing the articles reporting gene therapy in maxillofacial surgery to answer the question: Was gene therapy successfully applied to regenerate bone in the maxillofacial region? Electronic searching of online databases was performed in addition to hand-search of the references of the included articles. No language or time restrictions were enforced. Meta-analysis was done to assess significant bone formation after delivery of gene material in the surgically induced maxillofacial defects. The search identified 2081 articles of which 57 were included with 1726 animals. Bone morphogenetic proteins (BMPs) were commonly used proteins for gene therapy. Viral vectors were the universally used vectors. Sprague-Dawley rats were the frequently used animal model in experimental studies. The quality of the articles ranged from excellent to average. Meta-analysis results performed on 21 articles showed that defects favoured bone formation by gene therapy. Funnel plot showed symmetry with the absence of publication bias. Gene therapy is on the top list of innovative strategies that developed in the last 10 years with the hope of developing a simple chair-side protocol in the near future

	combining improvement of gene delivery as well as knowledge of the molecular basis of oral and maxillofacial structures.

ABBREVIATIONS

µCT: Micro computed tomography
911 helper: human embryonic retinoblasts
AAV: Adeno-associated virus,
ALP: Alkaline phosphatase
b-FGF: Basic fibroblast growth factor
BMD: Bone mineral density
BMP-2: Bone morphogenetic protein 2
BMP-7: Bone morphogenetic protein 7
CHA: Coral hydroxyapatite
CMPC: Calcium magnesium phosphate cement
EGFP: Enhanced green fluorescence protein
FACS: Fluorescence-activated cell sorting
FEA: Finite element analysis
HA/TCP: Hydroxyapatite/beta-tricalcium phosphate
HGF: Hepatocyte growth factor,
HVJ: hemagglutinating virus of Japan
IGF 1: Insulin growth factor
LMP-3: LIM mineralization protein 3,
MKP-1: Mitogen-activated protein kinase phosphatase 1
MBG: Mesoporous bioglass
N/R: Not reported
NGF-β: Nerve growth factor beta
NNB: Natural non-organic bone
OF: Orthodontic force
OSX: Osterix
OSTEOBONE: Calcium silicon phosphorus
pOBs: Periosteal derived osteoblasts
PBS: Phosphate buffered saline
PDGF-A: Platelet derived growth factor A
PDLSCs: Periodontal stem cells
PFU: plaque forming unit
Pg-LPS: lipopolysaccharide mediated bone loss
RANKL: Receptor activator of nuclear factor kappa-B ligand
Runx2: Runt-related transcription factor 2
SEM: Scanning electron microscope
TM: Tooth movement
TRAP: Tartrate resistance acid phosphatase
TU: Transduction units
VEGF: Vascular endothelial growth factor
WEHI 164: mouse skin fibroblast
JM 109: Escherichia Coli
β-TCP: beta tricalcium phosphate
293FT: human embryonic kidney cells with the SV40 large T antigen
ADSCs: Adipose derived stem cells
AV: Adenovirus
BGC: Bioactive glass ceramic
BMMSCs: Bone marrow mesenchymal stem cells
BMP-4: Bone morphogenetic protein 4
BMP-9: Bone morphogenetic protein 9
CFSE: Carboxyfluorescein diacetate succinimidyl ester
CRE8: Cre-expressing 293 cells
DPSCs: Dental pulp stem cells
ERR: External root resorption
ELISA: Enzyme linked immunosorbent assay
GAM: Gene activated matrix
HA/COL: Hydroxyapatite/ Collagen
HA/PA: hydroxyapatite/polyamide
HEK293: human embryonic kidney 293 cell line
HIF-1α: hypoxia-inducible factor-1 alpha
iPSCs: Induced pluripotent stem cells
IFU: infectious units per ml
LacZ: β-galactosidase
Luc: firefly luciferase
MOI: multiplicity of infection
mSS: Premineralized silk fibroin protein scaffolds
NB: Nano-bubbles
NIH3T3: mouse embryo fibroblast
NOD/SCID mice: non-obese/severe combined immunodeficient
OPG: Osteoprotegrin
PCR: Polymerase chain reaction
PDGF-B: Platelet derived growth factor B
PDLA: Poly D, L-lactide
PF127: Pluronic F127
PG13: mouse embryonic fibroblast
PLGA: Poly lactic co glycolic acid
RSV: respiratory syncytial virus
SDF: syngeneic dermal fibroblasts
TGF-b: Transforming growth factor beta
TNFR: Tumour necrosis factor alpha receptor
TSG-6: Tumour necrosis factor alpha-stimulated gene-6
US: Ultra-sound
WB: Western Blot

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INTRODUCTION

Craniofacial anomalies and bone defects resulting from bone loss due to trauma, reconstructive surgery, neoplasia, congenital defects, infection or periodontal disease present a difficult and challenging problem for maxillofacial surgeons and scientists over the years with the goal of restoring facial form, function and occlusion. Conventional therapies are directed towards maxillofacial surgery, the use of prostheses or bone grafts. However, the effectiveness of these techniques is constrained by donor site morbidity, high cost and insufficient tissue resources. Recently, it had been agreed on the urgent need for new strategies for craniofacial reconstruction to improve bone regeneration with complete healing of the defects regardless of size [1-3]. As an alternative to the traditional techniques, "tissue engineering" has developed as a new and promising multi-disciplinary technique in the field of maxillofacial reconstruction and surgery [4].

With the development of cell and molecular biology, DNA-based technology had appeared as a promising method to meet challenges of tissue engineering in different applications. The genetic principle is either applied individually or together with tissue engineering to be known as gene-enhanced tissue engineering that regenerates lost tissue by local delivery of cells that have been genetically-modified to deliver signalling factors at DNA-level [5]. To date, gene therapy is the leading technology in medicine providing hope for those individuals that are suffering genetic disorders.

Gene therapy is known to be transferring genetic material or functioning gene to replace a damaged one inducing individual's own cells to produce a therapeutic agent to improve the clinical outcome. It has several advantages over traditional treatments as the expression in host cells lasts longer for weeks to years than pharmaceutical compounds or recombinant protein which range from several hours to days. It reduces technical challenges associated with ex-vivo protein expression and purification. Finally, the delivery of genetic sequences could mimic the natural biologic healing response [6,7].

There have been a couple of advances in gene therapy relevant to dentistry since 1995. When applying the gene therapy principles, the maxillofacial region has significant advantages compared to other locations in the body including easy access and inspection. Gene-based tissue engineering in the oral and maxillofacial complex include treatment of salivary gland diseases, autoimmune diseases, cancerous and precancerous lesions, pain, caries, dermatological disorders, delivery of growth factors for periodontal and pulp regeneration, treatment of malignant neoplasms of the head and neck, bone regeneration of large osseous defects in the craniofacial region and articular cartilage repair [8,9].

Although gene therapy was originally accepted as a means of treating heritable genetic disorders, its application in the craniofacial region is more often directed at regenerating tissues by acting as a delivery system for therapeutic genes promoting healing directly to cells within the defect or by genetically engineering mesenchymal stem cell progenitors to produce factors prior to implantation resulting in higher and more constant levels of protein production [10-12].

Thus, we have conducted a systematic review summarizing the articles reporting trials of gene therapy worldwide in the field of oral and maxillofacial surgery.

MATERIAL AND METHODS

This study was registered in SYRCLE (SYstematic Review Centre for Laboratory animal Experimentation) systematic review protocol for animal intervention studies (www.syrcle.nl). The guidelines for reporting systematic reviews and meta-analyses of animal studies was proposed by Peters et al [13] that are akin to the PRISMA guidelines for the reporting of systematic reviews and meta-analyses of healthcare interventions in human clinical studies.[14]

Review questions

The following PICO question was mainly addressed: Was gene therapy successfully applied to regenerate bone or heal defects in the oral and maxillofacial region?

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Search strategy and selection criteria

A systematic review of the literature was performed to provide an overview of published articles describing gene therapy in the field of Oral and Maxillofacial Surgery. Medical databases were searched to 18th December 2015. The data search included a combination of the following keywords: ‘‘Gene therapy’’ ‘‘AND’’ ‘‘Maxillofacial surgery’’ ‘‘OR’’ ‘‘Gene therapy’’ ‘‘AND’’ ‘‘Bone tissue engineering’’, ‘‘Genetic Engineering’’ ‘‘AND’’ ‘‘Maxillofacial bone’’, ‘‘Gene therapy’’ ‘‘AND’’ “Distraction Osteogenesis” ‘‘OR’’ ‘‘Gene therapy’’ ‘‘AND’’ “Alveolar bone” ‘‘OR’’ ‘‘Gene therapy’’ ‘‘AND’’ “Periodontal tissue” ‘‘OR’’ ‘‘Gene therapy’’ ‘‘AND’’ “Temporomandibular joint”. All the possible combinations of these words were explored. Medical subject headings (MeSH terms) without subheading restrictions was used and the heading sequence was ‘‘Gene therapy’’ ‘‘AND’’ ‘‘Dentistry’’.

In addition, we performed hand-search to the references of the included articles, papers of interest and related systematic or non-systematic reviews. The International Journal of Oral and Maxillofacial Surgery, Journal of Craniomaxillofacial Surgery, Gene therapy, Molecular therapy and Human gene therapy journals were also screened to identify possible references not reported elsewhere. No language or time restriction was enforced. Relevant full publications and meeting abstracts were identified by electronic searching of three online databases (PubMed, Cochrane library and Web of Knowledge). After the identification of articles in the databases, the articles were imported into Endnote X7 software (Thompson Reuters, Philadelphia, PA, USA) to store, manage search results and remove duplicates regardless of whether the studies are eventually included or excluded in the systematic review. Titles and abstracts identified were screened resulting in a number of seemingly relevant studies for the systematic review. The abstracts of the articles were then reviewed and the full text was obtained for those articles with apparent relevance. The identified articles were selected based on the inclusion criteria and exclusion criteria.

Inclusion criteria

(1) Relevant data on Gene therapy, (2) Animal studies, (3) Defects performed in the Oral and Maxillofacial region, (4) Any language.

Exclusion criteria

(1) In vitro studies, (2) Gene therapy in bones other than maxillofacial, (3) Calvarial bones defects, (4) Review articles, (5) letters to the editor, editorials, poster or oral presentations or articles with only abstract, (6) Oral cancer or soft tissue lesions, (7) Studies based on the use of only growth factors or cell-based therapies.

To improve the sensitivity of the relevant studies, each publication identified in the electronic search were assessed independently by two independent reviewers (RF and SO) to make a decision on inclusion/exclusion criteria or data extraction and quality of the articles with differences resolved by discussion.

Data extraction

All information was extracted using a standardized data form created in Excel. Data extracted included: 1) Author, 2) Year, 3) Journal, 4) Country, 5) Language, 6) therapeutic gene, 7) Vector, 8) Control gene, 9) Virus Titres (Concentration), 10) Cell lines for generation of virus, 11) Experiment design, 12) Defect model, 13) Site, 14) Animal Model, 15) Sample size, 16) Defect size, 17) Carrier/Scaffold, 18) Gene delivery route, 19) Stem cells source, 20) Experimental groups, 21) Cell concentration to be used in the defect, 22) Analysis methods with main endpoint results.

Data was extracted from either text or tables in the results section of the included studies. Data that was presented as graphs was extracted electronically using WebPlotDigitizer software, version: 3.9 (WebPlotDigitizer, US, http://arohatgi.info/WebPlotDigitizer, 2015).

Methodological quality assessment

The quality assessment of all the included studies in this systematic review was performed based on ARRIVE (Animal Research: Reporting In Vivo Experiments) guidelines [15] and evaluated based on a predefined grading system [16] applied to the following items: (1) Title, (2) Abstract/Summary, (3) Introduction/Background, (4) Introduction/ Primary and secondary

objectives, (5) Methods/Ethical statement, (6) Methods/Study design, (7) Methods/Experimental procedure, (8) Methods/Experimental Animals, (9) Methods/Housing and husbandry, (10) Methods/Sample size, (11) Methods/Allocation animals to experimental groups, (12) Methods/Experimental outcomes, (13) Methods/Statistical methods, (14) Results/Baseline data, (15) Results/Numbers analysed, (16) Results/Outcomes and estimation, (17) Results/Adverse events, (18) Discussion/Interpretation and scientific implications, (19) Discussion/Generalisability and translation, (20) Discussion/ Funding.

Risk of bias assessment

Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) risk of bias tool was applied to assess the internal validity of the included studies using RevMan software (version 5.3) [17,18]. A modified 7-point-item check list was used to assess the risk of bias, including: (1) published in a peer-reviewed journal; (2) random allocation to treatment or control; (3) treatment allocation concealment; (4) blinded assessment of outcome; (5) reporting of a sample size calculation; (6) statement of compliance with animal welfare regulations and (7) statement of potential conflict of interest. Each trial was assessed by two independent observers (RF and SO) and any differences resolved by discussion.

Outcome measure

The primary outcome measure for this meta-analysis was significant new bone formation by histology (% of area and % of volume) or radiograph (bone volume fraction) between the experimental and control group.

Statistical Analysis

A qualitative data analysis was performed with the aim of summarizing the results of the studies included. Meta-analyses as well as forest and funnel plots were conducted using RevMan software (Review Manager [RevMan] Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014). Bone formation was assessed as continuous outcome variables by inverse variance (IV) method and recorded as the

standardized mean difference (SMD) with 95% confidence interval (CI). The effect size of the SMD was classified as follows: 0.2 represents a small effect, 0.5 a moderate effect, and 0.8 a large effect [19]. The I^2 indicating heterogeneity and Cochran's Q statistical test were calculated; a value of I^2: 0% to 40% might not be important, 30% to 60% may represent moderate heterogeneity; 50% to 90% may represent substantial heterogeneity and 75% to 100% shows considerable heterogeneity [20]. A weighted fixed-effect model was used to estimate the overall effect size. Results with a $p<0.0001$ were considered indicative of statistical significance. Potential publication bias was explored using funnel plot generated using RevMan.

RESULTS

Search results

The search identified a total of 2081 references from the different databases and hand search: PubMed (n= 2000), Web of science (n= 63), Cochrane library (n= 7), hand-search (n=11). After duplicates removal via Endnote duplicate function, 1509 articles were screened for titles/abstracts and resulted in only 148 studies for full-text evaluation with the exclusion of 1361 articles that were irrelevant to the topic or review articles. Further screening resulted in a total of 57 studies which were considered eligible for the systematic review and fulfilled the final selection criteria. **Figure 1** illustrates the search flow and the identification of eligible studies. **Supplementary Table 1** summarizes excluded articles with reasons for exclusion.

Study characteristics

The articles analysed were published between 1999 and 2015. Most of the studies were conducted in USA [21-31] and China [32-61]. However, few studies were conducted in Taiwan [62], Japan [63,64], Spain [65], Germany [66], Italy [67], Korea [68] or as a collaboration between two countries [55,69-76]. Almost all articles (91.3%) were published in English [21-41,43-47,49-53,55-59,61-71,73-77] while only 5 articles (8.7%) [42,48,51,54,60] were published in Chinese. Bone morphogenetic proteins (BMPs) were the most commonly

used proteins for gene therapy (n=28, 49.1%) [22,23,26,28,29,34,35,37,40,42,43,47,48,50,51,54-58,62,66,68-73] followed by Platelet-derived growth factors (PDGF; n=6, 10.5%) [21,24,25,30,55,56], while the remaining 23 articles (40.4%) were using various proteins as: Enhanced green fluorescent protein (EGFP) [33,53,63,74], Tumour necrosis factor alpha receptor (TNFR) [27], Hepatocyte growth factor (HGF) [32], Receptor activator of nuclear factor kappa-B ligand (RANKL) [64,78], Basic fibroblast growth factor (b-FGF) [36,49], β-galactosidase (LacZ) [77], Osterix (OSX) [38,39], LIM mineralization protein 3 (LMP-3) [67], Vastatin [41], Vascular endothelial growth factor (VEGF) [44], Osteoprotegrin (OPG) [45,59,60,76], Runt-related transcription factor 2 (Runx2) [46], Nerve growth factor beta (NGF-β) [52], Tumour necrosis factor alpha-stimulated gene-6 (TSG-6) [75], Mitogen-activated protein kinase (MAPK) phosphatase 1 (MKP-1) [31] and Hypoxia-inducible factor-1 alpha (HIF-1α) [61].

In 30 articles (52.6%) [21-25,28-32,35-37,40,43,46,47,54-58,62,66,67,69,72,73,77,79], adenovirus was the universally used vector. However, other vectors were used as: plasmid (n=12, 21%) [26,34,38,48-51,59,60,63,70,80], adeno-associated virus (n=4, 7%) [27,33,41,44], hemagglutinating virus of Japan (HJV; n=3, 5.3%) [64,65,76], liposome (n=2, 3.5%) [66,71], lentivirus (n= 5, 8.8%) [45,52,53,61,75] and retrovirus (n=1, 1.8%) [74]. For the control genes, Green fluorescent protein (GFP) were the most abundant control in 20 articles (35.1%) [29,32,34,36-38,40,41,44,46,49,52,54,56-58,61,67,69,70,73,80] followed by β-galactosidase (LacZ) in 9 articles (15.8%) [22,23,31,35,43,62,66,69,72] and Luciferase (Luc) in 6 articles (10.5%) [21,24,25,28,30,63] respectively. However, in 22 articles (38.6%), the control gene was not reported. Seven different packaging cell lines were used for replication of the viruses: HEK293 (human embryonic kidney 293 cell line) [23-25,27,29,31-33,41,44-47,54,56,57,68,69,77], 293FT (human embryonic kidney cells with the SV40 large T antigen) [53], WEHI 164 (mouse skin fibroblast) [40], NIH3T3 (mouse embryo fibroblast) [64,65], CRE8 (Cre-expressing 293 cells) [67], 911 helper (human embryonic retinoblasts

(HER) [66] and PG13 (mouse embryonic fibroblast) [74]. The experiments were performed either as in vitro/ in vivo in 38 articles (66.7%) [23,29-32,35-37,40,41,43-47,49-51,53,55-62,64-70,72-75] or were completely in vivo studies in 19 articles (33.3%) [21,22,24-28,33,34,38,39,42,48,52,54,63,71,76,77]. **Table 1** presents the characteristic of the included studies.

Alveolar bone defects with or without dental implant were the prevalent model used for gene therapy in 20 articles (35.1%) [21,22,25,26,28,35,40,45,47,50,51,56-58,61,66,67,69,70,72], periodontal disease with or without alveolar bone involvement (n=17, 29.8%) [24,27,29-32,42,48,49,53,55,59,60,62,63,68,75] followed by distraction osteogenesis (n=9, 15.8%) [23,34,36,38,39,43,46,52,71], temporomandibular joint (n=4, 7%) [33,41,44,77], orthodontic tooth movement (n= 3, 5.2%) [64,65,76], sinus floor elevation (n=2, 3.5%) [37,73], tooth restoration with bio-root regeneration (n=1, 1.8%) [74] and central fissures (cleft) (n=1, 1.8%) [54]. Most of the defects were in the mandible (n=39, 68.4%) [22-24,29,30,33-36,38-54,56-61,66-68,70-73,77] while in 16 articles (28%) [21,25-28,31,37,55,62-65,69,73,75,76] the defects were created in the maxilla. One article reported defects in both jaws (1.8%) [32] and the location was missing in one article (1.8%) [74]. The posterior mandible (premolar-molar area) was the most frequent region. However, some studies did the experiments in the anterior region.

Sprague-Dawley rats were the frequently used animal model in experimental studies of gene therapy (n=17, 29.8%) [21-25,27,28,30,31,33,34,41,44,50,51,53,75] followed by Wistar rats in 6 studies (10.5%) [63-67,76], Lewis Fisher in 3 studies (5.3%) [29,35,58] and ginue-pigs or mice in one article each (n=2, 3.5%) each [72,77]. White New Zealand rabbits were also used as a small animal model for the studies (n=14, 24.6%) [36-40,43,45-47,52,54,62,70,73]. For large animals models, dogs and pigs were commonly used in 11 (19.3%) [26,42,48,49,55-57,59-61,66] and 4 (7%) studies respectively [32,69,71,74]. Sample size ranged between 4

and 24 for large animal models. However, for small animal models, the sample size ranged between 11 and 144 animals.

Two different defect shapes were identified: circular (n=9, 15.8%) [22,35,48-51,58,66,72], rectangular (n=22, 38.6%) [24,26,28-30,32,37,40,45,47,53,55-57,59-62,67,69,70,73] and 26 studies (45.6%) [21,23,25,27,31,33,34,36,38,39,41-44,46,52,54,63,64,68,71,74-78] did not mention the shape of the defect. Variable diameters were recognised for the circular defects ranging from 1 to 6 mm. The rectangular defects were characterised by heterogeneity of dimensions.

Gene delivery route was ex-vivo in 35 articles (61.4%) [29,32-40,42,43,45-47,49-54,58-62,66-74], in-vivo in 21 articles (36.8%) [21-28,30,31,41,44,48,55-57,63-65,76,77] and both in only one article (1.8%) [75]. For in-vivo gene delivery route (direct injection or GAM), physiological saline, collagen gels or lipid bubbles were used to deliver the genetically modified cells or material to the defect. The scaffolds used for seeding the cells differed in each study. The used scaffolds were: beta-tricalcium phosphate (β-TCP) [37,45,58], Bioactive glass ceramic (BGC) [47], Coral hydroxyapatite (CHA) [50,51], Hydroxyapatite/ Collagen (HA/COL) [67,68], Hydroxyapatite/ beta-tricalcium phosphate (HA/TCP) [74], Premineralized silk fibroin protein scaffolds (mSS) [35], Natural non-organic bone (NNB) [70], Mesoporous bioglass/silk fibrin (MBG) [55], hydroxyapatite/polyamide (HA/PA) [40], Pluronic F127 (PF127) [62], Poly D, L-lactide (PDLA) [26], Poly lactic co glycolic acid (PLGA) [59,60], Calcium magnesium phosphate cement (CMPC) [61] and Calcium silicon phosphorus (OsteoBone) [73]. Regarding the source of transfected/transduced stem cells, mesenchymal stem cells (MSCs) were used in 25 experiments (43.8%) either from bone marrow or adipose tissue or induced pluripotent [34-40,42,43,49-52,58-62,66,70-73,75], while different type of cells such as syngeneic dermal fibroblasts (SDFs) [29,67], dental pulp stem cells (DPSCs) [32,74], periodontal derived stem cells (PDLSCs) [45,47,53,56,57,68,74] and periosteal derived osteoblast cells (pOBs) [54] were used in other studies with different

concentration of the cells. All the experiments had been divided into different study groups for comparing the efficiency of gene therapy in the disease model. **Table 2** shows the extracted data from the included studies with reference to the disease model and animals used. Different analysis methods were used for either the in-vitro or in-vivo experiments as: Western blot (n=12, 21%) [23,29,31,45,46,49,59-62,69,70], In-situ hybridization (n=6, 10.5%) [33,44,47,50,51,66], PCR (n=27, 47.3%) [21,24,25,27,29,30,33,36,37,40,41,44-46,48,49,53,55-58,61,66,67,72,75,77], Bioluminescence (n=5, 8.7%) [21,24,28,30,63], µCT (n= 20, 35%) [22,25,27,31,32,35,36,43,46,49,55,58,61,62,67,69,71,72,76], [74], Histology (n=48, 84.2%) [22-26,28-32,34-40,42,45-50,52-55,57-74,76,77], Staining (n=16, 28%) [23,31,32,35,37,45,50,53,58,62,66,67,69,71,72,77], Radiograph (n=18, 31.5%) [23,26,27,34-36,38-40,43,46,50,54,58,59,61,66,74], Histomorphometry (n=22, 38.5%) [23,26,28-30,34,35,37,40,42,45,47,50,52,54,55,57,58,61,70,73,74], SEM (n=14, 24.5%) [25,28,34,40,45,50,55,57,58,60,61,70,74,75], Biomechanical analysis (n=8, 14%) [25,36,40,43,46,54,69,74], Immunohistochemistry (n=25, 43.8%) [29-31,34-36,39-41,44,45,50,51,53,59-61,64,66,68,69,71,73,74,76], Confocal microscopy (n=7, 12.2%) [35,45,56,57,63,66,73], Bone resorption assay (n=2, 3.5%) [64,65], ALP activity (n=7, 12.2%) [29,45,47,53,55,57,70], Immunofluorescence (n=15, 26.3%) [36,38-41,44,45,49,53,58,65,67,70,72-74], FACS (n=12, 21%) [29,32,41,44,49,53,61,62,66,72,74,75], TRAP (n=3, 5.2%) [27,44,75], Cell proliferation (n=8, 14%) [29,40,45,47,49,53,55,68] or ELISA (n=16, 28%) [27,31,32,35,43,44,47,55-57,62,66,68,72,73,75]. **Table 3** summarizes the endpoint results of the main analytical methods used for the experiments either in vitro or in vivo.

Methodological quality assessment of included articles

The items included in the assessment of the quality of the articles are summarized in **Table 4**. The quality of finally selected studies was assessed by different categories [15,16]. A relationship was driven between the Quality Score/Maximum Score by dividing the maximum

score by category to the total score (T=36). Three possible quality coefficients were conducted: 0.8–1 Excellent, 0.5–0.8 Average, <0.5 Poor as reported elsewhere [15,16,81]. In the included articles, 21 articles were excellent articles fulfilling nearly all the criteria of the ARRIVE guidelines with coefficients 0.8-1. Thirty-five articles were qualified as average articles with coefficients 0.5-0.8 and only one article was categorized as being of poor quality with coefficients <0.5. All the titles of the manuscripts were accurate. The abstracts were clearly accurate in 24 articles (42.1%) and possibly accurate in 30 articles (52.6%) and clearly inaccurate in 3 articles (5.3%). Introduction (background, objectives, experimental approach and rationale, relevance to human biology) was clear and sufficient in all the articles. Introduction (Objectives-primary and secondary) was clear in nearly all the articles (n=56, 98.2%) while only one article was not clear (n=1, 1.8%). The methods (Ethical statement-nature of the review permission, relevant licenses, national and institutional guidelines for the care and use of animals) were clearly sufficient in 50 articles (87.7%), possibly sufficient in one article (1.8%) and clearly insufficient in 6 articles (10.5%). The methods (study design-number of experimental and control groups, any steps taken to minimize bias, that is, allocation concealment, randomization and blinding) were possibly sufficient in 12 articles (21%) and clearly sufficient in 45 articles (78.9%). The methods (experimental procedure-precise details, that is, how, when, where, why) were clearly sufficient in 34 article (59.6%), possibly sufficient in 22 articles (38.6%) and clearly insufficient in one article (1.8%) of the manuscripts. The methods (experimental animals-species, strain, sex, maturity, weight, source of animals) were possibly sufficient in 25 articles (43.8%) and clearly sufficient in 32 articles (56.1%). The methods (housing and animal-husbandry and welfare-related assessment interventions, that is, type of cage, bedding material, number of cage companions, light/dark cycle, temperature, access to food and water) were clearly insufficient in 41 articles (72%) and possibly sufficient in 16 articles (28%). The methods (sample size-total number of animals used in each experimental group, details of calculation methods) were clearly

adequate in 53 articles (93%), possibly adequate in 3 article (5.2%) and clearly inadequate in one article (1.8%). The methods (allocation of animals to experimental groups-randomization or matching, order in which animals were treated or assessed) were expressed in 52 articles (91.2%) and were not expressed in five article (8.8%). The methods (experimental outcomes-definition of primary and secondary outcomes) were unclear/incomplete in 6 articles (10.5%) and absent in one article (1.8%) while 50 articles (87.7%) showed complete outcomes. The methods (statistical methods-details and unit of analysis) were missing in 7 articles (12.3%) and were provided in 50 articles (87.7%).

The results (baseline data characteristics and health status of animals) were not provided in 33 articles (57.9%) and were provided in 24 articles (42.1%). Results (number analysed-absolute numbers in each group included in each analysis, explanation for exclusion) were clearly inadequate in 4 articles (7%), possibly adequate in 44 articles (79%) and clearly adequate in only 8 articles (14%). Results (outcomes and estimation results for each analysis with a measure of precision, as standard error or confidence interval) were not complete in 17 articles (30%) and complete in 40 articles (70%). Results (adverse events details and notifications for reduction) were missing in 6 articles (10.5%), not complete in 33 articles (58%) and clearly accurate in 18 article (31.5%). The discussions (interpretation/scientific implications-study limitations including animal model, implications for the 3Rs) were clearly inadequate in one article (1.8%), possibly adequate in 51 article (89.4%) and clearly adequate in 5 articles (8.8%). Discussions (generalizability/translation-relevance to human biology) were inadequate in 2 articles (3.5%), possibly adequate in 46 article (80.7%) and clearly adequate in 9 articles (15.8%). Discussions (funding-sources, role of the funders) were clearly inadequate in 3 articles (5.3%) and clearly adequate in 54 articles (94.7%). **Table 5** represents the assessment of the quality of the published articles included in the review.

Risk of bias assessment of the included articles

Overall, all the studies were having low risk of bias in publishing in peer-reviewed journals. Random allocation of the treatment or control were reported in 29 articles [22,29,30,32-39,41,43-45,49,52,53,58,59,61,63,67,70,73-76] and three studies [37,40,46] had a low risk of bias in random allocation concealment. Blinding of outcome assessment was performed in 13 studies [23,27-30,32-34,36,38,39,52,65] and only two studies [25,33] were reporting sample size calculation The statement of compliance with animal welfare regulations was reported in 51 studies [23-28,30-47,49-65,67,68,70-77] while conflict of interest were in 11 studies [24-26,31,32,45,52,53,73,74]. More details about possible risk of bias were presented in **Fig 2**.

Meta-analysis

Fourteen studies were included in the histological meta-analysis of percentage of area of newly formed bone by gene therapy whereas three studies were included in percentage of volume of newly formed bone. However, four studies were included in the radiographic meta-analysis of the bone formation by calculating the bone volume fraction. **Fig.3** summarizes the results of forest plot of gene therapy treatment versus control treatment.

Percentage of area of bone formation by histology (**Fig.3A**):

Pooled data from *gene vs reporter* comprising of 9 inter-group comparisons generated from 7 original studies involving 204 animals (102 treated and 102 control groups) was (SMD=1.74, 95% CI, I^2=64%, p<0.00001) while data from *gene vs scaffold* comprising of 5 inter-group comparisons generated from 4 original studies involving 68 animals (34 treated and 34 control groups) was (SMD=1.17, 95% CI, I^2=87%, P=0.0004).

Pooled data from *gene/scaffold vs reporter/scaffold* comprising of 6 inter-group comparisons generated from 4 original studies involving 48 animals (24 treated and 24 control groups) was (SMD=1.31, 95% CI, I^2=45%, P=0.0006). However, data from *gene/scaffold vs scaffold* comprising of 4 inter-group comparisons generated from 4 original studies involving 48 animals (24 treated and 24 control groups) was (SMD=2.12, 95% CI, I^2=82%, p<0.00001).

Finally, pooled data from *gene/scaffold vs untransfected cells/scaffold* comprising of 3 inter-group comparisons generated from 3 original studies involving 46 animals (23 treated and 23 control groups) was (SMD=1.62, 95% CI, I^2=67%, p<0.00001).

Percentage of volume of bone formation by histology (**Fig.3B**):

Pooled data from *gene vs reporter* comprising of 2 inter-group comparisons generated from 2 original studies involving 52 animals (36 treated and 36 control groups) was (SMD=1.71, 95% CI, I^2=48%, P<0.00001) while data from *gene vs saline* comprising of 3 inter-group comparisons generated from 3 original studies involving 108 animals (54 treated and 54 control groups) was (SMD=2.34, 95% CI, I^2=0%, p<0.00001).

Bone volume fraction for bone formation by radiograph (**Fig.3C**):

Pooled data from *gene vs reporter* comprising of 4 inter-group comparisons generated from 4 original studies was performed involving 84 animals (42 treated and 42 control groups), (SMD=1.36, 95% CI, I^2=86%, p<0.00001).

Publication bias

Funnel plots of the study results are shown in **Fig.4**. Symmetrical funnel plots were obtained in all the models. The funnel plot of the study standard error by effect size (SMD) was symmetric. The funnel plot of standard error versus effect size (standard mean difference) was symmetrical indicating the absence of potential publication bias among the meta-analysis of bone formation by histology (**Fig.4 A&B**) or radiograph (**Fig.4C**).

DISCUSSION

Several literature reviews have focused on gene therapy in bone tissue engineering, dentistry or oral and maxillofacial surgery [9,12,82,83]. However, there has been no systematic review or meta-analysis with a specific focus on research covering gene therapy in the field of Oral and Maxillofacial Surgery. Thus, we have conducted a comprehensive systematic review of the studies addressing efforts made in the field of gene therapy for healing of maxillofacial defects revealing the raised success rate during the recent years. Our meta-analysis results

provided evidence that gene therapy was beneficial in treating maxillofacial defects in terms of improving bone formation based on histological and radiographic measures.

Although gene therapy was initially considered as a means of correcting hereditary disorders by changing the genes that cause the disease [84], more recent research is applying gene therapy to produce continuous amounts of biologically active molecules in the defects such as its potent ability for alveolar bone regeneration, periodontal healing and dental implants osseo-integration [55]. Clinical trials using gene therapy are now underway in salivary gland regeneration for dental application (https://clinicaltrials.gov/ct2/show/NCT00004178) and bone regeneration (https://clinicaltrials.gov/ct2/show/NCT02293031). However, future clinical trials for the use of gene therapy in periodontal regeneration remain hopeful for the near future.

From our results, multiple genes were used as osteogenic factors for gene therapy in the maxillofacial region because of their potent induction of de novo bone formation in vivo with varying results as soluble growth factors (PDGF, FGFs), morphogens (BMPs), angiogenetic factors (VEGF), intracellular regulators (LIM mineralization protein-1: LMP-1), transcription factors (Runx2) associated with bone and cartilage-related gene expression [85,86]. All of these biological factors have been investigated for their potential use in bone tissue engineering and repair. However, BMPs were preferred candidates for local gene therapy for bone regeneration as they can initiate and sustain the entire bone formation cascade [87]. Some studies proved the feasibility of transferring BMP genes [88,89]. On the other hand, previous investigations had reported the effect of PDGF on osseous defect healing showing that PDGF signalling plays role in chemotaxis and proliferation of osteoblasts and fibroblasts [90]. However, PDGF's ability to induce osteogenic differentiation is less clear. Recently, LMP-1 proved the initiation of membranous bone formation *in vitro* and *in vivo* [91]. Unlike BMPs acting extracellularly through cell surface receptors, LMP-1 is an intracellular signalling molecule involved in osteoblast differentiation [83].

Another critical element of gene therapy is the vector which is the vehicle that facilitates the transfer of genetic material into the target cell nucleus without degradation or causing toxicity. Two kinds of vectors have been employed as vehicles: viral and non-viral vectors. Gene transfer via viral vectors is called transduction while transfer via the non-viral vectors is transfection. Different viral vectors have been introduced as DNA-based like adenoviruses, adeno-associated viruses or RNA-based viral vectors as retroviruses and lentiviruses. Non-viral vectors can be plasmids, liposomes or polyplexes. Each vector has its own advantages and disadvantages. Viral vectors have the advantage of its ability to carry the gene efficiently and ensure long-term expression but they can only trigger short-term gene expression and are highly immunogenic. Another advantage of viral vectors is that they are non-virulent due to their modified genome in which the essential viral genes are replaced by the therapeutic gene being unable to replicate in the absence of these critical gene products. Non-viral vectors could be also used due to their safety profile and minimal immunogenicity. However, the main disadvantage in their use is the insufficient transfection efficiencies. [85,92-96].

For viral-based gene therapy, it is necessary to allow continuous high-titre virus production. The viruses are replicated in either human or non-human cell lines. A whole panel of different cell lines has been used all-over the years to generate viral vector to be used as therapeutic product. HEK293 cells and their derivatives have been extensively used for production of different vectors due to the ease of handling and possibility to grow as adherent as well as suspension cells [97,98]. In line with our findings, several studies had proved the efficiency of viral vector in the transfer of the DNA [99-101] while other studies have used non-viral vectors [102,103].

Reporter gene assays have emerged as a rapid and sensitive strategy for indirectly monitoring transgene expression by cloning the promoter region of the gene of interest correlated to the reporter gene and measure reporter gene expression as a reflection of the expression of the gene of interest [104]. It is important to use a reporter gene that is not naturally expressed in

the cell or organism under study. Different strategies of making the fusion construct and their applications have been reported [105]. Commonly used reporter genes are green fluorescent protein (GFP) which is a fluorescent protein causes cells that express it to glow green under UV light, luciferase (Luc) [106] produces light by a catalytic reaction with luciferin. Another common reporter gene expressed in bacteria is the protein β-galactosidase (LacZ) causing bacteria expressing the gene to appear blue when grown on a medium that contains the substrate analogue X-gal. In our results, several reporter genes have been used which gives an add-on to the experiments being an internal control for the expression of the gene of interest.

Various biological delivery systems have been applied for transferring therapeutic gene to target cells. In the in-vivo approach, cells can be genetically modified in situ or the vector is administered to the defect via systemic or local direct injection associated with a biomaterial. The combination of vector and biomaterial is called gene activated matrix (GAM). GAMs are three-dimensional biomaterials acting as a scaffold for vectors introduced to a localised area and useful for avoiding unintended spread of transfection to local tissues. Regarding the ex vivo approach, cells are removed, genetically modified and re-implanted in the defect by direct injection or using a biomaterial as carrier [107-109].

Genetic modification of stem or progenitor cells serves as an important advancement in regenerative medicine to improve their in-vivo performance. By combining gene with cell therapy, stem cell function may be enhanced by improving proliferation or differentiation of the stem cells. Another important function of stem cells is for drug delivery exerting paracrine or endocrine actions. The most common cell source is mesenchymal stem cells (MSC) which can be isolated from bone marrow, muscle tissue, peripheral blood, umbilical cord, adipose tissue, liver, multiple dental tissues or induced pluripotent stem cells (iPSC) [110,111]. MSC are adult stem cells capable of self-renewal and differentiation into multiple lineages as cartilage, adipose and bone which have been used for treating bone-related diseases [112]. The induced pluripotent stem cells (iPSCs) is a new source of stem cell generated from

human somatic cells into a pluripotent stage [113]. Various cells such as gingival or dermal fibroblasts, periosteal cells, primary articulated joint chondroblasts, bone marrow stromal cells/ MSCs, muscle-derived stem cells, fat-derived stem cells, osteoblasts and myoblasts have been successfully transfected or transduced by different vector systems using in vivo or ex vivo techniques [114]. From our results, the most commonly used stem cells in the maxillofacial region were genetically modified bone marrow, adipose, periodontal and dental pulp stem cells. Other studies used the same cells for regeneration of bone and other organs: BMMSCs [115,116], ADSCs [117-119], PDLSCs [120], DPSCs [121].

Animal models are valuable tools in biomedical research in particular gene therapy to test the safety, efficacy, dosage and localization of transgene expression in models that closely resemble human diseases. Animal craniofacial models for gene therapy exist not only for bone [122] but also for periodontal ligaments [30], TMJ [44], cartilage [123] as well as salivary glands [124]. Such models have critical-size defects with the absence of spontaneous complete osseous regeneration of the created defects during the lifetime of the animals [125,126].

Considering limitation of our systematic review, meta-analysis was conducted for only few included studies due to the high level of heterogeneity in reporting the treatment outcomes. It is also important to consider variability in research methodologies, characteristics of laboratory animals, different interventions and measurement of outcome variables play role in meta-analysis of animal studies. Moreover, the studies which were included in our meta-analysis generally used animal models for gene therapy. Therefore, randomized clinical studies in humans are needed to confirm our conclusions. However, meta-analysis was performed only to articles that had clearly reported bone formation (primary outcome) either by percentage of area or volume histologically as well as radiographically.

CONCLUSION

Challenging approaches had emerged for oral and maxillofacial reconstruction in the last decade due to the complex nature of craniofacial defects. Tissue engineering is attracting the spotlights as a new paradigm for bone regeneration which requires the collaboration of multidisciplinary teams of surgeons, biologists and biomedical engineers. Gene therapy is on the top list of innovative strategies in tissue engineering that developed in the last 10 years. While significant progress has been made towards preclinical studies of gene therapy in the maxillofacial region building the scientific basis of this technique, gene therapy is still in the clinical trials phase in salivary glands and craniofacial defects.

CONFLICT OF INTEREST

There are no conflicts of interest associated with this research.

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REFERENCES

1. Bonadio J and ML Cunningham. (2002). Genetic Approaches to Craniofacial Tissue Repair. Annals of the New York Academy of Sciences 961:48-57.
2. Dai J, AB Rabie, U Hagg and R Xu. (2004). Alternative gene therapy strategies for the repair of craniofacial bone defects. Curr Gene Ther 4:469-485.
3. Kademani D, S Mardini and SL Moran. (2008). Reconstruction of head and neck defects: a systematic approach to treatment. Semin Plast Surg 22:141-155.
4. Katari RS, A Peloso and G Orlando. (2014). Tissue engineering. Adv Surg 48:137-154.
5. Bonadio J. (2000). Tissue engineering via local gene delivery: update and future prospects for enhancing the technology. Adv Drug Deliv Rev 44:185-194.
6. Rios HF, Z Lin, B Oh, CH Park and WV Giannobile. (2011). Cell- and gene-based therapeutic strategies for periodontal regenerative medicine. J Periodontol 82:1223-1237.
7. Chatterjee A, N Singh and M Saluja. (2013). Gene therapy in periodontics. J Indian Soc Periodontol 17:156-161.
8. Edwards PC and JM Mason. (2006). Gene-enhanced tissue engineering for dental hard tissue regeneration: (2) dentin-pulp and periodontal regeneration. Head Face Med 2:16.
9. Shilpashree HS and S Sarapur. (2013). Gene therapy in dentistry: a review. N Y State Dent J 79:60-64.
10. Nussenbaum B and PH Krebsbach. (2006). The role of gene therapy for craniofacial and dental tissue engineering. Advanced Drug Delivery Reviews 58:577-591.

11. Mahale S, N Dani, SS Ansari and T Kale. (2009). Gene therapy and its implications in Periodontics. J Indian Soc Periodontol 13:1-5.
12. Scheller EL, LG Villa-Diaz and PH Krebsbach. (2012). Gene therapy: implications for craniofacial regeneration. J Craniofac Surg 23:333-337.
13. Peters JL, AJ Sutton, DR Jones, L Rushton and KR Abrams. (2006). A systematic review of systematic reviews and meta-analyses of animal experiments with guidelines for reporting. J Environ Sci Health B 41:1245-1258.
14. Moher D, A Liberati, J Tetzlaff and DG Altman. (2010). Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. Int J Surg 8:336-341.
15. Kilkenny C, WJ Browne, IC Cuthill, M Emerson and DG Altman. (2010). Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. PLoS Biol 8:e1000412.
16. Schwarz F, G Iglhaut and J Becker. (2012). Quality assessment of reporting of animal studies on pathogenesis and treatment of peri-implant mucositis and peri-implantitis. A systematic review using the ARRIVE guidelines. J Clin Periodontol 39 Suppl 12:63-72.
17. Sena E, HB van der Worp, D Howells and M Macleod. (2007). How can we improve the pre-clinical development of drugs for stroke? Trends in Neurosciences 30:433-439.
18. Macleod MR, T O'Collins, DW Howells and GA Donnan. (2004). Pooling of animal experimental data reveals influence of study design and publication bias. Stroke 35:1203-1208.
19. Cohen J. *Statistical Power Analysis for the Behavioral Sciences.* . (1988). L. Erlbaum Associates., Hillsdale, NJ.
20. Higgins JP, SG Thompson, JJ Deeks and DG Altman. (2003). Measuring inconsistency in meta-analyses. BMJ 327:557-560.
21. ZR A, P-C Chang, JA Cirelli, Q Jin, J Sugai and WV Giannobile. (2007). PDGF Gene Therapy to Promote Oral Implant Osseointegration Eur Cell Mater 13:43.
22. Alden TD, EJ Beres, JS Laurent, JA Engh, S Das, SD London, JA Jane, Jr., SB Hudson and GA Helm. (2000). The use of bone morphogenetic protein gene therapy in craniofacial bone repair. J Craniofac Surg 11:24-30.
23. Ashinoff RL, CL Cetrulo, Jr., RD Galiano, M Dobryansky, KA Bhatt, DJ Ceradini, Jt Michaels, JG McCarthy and GC Gurtner. (2004). Bone morphogenic protein-2 gene therapy for mandibular distraction osteogenesis. Ann Plast Surg 52:585-590; discussion 591.
24. Chang PC, JA Cirelli, Q Jin, YJ Seol, JV Sugai, NJ D'Silva, TE Danciu, LA Chandler, BA Sosnowski and WV Giannobile. (2009). Adenovirus encoding human platelet-derived growth factor-B delivered to alveolar bone defects exhibits safety and biodistribution profiles favorable for clinical use. Hum Gene Ther 20:486-496.
25. Chang PC, YJ Seol, JA Cirelli, G Pellegrini, Q Jin, LM Franco, SA Goldstein, LA Chandler, B Sosnowski and WV Giannobile. (2010). PDGF-B gene therapy accelerates bone engineering and oral implant osseointegration. Gene Ther 17:95-104.
26. Chen JC, SR Winn, X Gong and WH Ozaki. (2007). rhBMP-4 gene therapy in a juvenile canine alveolar defect model. Plast Reconstr Surg 120:1503-1509.
27. Cirelli JA, CH Park, K MacKool, M Taba, Jr., KH Lustig, H Burstein and WV Giannobile. (2008). AAV2/1-TNFR:Fc gene delivery prevents periodontal disease progression. Gene Ther 16:426-436.
28. Dunn CA, Q Jin, M Taba, Jr., RT Franceschi, R Bruce Rutherford and WV Giannobile. (2005). BMP gene delivery for alveolar bone engineering at dental implant defects. Mol Ther 11:294-299.

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

29. Jin QM, O Anusaksathien, SA Webb, RB Rutherford and WV Giannobile. (2003). Gene therapy of bone morphogenetic protein for periodontal tissue engineering. J Periodontol 74:202-213.
30. Jin Q, O Anusaksathien, SA Webb, MA Printz and WV Giannobile. (2004). Engineering of tooth-supporting structures by delivery of PDGF gene therapy vectors. Mol Ther 9:519-526.
31. Yu H, Q Li, B Herbert, R Zinna, K Martin, CR Junior and KL Kirkwood. (2011). Anti-inflammatory effect of MAPK phosphatase-1 local gene transfer in inflammatory bone loss. Gene Ther 18:344-353.
32. Cao Y, Z Liu, Y Xie, J Hu, H Wang, Z Fan, C Zhang, J Wang, CT Wu and S Wang. (2015). Adenovirus-mediated transfer of hepatocyte growth factor gene to human dental pulp stem cells under good manufacturing practice improves their potential for periodontal regeneration in swine. Stem Cell Res Ther 6:249.
33. Dai J and AB Rabie. (2007). Direct AAV-mediated gene delivery to the temporomandibular joint. Front Biosci 12:2212-2220.
34. Hu J, MC Qi, SJ Zou, JH Li and E Luo. (2007). Callus formation enhanced by BMP-7 ex vivo gene therapy during distraction osteogenesis in rats. J Orthop Res 25:241-251.
35. Jiang X, J Zhao, S Wang, X Sun, X Zhang, J Chen, DL Kaplan and Z Zhang. (2009). Mandibular repair in rats with premineralized silk scaffolds and BMP-2-modified bMSCs. Biomaterials 30:4522-4532.
36. Jiang X, S Zou, B Ye, S Zhu, Y Liu and J Hu. (2010). bFGF-Modified BMMSCs enhance bone regeneration following distraction osteogenesis in rabbits. Bone 46:1156-1161.
37. Jiang XQ, XJ Sun, HC Lai, J Zhao, SY Wang and ZY Zhang. (2009). Maxillary sinus floor elevation using a tissue-engineered bone complex with beta-TCP and BMP-2 gene-modified bMSCs in rabbits. Clin Oral Implants Res 20:1333-1340.
38. Lai QG, SL Sun, XH Zhou, CP Zhang, KF Yuan, ZJ Yang, SL Luo, XP Tang and JB Ci. (2014). Adipose-derived stem cells transfected with pEGFP-OSX enhance bone formation during distraction osteogenesis. J Zhejiang Univ Sci B 15:482-490.
39. Lai QG, KF Yuan, X Xu, DR Li, GJ Li, FL Wei, ZJ Yang, SL Luo, XP Tang and S Li. (2011). Transcription factor osterix modified bone marrow mesenchymal stem cells enhance callus formation during distraction osteogenesis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 111:412-419.
40. Li J, Y Li, S Ma, Y Gao, Y Zuo and J Hu. (2010). Enhancement of bone formation by BMP-7 transduced MSCs on biomimetic nano-hydroxyapatite/polyamide composite scaffolds in repair of mandibular defects. J Biomed Mater Res A 95:973-981.
41. Favia G, GP Pilolli and E Maiorano. (2009). Osteonecrosis of the Jaw Correlated to Bisphosphonate Therapy in Non-oncologic Patients: Clinicopathological Features of 24 Patients. Journal of Rheumatology 36:2780-2787.
42. Li YF, FH Yan, Q Zhong and X Zhao. (2010). [Effect of hBMP-7 gene modified bone marrow stromal cells on periodontal tissue regeneration]. Zhonghua Yi Xue Za Zhi 90:1427-1430.
43. Long J, P Li, HM Du, L Liu, XH Zheng, YF Lin, H Wang, W Jing, W Tang, WH Chen and WD Tian. (2011). Effects of bone morphogenetic protein 2 gene therapy on new bone formation during mandibular distraction osteogenesis at rapid rate in rabbits. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 112:50-57.
44. Rabie AB, J Dai and R Xu. (2007). Recombinant AAV-mediated VEGF gene therapy induces mandibular condylar growth. Gene Ther 14:972-980.
45. Su F, SS Liu, JL Ma, DS Wang, LL E and HC Liu. (2015). Enhancement of periodontal tissue regeneration by transplantation of osteoprotegerin-engineered periodontal ligament stem cells. Stem Cell Res Ther 6:22.

46. Sun JJ, XH Zheng, LY Wang, L Liu, W Jing, YF Lin, W Tian, W Tang and J Long. (2014). New bone formation enhanced by ADSCs overexpressing hRunx2 during mandibular distraction osteogenesis in osteoporotic rabbits. J Orthop Res 32:709-720.
47. Sun M, W Tan, K Wang, Z Dong, H Peng and F Wei. (2013). Effects of Allogenous Periosteal-Derived Cells Transfected With Adenovirus-Mediated BMP-2 on Repairing Defects of the Mandible in Rabbits. Journal of Oral and Maxillofacial Surgery 71:1789-1799.
48. Sun QF, XM Zhu, PS Yang, Y Liu and F Du. (2007). [Gene therapy of bone morphogenetic protein-2 for periodontal tissue regeneration in vivo]. Shanghai Kou Qiang Yi Xue 16:211-214.
49. Tan Z, Q Zhao, P Gong, Y Wu, N Wei, Q Yuan, C Wang, D Liao and H Tang. (2009). Research on promoting periodontal regeneration with human basic fibroblast growth factor-modified bone marrow mesenchymal stromal cell gene therapy. Cytotherapy 11:317-325.
50. Tang Y, W Tang, Y Lin, J Long, H Wang, L Liu and W Tian. (2008). Combination of bone tissue engineering and BMP-2 gene transfection promotes bone healing in osteoporotic rats. Cell Biol Int 32:1150-1157.
51. Tang YC, W Tang, WD Tian, XZ Chen and SW Li. (2006). [A study on repairing mandibular defect by means of tissue-engineering and human bone morphogenetic protein-2 gene transfection in osteoporotic rats]. Zhonghua Kou Qiang Yi Xue Za Zhi 41:430-431.
52. Wang L, Y Zhao, J Cao, X Yang and D Lei. (2015). Mesenchymal stem cells modified with nerve growth factor improve recovery of the inferior alveolar nerve after mandibular distraction osteogenesis in rabbits. Br J Oral Maxillofac Surg 53:279-284.
53. Wen Y, J Lan, H Huang, M Yu, J Cui, J Liang, B Jiang and X Xu. (2012). Application of eGFP to label human periodontal ligament stem cells in periodontal tissue engineering. Arch Oral Biol 57:1241-1250.
54. Ye ZC, FC Wei, KT Wang, SZ Sun, HQ Zhao and GJ Li. (2006). [Repair of mandibular central fissures in rabbits with hBMP-2 gene modified tissue engineered bone]. Shanghai Kou Qiang Yi Xue 15:42-47.
55. Zhang Y, RJ Miron, S Li, B Shi, A Sculean and X Cheng. (2015). Novel MesoPorous BioGlass/silk scaffold containing adPDGF-B and adBMP7 for the repair of periodontal defects in beagle dogs. J Clin Periodontol 42:262-271.
56. Zhang Y, B Shi, C Li, Y Wang, Y Chen, W Zhang, T Luo and X Cheng. (2009). The synergetic bone-forming effects of combinations of growth factors expressed by adenovirus vectors on chitosan/collagen scaffolds. J Control Release 136:172-178.
57. Zhang Y, J Song, B Shi, Y Wang, X Chen, C Huang, X Yang, D Xu, X Cheng and X Chen. (2007). Combination of scaffold and adenovirus vectors expressing bone morphogenetic protein-7 for alveolar bone regeneration at dental implant defects. Biomaterials 28:4635-4642.
58. Zhao J, J Hu, S Wang, X Sun, L Xia, X Zhang, Z Zhang and X Jiang. (2010). Combination of beta-TCP and BMP-2 gene-modified bMSCs to heal critical size mandibular defects in rats. Oral Dis 16:46-54.
59. Zhou W and L Mei. (2012). Effect of autologous bone marrow stromal cells transduced with osteoprotegerin on periodontal bone regeneration in canine periodontal window defects. Int J Periodontics Restorative Dent 32:e174-181.
60. Zhou W, CH Zhao and LX Mei. (2010). [Effect of the compound of poly lactic-co-glycolic acid and bone marrow stromal cells modified by osteoprotegerin gene on the periodontal regeneration in Beagle dog periodontal defects]. Hua Xi Kou Qiang Yi Xue Za Zhi 28:324-329.

61. Zou D, J He, K Zhang, J Dai, W Zhang, S Wang, J Zhou, Y Huang, Z Zhang and X Jiang. (2012). The bone-forming effects of HIF-1alpha-transduced BMSCs promote osseointegration with dental implant in canine mandible. PLoS One 7:e32355.
62. Chen YL, PK Chen, LB Jeng, CS Huang, LC Yang, HY Chung and SC Chang. (2008). Periodontal regeneration using ex vivo autologous stem cells engineered to express the BMP-2 gene: an alternative to alveolaplasty. Gene Ther 15:1469-1477.
63. Chen R, M Chiba, S Mori, M Fukumoto and T Kodama. (2009). Periodontal gene transfer by ultrasound and nano/microbubbles. J Dent Res 88:1008-1013.
64. Kanzaki H, M Chiba, K Arai, I Takahashi, N Haruyama, M Nishimura and H Mitani. (2006). Local RANKL gene transfer to the periodontal tissue accelerates orthodontic tooth movement. Gene Ther 13:678-685.
65. Iglesias-Linares A, AM Moreno-Fernandez, R Yanez-Vico, A Mendoza-Mendoza, M Gonzalez-Moles and E Solano-Reina. (2011). The use of gene therapy vs. corticotomy surgery in accelerating orthodontic tooth movement. Orthod Craniofac Res 14:138-148.
66. Park J, J Ries, K Gelse, F Kloss, K von der Mark, J Wiltfang, FW Neukam and H Schneider. (2003). Bone regeneration in critical size defects by cell-mediated BMP-2 gene transfer: a comparison of adenoviral vectors and liposomes. Gene Ther 10:1089-1098.
67. Lattanzi W, C Parrilla, A Fetoni, G Logroscino, G Straface, G Pecorini, E Stigliano, A Tampieri, R Bedini, R Pecci, F Michetti, A Gambotto, PD Robbins and E Pola. (2008). Ex vivo-transduced autologous skin fibroblasts expressing human Lim mineralization protein-3 efficiently form new bone in animal models. Gene Ther 15:1330-1343.
68. Park SY, KH Kim, EH Gwak, SH Rhee, JC Lee, SY Shin, KT Koo, YM Lee and YJ Seol. (2015). Ex vivo bone morphogenetic protein 2 gene delivery using periodontal ligament stem cells for enhanced re-osseointegration in the regenerative treatment of peri-implantitis. J Biomed Mater Res A 103:38-47.
69. Chang SC, HL Chuang, YR Chen, JK Chen, HY Chung, YL Lu, HY Lin, CL Tai and J Lou. (2003). Ex vivo gene therapy in autologous bone marrow stromal stem cells for tissue-engineered maxillofacial bone regeneration. Gene Ther 10:2013-2019.
70. Jiang X, SA Gittens, Q Chang, X Zhang, C Chen and Z Zhang. (2006). The use of tissue-engineered bone with human bone morphogenetic protein-4-modified bone-marrow stromal cells in repairing mandibular defects in rabbits. Int J Oral Maxillofac Surg 35:1133-1139.
71. Kroczek A, J Park, T Birkholz, FW Neukam, J Wiltfang and P Kessler. (2010). Effects of osteoinduction on bone regeneration in distraction: results of a pilot study. J Craniomaxillofac Surg 38:334-344.
72. Steinhardt Y, H Aslan, E Regev, Y Zilberman, I Kallai, D Gazit and Z Gazit. (2008). Maxillofacial-derived stem cells regenerate critical mandibular bone defect. Tissue Eng Part A 14:1763-1773.
73. Sun XJ, LG Xia, LL Chou, W Zhong, XL Zhang, SY Wang, J Zhao, XQ Jiang and ZY Zhang. (2010). Maxillary sinus floor elevation using a tissue engineered bone complex with BMP-2 gene modified bMSCs and a novel porous ceramic scaffold in rabbits. Arch Oral Biol 55:195-202.
74. Wei F, T Song, G Ding, J Xu, Y Liu, D Liu, Z Fan, C Zhang, S Shi and S Wang. (2013). Functional tooth restoration by allogeneic mesenchymal stem cell-based bio-root regeneration in swine. Stem Cells Dev 22:1752-1762.
75. Yang H, RM Aprecio, X Zhou, Q Wang, W Zhang, Y Ding and Y Li. (2014). Therapeutic effect of TSG-6 engineered iPSC-derived MSCs on experimental periodontitis in rats: a pilot study. PLoS One 9:e100285.

76. Zhao N, Y Liu, H Kanzaki, W Liang, J Ni and J Lin. (2012). Effects of local osteoprotegerin gene transfection on orthodontic root resorption during retention: an in vivo micro-CT analysis. Orthod Craniofac Res 15:10-20.

77. Kuboki T, T Nakanishi, M Kanyama, W Sonoyama, T Fujisawa, K Kobayashi, T Ikeda, T Kubo, A Yamashita and M Takigawa. (1999). Direct adenovirus-mediated gene delivery to the temporomandibular joint in guinea-pigs. Archives of Oral Biology 44:701-709.

78. Iglesias-Linares A, R Maria Yanez-Vico, A Maria Moreno-Fernandez, A Mendoza-Mendoza and E Solano-Reina. (2012). Corticotomy-Assisted Orthodontic Enhancement by Bone Morphogenetic Protein-2 Administration. Journal of Oral and Maxillofacial Surgery 70:E124-E132.

79. Abbayya K, SA Zope, S Naduwinmani, A Pisal and N Puthanakar. (2015). Cell- and Gene- Based Therapeutics for Periodontal Regeneration. Int J Prev Med 6:110.

80. Aghaloo T, X Jiang, C Soo, Z Zhang, X Zhang, J Hu, H Pan, T Hsu, B Wu, K Ting and X Zhang. (2007). A study of the role of nell-1 gene modified goat bone marrow stromal cells in promoting new bone formation. Mol Ther 15:1872-1880.

81. Delgado-Ruiz RA, JL Calvo-Guirado and GE Romanos. (2015). Critical size defects for bone regeneration experiments in rabbit calvariae: systematic review and quality evaluation using ARRIVE guidelines. Clin Oral Implants Res 26:915-930.

82. Gupta K, S Singh and KN Garg. (2015). Gene therapy in dentistry: tool of genetic engineering. Revisited. Arch Oral Biol 60:439-446.

83. Betz VM, OB Betz, MB Harris, MS Vrahas and CH Evans. (2008). Bone tissue engineering and repair by gene therapy. Front Biosci 13:833-841.

84. Friedmann T. (1996). Human gene therapy--an immature genie, but certainly out of the bottle. Nat Med 2:144-147.

85. Fischer J, A Kolk, S Wolfart, C Pautke, PH Warnke, C Plank and R Smeets. (2011). Future of local bone regeneration – Protein versus gene therapy. Journal of Cranio-Maxillofacial Surgery 39:54-64.

86. Franceschi RT, S Yang, RB Rutherford, PH Krebsbach, M Zhao and D Wang. (2004). Gene therapy approaches for bone regeneration. Cells Tissues Organs 176:95-108.

87. Luo J, MH Sun, Q Kang, Y Peng, W Jiang, HH Luu, Q Luo, JY Park, Y Li, RC Haydon and TC He. (2005). Gene therapy for bone regeneration. Curr Gene Ther 5:167-179.

88. Jane JA, Jr., BA Dunford, A Kron, DD Pittman, T Sasaki, JZ Li, H Li, TD Alden, H Dayoub, GR Hankins, DF Kallmes and GA Helm. (2002). Ectopic osteogenesis using adenoviral bone morphogenetic protein (BMP)-4 and BMP-6 gene transfer. Mol Ther 6:464-470.

89. Blum JS, MA Barry, AG Mikos and JA Jansen. (2003). In vivo evaluation of gene therapy vectors in ex vivo-derived marrow stromal cells for bone regeneration in a rat critical-size calvarial defect model. Hum Gene Ther 14:1689-1701.

90. Hsieh SC and DT Graves. (1998). Pulse application of platelet-derived growth factor enhances formation of a mineralizing matrix while continuous application is inhibitory. J Cell Biochem 69:169-180.

91. Viggeswarapu M, SD Boden, Y Liu, GA Hair, J Louis-Ugbo, H Murakami, HS Kim, MT Mayr, WC Hutton and L Titus. (2001). Adenoviral delivery of LIM mineralization protein-1 induces new-bone formation in vitro and in vivo. J Bone Joint Surg Am 83-A:364-376.

92. Mali S. (2013). Delivery systems for gene therapy. Indian J Hum Genet 19:3-8.

93. Gardlik R, R Palffy, J Hodosy, J Lukacs, J Turna and P Celec. (2005). Vectors and delivery systems in gene therapy. Med Sci Monit 11:RA110-121.

Mary Ann Liebert Inc., 140 Huguenot Street, New Rochelle, NY 10801

94. Ibraheem D, A Elaissari and H Fessi. (2014). Gene therapy and DNA delivery systems. Int J Pharm 459:70-83.
95. Guan X, MA Goddard, DL Mack and MK Childers. (2016). Gene therapy in monogenic congenital myopathies. Methods 99:91-98.
96. Tilemann L, K Ishikawa, T Weber and RJ Hajjar. (2012). Gene Therapy for Heart Failure. Circulation Research 110:777-793.
97. Stacey GN and OW Merten. (2011). Host cells and cell banking. Methods Mol Biol 737:45-88.
98. Schucht R, AS Coroadinha, MA Zanta-Boussif, E Verhoeyen, MJ Carrondo, H Hauser and D Wirth. (2006). A new generation of retroviral producer cells: predictable and stable virus production by Flp-mediated site-specific integration of retroviral vectors. Mol Ther 14:285-292.
99. Carr DJ, JM Wallace, RP Aitken, JS Milne, JF Martin, IC Zachary, DM Peebles and AL David. (2016). Peri- and Postnatal Effects of Prenatal Adenoviral VEGF Gene Therapy in Growth-Restricted Sheep. Biol Reprod.
100. Teos LY, CY Zheng, X Liu, WD Swaim, CM Goldsmith, AP Cotrim, BJ Baum and IS Ambudkar. (2016). Adenovirus-mediated hAQP1 expression in irradiated mouse salivary glands causes recovery of saliva secretion by enhancing acinar cell volume decrease. Gene Ther.
101. Song K, NJ Rao, ML Chen, ZJ Huang and YG Cao. (2011). Enhanced bone regeneration with sequential delivery of basic fibroblast growth factor and sonic hedgehog. Injury 42:796-802.
102. Kaur H, H Uludag and T El-Bialy. (2014). Effect of nonviral plasmid delivered basic fibroblast growth factor and low intensity pulsed ultrasound on mandibular condylar growth: a preliminary study. Biomed Res Int 2014:426710.
103. Apaolaza PS, A Del Pozo-Rodriguez, J Torrecilla, A Rodriguez-Gascon, JM Rodriguez, U Friedrich, BH Weber and MA Solinis. (2015). Solid lipid nanoparticle-based vectors intended for the treatment of X-linked juvenile retinoschisis by gene therapy: In vivo approaches in Rs1h-deficient mouse model. J Control Release 217:273-283.
104. Kain SR and S Ganguly. (2001). Overview of genetic reporter systems. Curr Protoc Mol Biol Chapter 9:Unit9 6.
105. Thibodeau SA, R Fang and JK Joung. (2004). High-throughput beta-galactosidase assay for bacterial cell-based reporter systems. Biotechniques 36:410-415.
106. Phippard D and AM Manning. (2003). Screening for inhibitors of transcription factors using luciferase reporter gene expression in transfected cells. Methods Mol Biol 225:19-23.
107. Tarassoli P, WS Khan, A Hughes and N Heidari. (2013). A review of techniques for gene therapy in bone healing. Curr Stem Cell Res Ther 8:201-209.
108. Balmayor ER and M van Griensven. (2015). Gene therapy for bone engineering. Front Bioeng Biotechnol 3:9.
109. Evans CH. (2010). Gene therapy for bone healing. Expert Rev Mol Med 12:e18.
110. Huang GT, S Gronthos and S Shi. (2009). Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J Dent Res 88:792-806.
111. Sheyn D, O Mizrahi, S Benjamin, Z Gazit, G Pelled and D Gazit. (2010). Genetically modified cells in regenerative medicine and tissue engineering. Adv Drug Deliv Rev 62:683-698.
112. Park JS, S Suryaprakash, YH Lao and KW Leong. (2015). Engineering mesenchymal stem cells for regenerative medicine and drug delivery. Methods 84:3-16.

113. Estrela C, AH Alencar, GT Kitten, EF Vencio and E Gava. (2011). Mesenchymal stem cells in the dental tissues: perspectives for tissue regeneration. Braz Dent J 22:91-98.

114. Fischer J, A Kolk, S Wolfart, C Pautke, PH Warnke, C Plank and R Smeets. (2011). Future of local bone regeneration - Protein versus gene therapy. J Craniomaxillofac Surg 39:54-64.

115. Tracy CJ, DN Sanders, JN Bryan, CA Jensen, LJ Castaner, MD Kirk and ML Katz. (2016). Intravitreal Implantation of Genetically Modified Autologous Bone Marrow-Derived Stem Cells for Treating Retinal Disorders. Adv Exp Med Biol 854:571-577.

116. Yin C, J Chen, Z Chen, Z Zeng and J Qiu. (2015). hBMP-2 and hTGF-beta1 expressed in implanted BMSCs synergistically promote the repairing of segmental bone defects. J Orthop Sci 20:717-727.

117. Xie Q, Z Wang, H Zhou, Z Yu, Y Huang, H Sun, X Bi, Y Wang, W Shi, P Gu and X Fan. (2016). The role of miR-135-modified adipose-derived mesenchymal stem cells in bone regeneration. Biomaterials 75:279-294.

118. Yang LY, JK Zheng, GZ Hui and LH Guo. (2004). [Adipose tissue-derived stromal cells as vector for gene therapy in central nervous system]. Sichuan Da Xue Xue Bao Yi Xue Ban 35:463-465.

119. Watanabe N, K Ohashi, K Tatsumi, R Utoh, IK Shim, K Kanegae, Y Kashiwakura, T Ohmori, Y Sakata, M Inoue, M Hasegawa and T Okano. (2013). Genetically modified adipose tissue-derived stem/stromal cells, using simian immunodeficiency virus-based lentiviral vectors, in the treatment of hemophilia B. Hum Gene Ther 24:283-294.

120. Mi HW, MC Lee, E Fu, LP Chow and CP Lin. (2011). Highly efficient multipotent differentiation of human periodontal ligament fibroblasts induced by combined BMP4 and hTERT gene transfer. Gene Ther 18:452-461.

121. Nakashima M, K Iohara, M Ishikawa, M Ito, A Tomokiyo, T Tanaka and A Akamine. (2004). Stimulation of reparative dentin formation by ex vivo gene therapy using dental pulp stem cells electrotransfected with growth/differentiation factor 11 (Gdf11). Hum Gene Ther 15:1045-1053.

122. Krebsbach PH, K Gu, RT Franceschi and RB Rutherford. (2000). Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone in vivo. Hum Gene Ther 11:1201-1210.

123. Palmer G, A Pascher, E Gouze, JN Gouze, O Betz, M Spector, PD Robbins, CH Evans and SC Ghivizzani. (2002). Development of gene-based therapies for cartilage repair. Crit Rev Eukaryot Gene Expr 12:259-273.

124. Cotrim AP, F Mineshiba, T Sugito, Y Samuni and BJ Baum. (2006). Salivary gland gene therapy. Dent Clin North Am 50:157-173, vii.

125. Li Y, S-K Chen, L Li, L Qin, X-L Wang and Y-X Lai. (2015). Bone defect animal models for testing efficacy of bone substitute biomaterials. Journal of Orthopaedic Translation 3:95-104.

126. Ward BB, SE Brown and PH Krebsbach. (2010). Bioengineering strategies for regeneration of craniofacial bone: a review of emerging technologies. Oral Dis 16:709-716.

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FIGURE LEGENDS

Fig.1: **Flow-chart of the process of literature search and studies included in the review**.

Fig.2: **Risk of bias graph for the studies included in this systematic review**. Assessment of risk of bias using modified CAMARADES tool. Panel (A) Risk of bias of all included studies with the percentage of risk of bias for each item of assessment; Panel (B) Author name of each study and with their respective result in each item of assessment. Item (1) published in a peer-reviewed journal; (2) random allocation to treatment or control; (3) treatment allocation concealment; (4) blinded assessment of outcome; (5) reporting of a sample size calculation; (6) statement of compliance with animal welfare regulations and (7) statement of potential conflict of interest respectively.

Fig.3: Forest plot of standard mean difference (SMD), with 95% Confidence Interval (CI) in bone formation by histology and radiograph comparing different subgroups. Panel (A) represents forest plot of percentage area of bone formation by histology. Several subgroups were analysed as: Gene vs Reporter gene, Gene vs Scaffold, Gene/Scaffold vs Reporter/Scaffold, Gene/Scaffold vs Scaffold, Gene/Scaffold vs Untransfected cells/Scaffold. Panel (B) represents forest plot of percentage volume of bone formation by histology. Panel (C) represents forest plot of bone volume fraction detected by 3D μCT. the diamond represents the overall effect within each subgroup.

Fig.4: Funnel plot showing publication bias among the studies. The symmetry of the funnel plot shows there was no evidence of publication bias among the studies. Each symbol on the funnel plot represents an individual study estimate included in the meta-analysis. The y-axis displays the standard error and the x-axis displays the standardized mean difference. SE: Standard Error; SMD: Standardized mean difference.

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TABLE LEGENDS

Table 1: Summary of essential features of all studies included in the systematic review.

Table 2: Extracted data from included studies with description of disease model and animal model used.

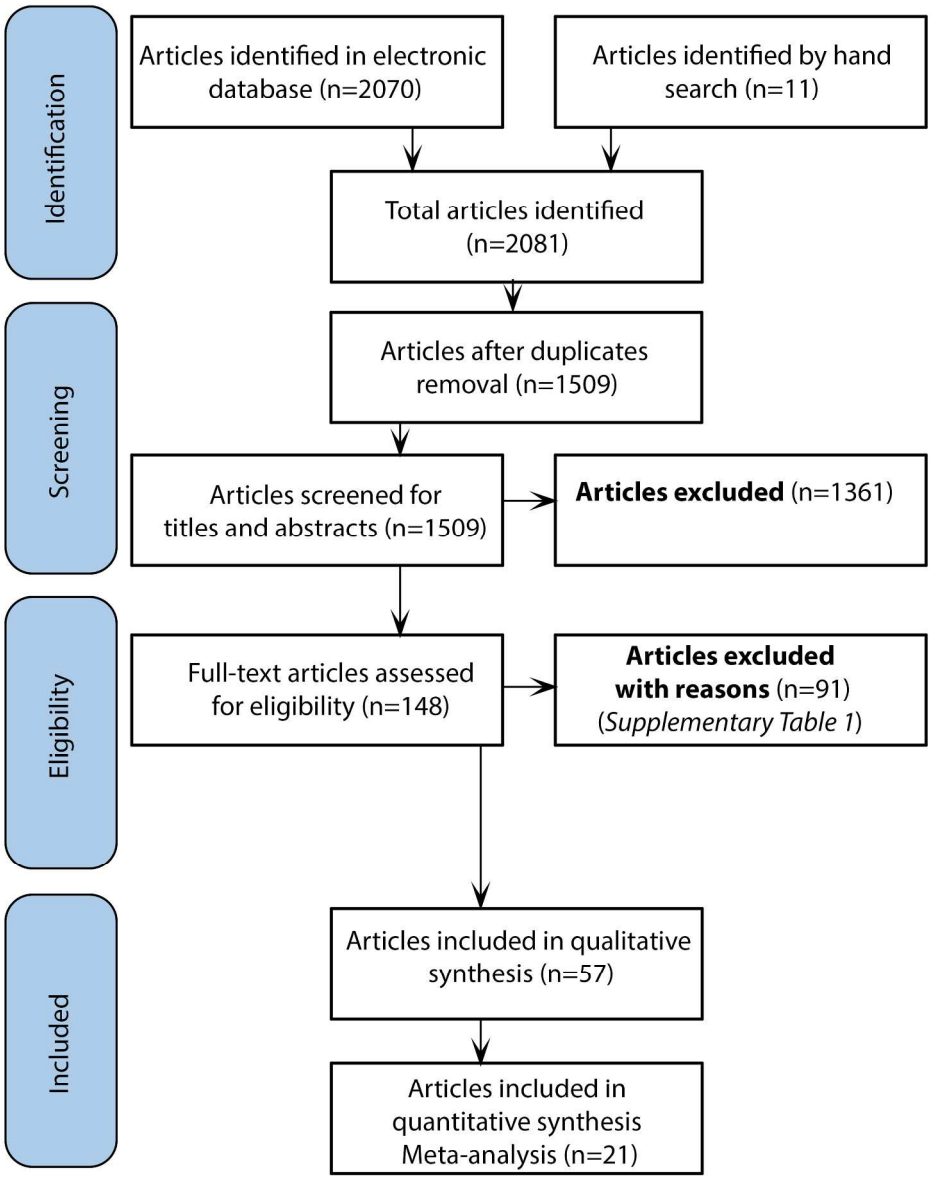
Table 3: Endpoint results of the main analytical methods used for the experiments.

Table 4: Categories and grading used to assess the quality of the selected studies.

Table 5: Quality assessment of articles included using ARRIVE guidelines.

Supplementary Table 1: Excluded articles with the reasons of exclusion.

Supplementary Table 2: SYRCLE protocol for registering systematic review for animal intervention studies.



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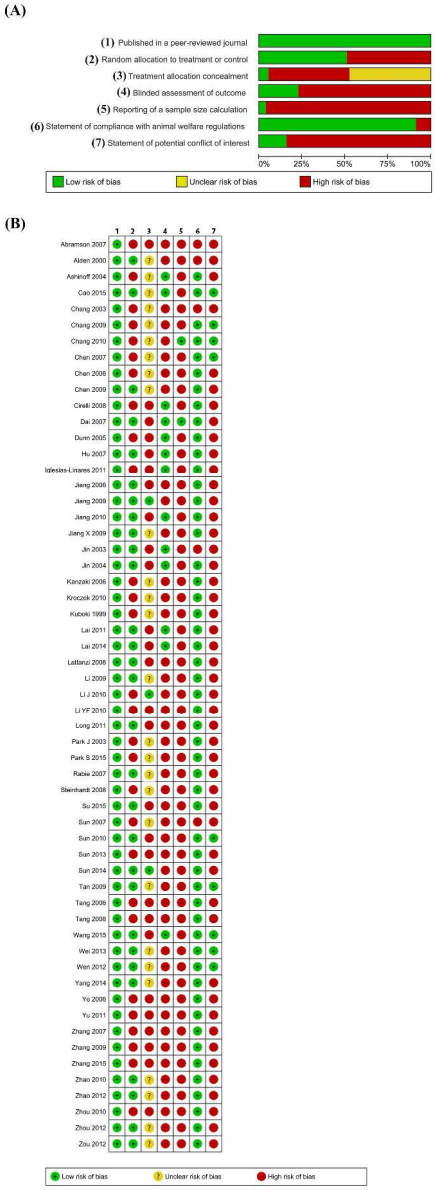

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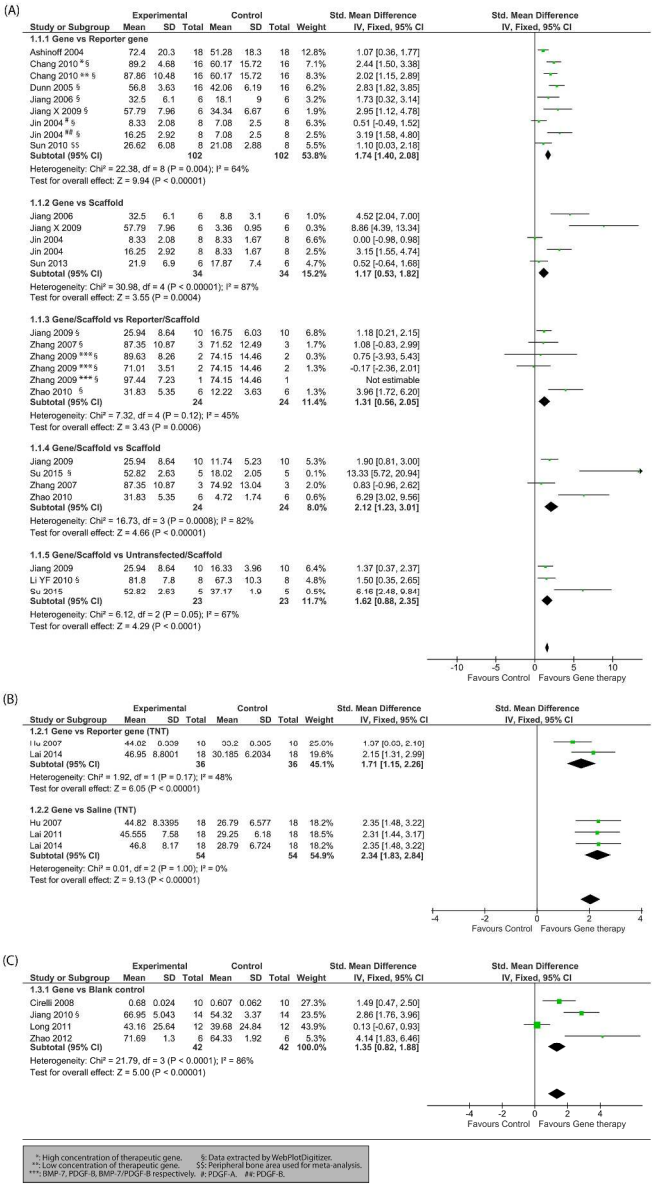

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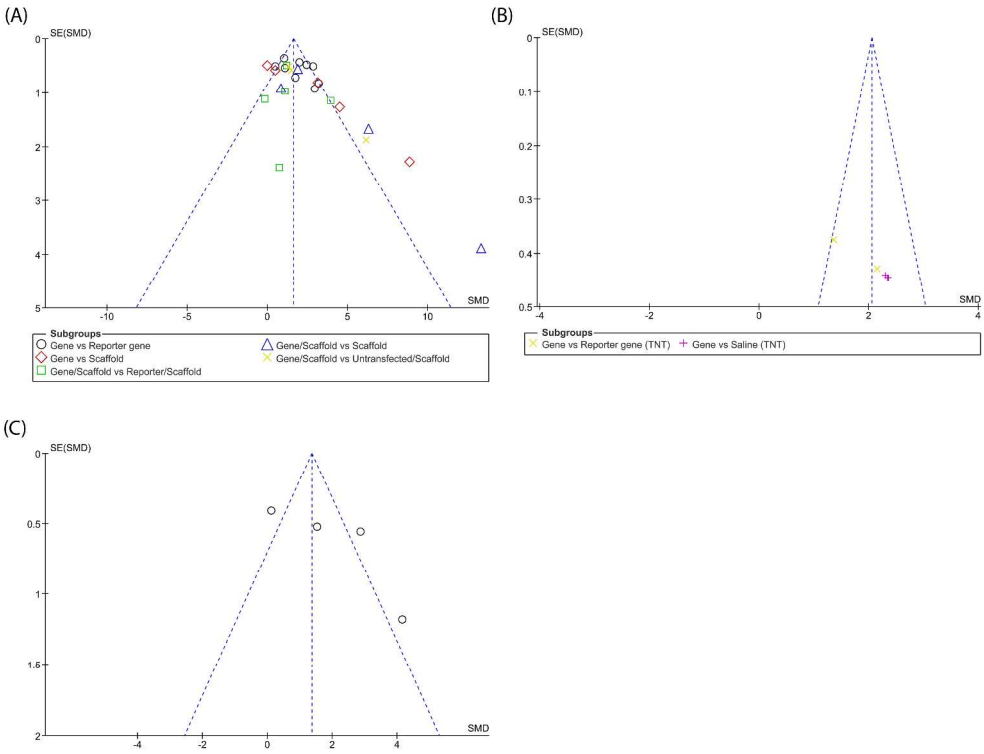

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Table 1: Summary of essential features of all studies included in the systematic review

Author	Year	Journal	Country	Language	Therapeutic Gene	Vector	Control	Virus Titres (Concentration)	Cell lines for generation of virus	Experiment design
Abramson [1]	2007	Eur Cell Mater	USA	English	PDGF-B	AV	Luc	N/R	N/R	In vivo
Alden [2]	2000	J Craniofac Surg	USA	English	BMP-2/BMP-9	AV	LacZ	5×10^7 particle/μl	N/R	In vivo
Ashinoff [3]	2004	Ann Plast Surg	USA	English	BMP-2	AV	LacZ	N/R	HEK293	In vitro/In vivo
Chang [4]	2009	Hum Gene Ther	USA	English	PDGF-B	AV	Luc	N/R	HEK293	In vivo
Chang [5]	2010	Gene Ther	USA	English	PDGF-B	AV	Luc	N/R	HEK293	In vivo
Chang [6]	2003	Gene Ther	Taiwan/USA	English	BMP-2	AV	LacZ	N/R	HEK293	In vitro/In vivo
Chen [7]	2009	J Dent Res	Japan	English	EGFP	Plasmid	Luc	N/R	JM 109	In vivo
Chen[8]	2007	Plast Reconstr Surg	USA	English	BMP-4	Plasmid	N/R	N/R	N/R	In vivo
Chen[9]	2008	Gene Ther	Taiwan	English	BMP-2	AV	LacZ	50 MOI	N/R	In vitro/In vivo
Cirelli [10]	2009	Gene Ther	USA	English	TNFR	AAV	N/R	$5\text{-}20\times10^{12}$ DRP/ml	HEK293	In vivo
Cao[11]	2015	Stem Cell Res Ther	China	English	HGF	AV	GFP	50-400 MOI	HEK293	In vitro/In vivo
Dai [12]	2007	Front Biosci	China	English	EGFP	AAV	N/R	N/R	HEK293	In vivo
Dunn [13]	2005	Mol Ther	USA	English	BMP-7	AV	Luc	N/R	N/R	In vivo
Hu [14]	2007	J Orthop Res	China	English	BMP-7	Plasmid	GFP	N/R	N/R	In vivo
Iglesias-Linares [15]	2011	Orthod Craniofac Res	Spain	English	RANKL	HVJ	N/R	N/R	NIH3T3	In vitro/In vivo
Jiang [16]	2006	Int J Oral Maxillofac Surg	China/Canada	English	BMP-4	Plasmid	GFP	N/R	JM 109	In vitro/In vivo
Jiang [17]	2009	Clin Oral Implants Res	China	English	BMP-2	AV	GFP	50 PFU/cell (MOI)	N/R	In vitro/In vivo
Jiang [18]	2009	Biomaterials	China	English	BMP-2	AV	LacZ	80 PFU/cell (MOI)	N/R	In vitro/In vivo
Jiang [19]	2010	Bone	China	English	b-FGF	AV	GFP	N/R	N/R	In vitro/In vivo
Jin [20]	2003	J Periodontol	USA	English	BMP-7/Noggin	AV	GFP	200 PFU/cell (MOI)	HEK293	In vitro/In vivo
Jin [21]	2004	Mol Ther	USA	English	PDGF-B /PDGF-A	AV	Luc	200 PFU/cell (MOI)	N/R	In vitro/In vivo
Kanzaki [22]	2006	Gene Ther	Japan	English	RANKL	HVJ	N/R	N/R	NIH3T3	In vitro/In vivo
Kroczek [23]	2010	J Craniomaxillofac Surg	Germany Netherlands	English	BMP-2	Liposome	N/R	N/R	N/R	In vivo
Kuboki [24]	1999	Arch Oral Biol	Japan	English	LacZ	AV	N/R	N/R	HEK293	In vivo
Lai [25]	2014	J Zhejiang Univ Sci B	China	English	OSX	Plasmid	GFP	N/R	N/R	In vivo
Lai [26]	2011	Oral Surg Oral Med Oral Pathol Oral Radiol Endod	China	English	OSX	Plasmid	N/R	N/R	N/R	In vivo
Lattanzi [27]	2008	Gene Ther	Italy	English	LMP-3	AV	GFP	N/R	CRE8	In vitro/In vivo
Li [28]	2010	J Biomed Mater Res A	China	English	BMP-7	AV	GFP	N/R	WEHI 164	In vitro/In vivo
Li [29]	2010	Zhonghua Yi Xue Za Zhi	China	Chinese	BMP-7	N/R	N/R	N/R	N/R	In vivo
Li [30]	2009	Arch Oral Biol	China	English	Vastatin	AAV	GFP	5×10^3, 1×10^4-5×10^4 PFU/cell (MOI)	HEK293	In vitro/In vivo
Long [31]	2011	Oral Surg Oral Med Oral Pathol Oral Radiol Endod	China	English	BMP-2	AV	LacZ	100 PFU/cell (MOI)	N/R	In vitro/In vivo

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Table 1: Summary of essential features of all studies included in the systematic review

Author	Year	Journal	Country	Language	Therapeutic Gene	Vector	Control	Virus Titres (Concentration)	Cell lines for generation of virus	Experiment design
Park J [32]	2003	Gene Ther	Germany	English	BMP-2	AV Liposome	LacZ	1-3×10^{10} PFU/ml	911 helper	In vitro/In vivo
Park S [33]	2015	J Biomed Mater Res A	Korea	English	BMP-2	AV	N/R	100 PFU/cell (MOI)	HEK293	In vitro/In vivo
Rabie [34]	2007	Gene Ther	China	English	VEGF	AAV	GFP	N/R	HEK293	In vitro/In vivo
Steinhardt [35]	2008	Tissue Eng Part A	Israel/USA	English	BMP-2	AV	LacZ	3000 PFU/cell	N/R	In vitro/In vivo
Su [36]	2015	Stem Cell Res Ther	China	English	OPG	Lentivirus	N/R	1.5×10^{6} TU/ml	HEK293	In vitro/In vivo
Sun [37]	2010	Arch Oral Biol	China/USA	English	BMP-2	AV	GFP	50 PFU/cell	N/R	In vitro/In vivo
Sun [38]	2013	J Oral Maxillofac Surg	China	English	BMP-2	AV	N/R	N/R	HEK293	In vitro/In vivo
Sun [39]	2014	J Orthop Res	China	English	Runx2	AV	GFP	5×10^{9} PFU/ml Runx2 2×10^{10} PFU/ml GFP	HEK293	In vitro/In vivo
Sun[40]	2007	Shanghai Kou Qiang Yi Xue	China	Chinese	BMP-2	Plasmid	N/R	N/R	N/R	In vivo
Tan [41]	2009	Cytotherapy	China	English	b-FGF	Plasmid	GFP	N/R	N/R	In vitro/In vivo
Tang [42]	2008	Cell Biol Int	China	English	BMP-2	Plasmid	N/R	N/R	N/R	In vitro/In vivo
Tang [43]	2006	Zhonghua Kou Qiang Yi Xue Za Zhi	China	Chinese	BMP-2	Plasmid	N/R	N/R	N/R	In vitro/In vivo
Wang [44]	2015	Br J Oral Maxillofac Surg	China	English	NGF-β	Lentivirus	GFP	N/R	N/R	In vivo
Wei [45]	2013	Stem Cells Dev	USA/China	English	EGFP	Retrovirus	N/R	N/R	PG13	In vitro/In vivo
Wen [46]	2012	Arch Oral Biol	China	English	EGFP	Lentivirus	N/R	N/R	293FT	In vitro/In vivo
Yang [47]	2014	PLoS One	USA/China	English	TSG-6	Lentivirus	N/R	N/R	N/R	In vitro/In vivo
Ye [48]	2006	Shanghai Kou Qiang Yi Xue	China	Chinese	BMP-2	AV	GFP	100 MOI	HEK293	In vivo
Yu [49]	2011	Gene Ther	USA	English	MKP-1	AV	LacZ	N/R	HEK293	In vitro/In vivo
Zhang [50]	2007	Biomaterials	China	English	BMP-7	AV	GFP	2×10^{10} particles/ml	HEK293	In vitro/In vivo
Zhang [51]	2009	J Control Release	China	English	BMP-7/PDGF-B	AV	GFP	2×10^{10} particles/ml	HEK293	In vitro/In vivo
Zhang[52]	2015	J Clin Periodontol	Switzerland China	English	BMP-7/PDGF-B	AV	N/R	1.4×10^{10} PFU/ml	N/R	In vitro/In vivo
Zhao [53]	2010	Oral Dis	China	English	BMP-2	AV	GFP	80 PFU/cell (MOI)	N/R	In vitro/In vivo
Zhao [54]	2012	Orthod Craniofac Res	China/Japan	English	OPG	HVJ	N/R	N/R	N/R	In vivo
Zhou [55]	2010	Hua Xi Kou Qiang Yi Xue Za Zhi	China	Chinese	OPG	Plasmid	N/R	N/R	N/R	In vitro/In vivo
Zhou[56]	2012	Int J Periodontics Restorative Dent	China	English	OPG	Plasmid	N/R	N/R	N/R	In vitro/In vivo
Zou [57]	2012	PLoS One	China	English	HIF-1α	Lentivirus	GFP	7 MOI	N/R	In vitro/In vivo

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Table 2: Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Abramson [1]	Alveolar bone defect with dental implant	Maxilla (bilateral: first molars)	Male Sprague Dawley rats	16	N/R	2.6% collagen gel	In-vivo (GAM)	----------	High dose Low dose Collagen alone Untreated control	8×10^{11} particles/ml 8×10^{10} particles/ml
Alden [2]	Alveolar bone defect	Mandible (bilateral: angle)	Sprague Dawley rats	13	4 mm circular	Physiological saline	In-vivo (local injection)	----------	BMP 2 BMP 9 LacZ	3.75×10^{8} particles/7.5 µl
Ashinoff [3]	Distraction Osteogenesis	Mandible (Right side: body)	Male Sprague Dawley rats	54	N/R	N/R	In-vivo (local injection)	----------	Untreated control BMP2 LacZ	1×10^{10} IFU
Chang [4]	Periodontal alveolar bone defect	Mandible (Buccal plate:1st and 2nd molars roots)	Sprague Dawley rats	144	$3\times2\times1$ mm^3	2.6% collagen gel	In-vivo (GAM)	----------	High dose Low dose Collagen alone	5.5×10^{8} PFU/ml 5.5×10^{9} PFU/ml in 20µl collagen
Chang [5]	Alveolar bone defect with dental implant	Maxilla (Bilateral: first molars)	Male Sprague Dawley rats	100	N/R	2.6% collagen gel	In-vivo (GAM)	----------	High dose Low dose Luc rhPDGF-BB Collagen alone	5.5×10^{8} PFU/ml 5.5×10^{9} PFU/ml
Chang [6]	Alveolar bone defect	Maxilla (Bilateral: infraorbital rim)	Female miniature swine	20	3×1.2 cm^2	Collagen Type I	Ex-vivo	----------	BMP2 LacZ	N/R
Chen [7]	Periodontal Disease	Maxilla (labial PDL: incisors)	Male Wistar rats	29	N/R	Lipid bubbles	In-vivo (GAM)	----------	DNA DNA/US DNA/NB DNA/US/NB	N/R
Chen[8]	Alveolar bone defect	Maxilla (Bilateral: anterior)	Foxhound dogs	N/R	2 cm	PDLA	In-vivo (GAM)	----------	BMP-4 scaffold Autograft Scaffold only Blank control	N/R
Chen[9]	Periodontal alveolar bone defect	Maxilla (Bilateral: incisors)	Male New Zealand White rabbits	12	$15\times7\times5$ mm^3	PF127	Ex-vivo	BMMSCs	BMP-2 transfected MSCs/PF127 Bgal transfected MSCs/PF127 Untransfected MSCs/PF127 PF127 only	50×10^{6}cell/ml
Cirelli [10]	Periodontal Disease	Maxilla (Bilateral: palatal gingival tissue between molars)	Male Sprague Dawley rats	45	N/R	Physiological saline	In-vivo (local injection)	----------	Vehicle Pg-LPS TNFR:Fc TNFR:Fc + Pg-LPS	1×10^{11}DRP/100ml

Table 2: Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Cao[11]	Periodontal Disease	Maxilla&Mandible (First molars)	Male Wuzhishan mini-pigs	20	5×7×3m m^3	Physiological saline	Ex-vivo	DPSCs	DPSCs HGF-DPSCs DPSCs sheet HGF-DPSCs sheet Blank control	1×10^7cells/0.6 ml
Dai [12]	Tempromandibular joint (Mandibular Condylar growth)	Mandibular condyles	Female Sprague Dawley rats	60	N/R	N/R	Ex-vivo	----------	EGFP PBS only	2×10^{11} genome copies/50 µl
Dunn [13]	Alveolar bone defect with dental implant	Maxilla (First molars)	Sprague–Dawley rats	44	2×1 mm^2	2.6% collagen gel	In-vivo (GAM)	----------	Luc BMP 7	2.5×10^{11} particles
Hu [14]	Distraction Osteogenesis	Mandible (Right side)	Male Sprague-Dawley rats	44	N/R	Physiological saline	Ex-vivo	BMMSCs	BMP 7 EGFP-N1 physiological saline	1×10^6 cell/0.15 ml
Iglesias-Linares [15]	Orthodontic tooth movement	Maxilla (Bilateral:Second molars)	Wistar rats	72	N/R	Solution	In-vivo (local injection)	----------	TM force + PBS (R&L) TM force + Corticotomy (R)/TM force + Flap surgery(L) TM force + RANKL (R)/TM force + Plasmid without RANKL insert (L) Cortictomy (R)/Flap Surgery (L) RANKL (R)/Plasmid without RANKL insert (L)	N/R
Jiang [16]	Alveolar bone defect	Mandible (Bilateral)	Female New Zealand White rabbits	14	15×6 mm^2	NNB	Ex-vivo	BMMSCs	NNB/EGFP-BMP 4 NNB/EGFP NNB/untransfected bMSCs NNB alone Blank control	50×10^6 cell/scaffold
Jiang [17]	Sinus floor elevation	Maxilla (Bilateral)	Male New Zealand rabbits	20	13×3×5 mm^3	β-TCP	Ex-vivo	BMMSCs	β-TCP alone Untransduced bMSCs/ β-TCP EGFP–bMSCs/ β-TCP BMP-2–bMSCs/ β-TCP	2×10^7 cell/scaffold
Jiang [18]	Alveolar bone defect	Mandible (Ascending ramus)	Male Fisher 344 rats	24	5mm circular	mSS	Ex-vivo	BMMSCs	mSS/bMSCs transduced BMP 2 mSS/bMSCs transduced LacZ mSS/bMSCs mSS alone	2×10^7 cell/scaffold
Jiang [19]	Distraction Osteogenesis	Mandible (Right side: between 1st premolar and mental foramen)	Male New Zealand rabbits	42	N/R	Physiological saline	Ex-vivo	BMMSCs	b-FGF transfected MSCs in physiological saline, EGFP transfected MSCs in physiological saline. Physiological saline	1×10^7cell/0.15 ml

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Table 2: Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Jin [20]	Periodontal alveolar bone defect	Mandible (Bilateral: mandibular 1st and 2nd molar:buccal root PDL)	Lewis rats	25	0.3×0.2 cm^2	Gelatin sponge	Ex-vivo	SDFs	GFP control-treated Noggin-treated BMP 7	1×10^6 cell/scaffold
Jin [21]	Periodontal alveolar bone defect	Mandible (Bilateral buccal plate of 1st and 2nd molars)	Sprague–Dawley rats	30	0.3×0.2 cm^2	2.6% collagen gel	In-vivo (GAM)	----------	Luc PDGF-B PDGF-A Collagen matrix alone	2.5×10^{11} viral particles(PN)/ml
Kanzaki [22]	Orthodontic tooth movement	Maxilla (Right 1st molar of OF group)	Male Wistar rats	25	N/R	Vector solution	In-vivo (local injection)	----------	Control group OF group with or without RANKL Mock group	N/R
Kroczek [23]	Distraction Osteogenesis	Mandible (Right side)	Female Goettingen mini-pigs	24	N/R	Aqueous solution	Ex-vivo	BMMSCs	BMP 2 group BMP 7 group TGF-b group IGF 1 group Liposome vector group No induction group	4×10^5cell/dish
Kuboki [24]	Tempromandibular joint	Mandibular condyles (Bilateral)	Hartley guinea-pigs	16	N/R	Physiological saline	In-vivo (local injection)	----------	Gene Placebo Control	4.8×10^7 PFU/cell
Lai [25]	Distraction Osteogenesis	Mandible (Right side)	Male New Zealand rabbits	44	N/R	Physiological saline	Ex-vivo	ADSCs	transfected ADSCs EGFP-N1transfected ADSCs physiological saline only	1×10^7cell/0.2 ml
Lai [26]	Distraction Osteogenesis	Mandible (Left side: anterior to 1st molar)	Male New Zealand rabbits	44	N/R	Physiological saline	Ex-vivo	BMMSCs	transfected BMMSCs, autologous BMMSCs physiological saline only	1×10^7cell/0.2 ml
Lattanzi [27]	Alveolar bone defect	Mandible (behind the root of the incisor)	Wistar rats	36	5×5 mm^2	HA/COL	Ex-vivo	SDFs	LMP-3 transduced SDF on HA/COL Untransduced SDF on HA/COL HA/COL scaffold without cells Control group EGFP	N/R
Li [28]	Alveolar bone defect	Mandible (Bilateral)	New Zealand rabbits	44	12×8 mm^2	HA/PA	Ex-vivo	BMMSCs	Scaffold seeded with BMP 7 transduced MSCs Scaffolds seeded with osteogenically cultured MSCs. Pure HA/PA scaffolds	2×10^6cell/scaffold
Li [29]	Periodontal Disease	Mandible (Bilateral:premolar teeth)	Adult Beagle dogs	5	N/R	collagen membrane	Ex-vivo	BMMSCs	Pure collagen membrane Collagen membrane / transfected cells Collagen membrane / untransfected cells	1×10^7 cell/scaffold

Table 2: Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Li [30]	Tempromandibular joint	Mandibular condyles (Bilateral)	Female Sprague–Dawley rats	30	N/R	N/R	In-vivo (local injection)	----------	Vastatin EGFP	2×10^{11} genome copies/50µl
Long [31]	Distraction Osteogenesis	Mandible (Right side: between anterior teeth and 1st premolar)	Male Japanese rabbits	36	N/R	Physiological saline	Ex-vivo	BMMSCs	Distraction of 0.8 mm/d for 12 days. Distraction of 2.4mm/d for 4 days, with MSCs transfected with lacZ Distraction of 2.4 mm/d for 4 days with MSCs transfected with BMP-2.	1×10^{7}cell/ ml
Park J [32]	Alveolar bone defect	Mandible (Left ramus)	Wistar rats	56	6mm circular	Collagen sponge	Ex-vivo	BMMSCs	BMP-2-infected BMSC LacZ-infected BMSC Untreated BMSC Empty collagen sponges	1×10^{6} cell/scaffold
Park S [33]	Periodontal alveolar bone defect with dental implant (Peri-implantitis wound)	Mandible (Bilateral: premolars and 1st molar)	Adult Beagle dogs	6	N/R	HA/COL hydrogel	Ex-vivo	PDLSCs	HA with collagen gel (control group,) HA with collagen gel/ PDLSCs HA with collagen gel/BMP2/PDLSC	N/R
Rabie [34]	Tempromandibular joint	Mandibular condyles (Bilateral)	Female Sprague–Dawley rats	90	N/R	Physiological saline	In-vivo (local injection)	----------	VEGF EGFP PBS	2×10^{11} genome copies/50µl
Steinhardt [35]	Alveolar bone defect	Mandible (Right side)	NOD/SCID mice	N/R	1mm circular	Collagen sponge	Ex-vivo	BMMSCs	MSC-BMP2 MSC-lacZ Control group (no implant)	5×10^{6} cell/scaffold
Su [36]	Alveolar bone defect	Mandible (Left side: alveolar bone of incisors)	Male New Zealand rabbits	20	$5\times10\times4$ mm^3	β-TCP	Ex-vivo	PDLSCs	Control β-TCP PDLSCs/β-TCP OPG-PDLSCs/β-TCP	5×10^{6} cell/scaffold
Sun [37]	Sinus floor elevation	Maxilla (Bilateral)	Male New Zealand rabbits	8	$13\times3\times5$ mm^3	OsteoBone	Ex-vivo	BMMSCs	BMP-2-infected BMSC/Scaffold EGFP-infected BMSC/Scaffold	2×10^{7} cell/scaffold
Sun [38]	Alveolar bone defect	Mandible (Bilateral)	Adult New Zealand rabbits	18	10×6 mm^2	BGC	Ex-vivo	PDLSCs	BMP-2–modified tissue-engineered bone Unmodified tissue-engineered bone Single BGC graft Defects without any implantation	2×10^{7} cell/scaffold
Sun [39]	Distraction Osteogenesis	Mandible (Right side: anterior to 1st premolar)	Female New Zealand rabbits	90	N/R	Physiological saline	Ex-vivo	ADSCs	Runx2 transfected ADSCs GFP-transfected ADSCs Ovariectomized control Sham surgery control	1×10^{7} cell/ml

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Table 2: Extracted data from included studies with description of disease model and animal model used

Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Sun[40]	Periodontal alveolar bone defect	Mandible (Bilateral: premolars)	Adult beagle dogs	6	5mm	Collagen sponge	In-vivo (GAM)	----------	BMP-2 plasmid group BMP-2 group PBS	N/R
Tan [41]	Periodontal Disease	Mandible (Bilateral: 1st, 2nd and 3rd premolars)	Male beagle dogs	4	5mm vertical	Sodium alginate	Ex-vivo	BMMSCs	bFGF transfected BMSCs Untransfected BMSCs	2×10^7 cell
Tang [42]	Alveolar bone defect	Mandible (Left ramus)	Female Sprague Dawley rats	40	4mm circular	CHA	Ex-vivo	BMMSCs	Control groups: empty defect CHA/autologous transfected BMP-2 CHA/untreated autologous BMSCs	5×10^6 cell/scaffold
Tang [43]	Alveolar bone defect	Mandible (Ramus)	Female Sprague-Dawley rats	24	4mm circular	CHA	Ex-vivo	BMMSCs	Control groups: left untreated BMSCs that transfected with BMP-2	5×10^9 cell/scaffold
Wang [44]	Distraction Osteogenesis	Mandible (Bilateral)	Male New Zealand rabbits	20	N/R	Physiological saline	Ex-vivo	BMMSCs	MSC transduced with NGF-b Control: EGFP	5×10^6 cell/0.1ml
Wei [45]	Tooth restoration/Bio-Root regeneration	N/R	Inbred miniature pigs	18	N/R	HA/TCP	Ex-vivo	DPSCs PDLSCs	HA/TCP Autologous Vc-induced PDLSCs in HA/TCP/DPSC Allogeneic Vc-induced PDLSCs in HA/TCP/DPSC	1×10^6 cell/scaffold
Wen [46]	Periodontal alveolar bone defect	Mandible (Right 1st molars)	Sprague-Dawley rats	6	1×3 mm^2	Collagen gel	Ex-vivo	PDLSCs	eGFP transfected PDLSCs untransfected PDLSCs Empty defect	5×10^5 cell
Yang [47]	Periodontal Disease	Maxilla (Bilateral: 1st molar)	Female Sprague–Dawley rats	30	N/R	**systemic:** culture media **Local:** Matrigel	Ex-vivo/ In vivo (systemic)	iPSC-derived MSCs	Healthy control Untreated periodontitis iPSC-MSCs-treated periodontitis iPSC- MSCs/TSG-6-treated periodontitis	5×10^6 cell/200µl media L: 1×10^6 cell/20µl gel
Ye [48]	Central fissures	Mandible	New Zealand rabbits	45	N/R	Bioglass	Ex-vivo	pOBs	BMP-2 transfected POBs/bioglass EGFP transfected POBs/bioglass Untransfected POBs/bioglass Bioglass only Blankcontrol	2×10^7 cell/scaffold
Yu [49]	Periodontal Disease	Maxilla (1st and 2nd molars)	Male Sprague–Dawley rats	51	N/R	HEPES	In-vivo (local injection)	----------	MKP-1 LacZ HEPES- buffered saline	1×10^9 PFU
Zhang [50]	Alveolar bone defect with dental implant	Mandible (Bilateral: Premolar region)	Adult hybrid dogs	9	$6\times5\times4$ mm^3	Chitosan/ Collagen	In-vivo (GAM)	PDLSCs	Pure scaffold Scaffolds with BMP7 Scaffolds with Easy1	1×10^7 cell/scaffold
Zhang [51]	Alveolar bone defect with dental implant	Mandible (Bilateral: Premolar region)	Adult hybrid dogs	6	$6\times5\times4$ mm^3	Chitosan/ Collagen	In-vivo (GAM)	PDLSCs	Scaffolds with Easy1: control Scaffolds with BMP 7 Scaffolds with PDGF-B Scaffolds with BMP-7/PDGF-B	1×10^7 cell/scaffold

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Author	Disease Model	Site	Animal Model	Sample size	Defect size	Carrier/ Scaffold	Gene Delivery route	Stem cell source	Experimental groups	Cell concentration
Zhang [52]	Periodontal Disease	Maxilla (2^{nd} & 3^{rd} premolars)	Male beagle dogs	5	5×5 mm^2	MBG/silk fibrin	In-vivo (GAM)	----------	Control non-filled defects scaffold alone PDGF-B scaffold BMP7 scaffold PDGF-B + BMP7 scaffold	5×10^5 cell/scaffold
Zhao [53]	Alveolar bone defect	Mandible (Bilateral: ramus)	Male Fisher 344 rats	11	5mm circular	β-TCP	Ex-vivo	BMMSCs	b-TCP alone b-TCP with untreated bMSCs b-TCP with bMSCs transduced with EGFP b-TCP with bMSCs transduced with BMP-2	2×10^7 cell/scaffold
Zhao [54]	Orthodontic tooth movement	Maxilla (Right 1st molars)	Male Wister rats	18	N/R	Vector solution	In-vivo (local injection)	----------	OPG transfection group Mock vector transfection group Control group	N/R
Zhou [55]	Periodontal alveolar bone defect	Mandible (Bilateral: premolars)	Male purebred beagle dogs	4	4×4×3 mm^3	PLGA	Ex-vivo	BMMSCs	BMSCs/OPG-PLGA BMSCS-PLGA	1×10^6 cell/scaffold
Zhou[56]	Periodontal alveolar bone defect	Mandible (Bilateral: premolars)	Male purebred beagle dogs	4	4x4x3 mm^3	PLGA	Ex-vivo	BMMSCs	BMSCs/OPG-PLGA BMSCS-PLGA PLGA Negative control: root planing only	1×10^6 cell/scaffold
Zou [57]	Alveolar bone defect with dental implant	Mandible (Bilateral: premolars region)	Adult male labrador retriever dogs	5	6×5×4 mm^3	CMPC	Ex-vivo	BMMSCs	Blank CMPC CMPC/BMSCs/GFP CMPC/BMSCs/HIF CMPC/BMSCs/cHIF	2×10^5 cell/scaffold

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Table 3: Endpoint results of the main analytical methods used for the experiments

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	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Abramson [1]		PDGFB demonstrated more mineralized tissue at 4 weeks than 2 weeks. Viral copies in blood and organs not significantly different between treated and untreated rats at all time points			
Alden [2]				3D CT showed marked osteogenesis and bony healing in BMP-2 and BMP-9 treatment groups while control did not show notable healing.	Slight healing of the defect in control. However, BMP-2 and BMP-9 showed marked bony regeneration across the defect site. BMP-2-treated defect demonstrated almost complete regeneration of the mandible indistinguishable from the normal mandible.
Ashinoff [3]				Increased radio-density in BMP-2-treated animals with increased new bone formation compared to control	
Chang [4]		Viral vector of PDGFB was detected within the first week in DNA and gradually decreased to undetectable levels after 2 weeks.	Luc/collagen showed high level in animals receiving high-dose Luc compared with low-dose.		Two weeks after surgery, nearly complete bone bridging of the alveolar bone in both PDGF-B groups whereas limited bridging in collagen-only animals. At 35 days, bone had completely bridged all of the defect area.
Chang [5]		Absence of PDGF-B in bloodstream.		µCT showed higher bone volume fraction in PDGF-B and rhPDGF-BB groups than low dose PDGF-B and Luc groups.	Bone was noted at coronal margin in Luc group and thicker bone trabeculae were evident in all PDGF-treated specimens. At day 14, near-complete defect fill was noted for all PDGF groups
Chang [6]				3D CT revealed complete repair of defects implanted with BMP-2. However, small islands of bone formation were observed in the βgal. Immunohistochemistry results revealed positive staining in BMP-2 cell constructs.	cancellous bone formation at defects implanted with BMP-2. Visible bone formation was noted at defect site implanted with BMP-2 cell constructs while βgal control had islands of bone formation with variable thickness and marked notching in the infraorbital rim.
Chen [7]			At day 1 after treatments: DNA+NB and DNA+US treatments were as low as with DNA alone treatment. Ultrasonication after DNA + NB injection significantly increased luciferase activity. Rats with removed gingivae exhibited weak luciferase activity in labial tissues of maxillary incisors		Histology showed no haemorrhage or inflammation, while fluorescence images showed EGFP expression mostly confined to labial gingival tissues of maxillary incisors

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Chen [8]				At week 4, rhBMP-4 and autograft-treated groups showed a significant increase in bone regeneration when compared with the defect-only group and the scaffold only groups. No tooth eruption was seen at the 4-week time point in any of the four groups. New bone could be differentiated from grafted bone. By week 12, the entire defect had been filled.	The difference between new and grafted bone could not be seen. All four groups exhibited tooth eruption at 12 weeks.
Chen [9]	Showed that the adenovirus mediated BMP-2 gene was positively expressed and processed in MSCs of the defect.			3D CT showed that BMP-2 group had the highest mean regenerated bone volume and there were no significant differences between the other three groups.	6 weeks, BMP-2 group exhibited greater amounts of new bone formation. The newly regenerated trabecular bone was covered by a thick layer of osteoid and osteoblasts with continual bone-forming activity. The β-gal group exhibited woven bone formation, from the apical aspect of the defect to the middle of the root. The PF127 group displayed minimal amounts of bone formation at the apical third
Cirelli [10]	TNFR:Fc protein 4 weeks before Pg-LPS delivery showed high level which were sustained during 8-week experimental period compared to Pg-LPS, vehicle or no treatment.	High expression of IL-6, IL-10, RANKL and OPG observed at 4 weeks in Pg-LPS-exposed animals, but not in TNRF:Fc.		2Dand 3D µCT of maxillae showed linear bone loss. Significant alveolar bone destruction was observed in Pg-LPS group continuously over 8 weeks. Administration of TNFR:Fc prevented linear bone resorption during entire study compared with Pg-LPS only treated group.	An intense inflammatory cell infiltrate observed in subepithelial connective tissue and surrounding alveolar bone of periodontia of Pg-LPS- animals but a significantly less intense inflammatory reaction was observed in TNFR:Fc+Pg-LPS animals. Control animals did not show evidence of inflammatory cell infiltrates.
Cao [11]	Increased expression of HGF in transfected MSCs.			3D CT indicated limited bone formation in the control group. In contrast, marked bone regeneration occurred in the hDPSC, HGF-hDPSC, hDPSC sheet and HGF-hDPSC sheet groups. The heights of newly regenerated bone were significantly higher in all treatment groups compared with control group. The bone volumes in all treatment groups were significantly larger than the volume in the control group.	At week 12, new periodontal tissue regeneration within the periodontal defects was significantly less pronounced in the control group compared with the regeneration in the treatment groups. Alveolar bone regeneration was also more pronounced in the HGF-hDPSC, hDPSC sheet group and HGF-hDPSC sheet group than in the control group. The percentages of periodontal bone in the hDPSC injection, HGF-hDPSC injection, hDPSC sheet, and HGFhDPSC sheet groups were significantly higher than that of the control group.

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Dai [12]		PCR of eGFP in heart, kidney, spleen and liver, mRNA was not detected reducing the prospects of systemic adverse effects. RT-PCR of transgene expression in the mandibular condyle revealed constant expression throughout the experiment. At day 21, there was a substantial increase in transgene expression.			
Dunn [13]			Showed sustained release of the gene product. All implants displayed the localized nature of expression in the near vicinity of the oral implants. The gene was expressed strongly for the first few days with peak expression at day 4 then declined by 2–5 weeks.		BMP-7-treated defects displayed tissue consistent with early osteoid formation throughout the defect area. Ad/Luc group exhibited normal bone healing, with most specimens showing minimal bone formation at the defect borders. At 28 days, bone formation was heightened both at the defect margins and along the dental implant surface in Ad/BMP-7-treated sites.
Hu [14]		Confirmed transcription of BMP-7 in transfected MSCs in contrast with negative signal in MSCs transfected with N1.		Radiodensity of callus in group A at 2 weeks was greater than in group B which was higher than group C. After 6 weeks of healing, more mineralization of distraction zone was seen in all three groups, but group A had greater radiodensity.	Immunocytochemistry showed BMP-7 expression in transfected MSCs while MSCs transfected with N1 exhibited negative signals. Bone regeneration in the distraction gaps was intramembranous ossification. At 2 weeks, positive signals for BMP-7 were found in the distraction zones in all three groups. Strong BMP-7 expression of was observed in group A, moderate in group B, and weak in group C. At 6 weeks, very weak BMP-7 positive staining was seen and a similar pattern and intensity was noted among the three groups.
Iglesias-Linares [15]					TM force groups with corticotomy or RANKL transfection showed a larger bone resorption area than control groups. Transfection group under orthodontic force maintained a higher bone resorption rate than corticotomy group under force throughout the experiment.
Jiang [16]					No inflammation or giant cell-type reaction was observed in any of the groups in immunohistochemistry.

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Jiang [17]					At week 2, newly formed trabeculae were found in the four groups. Just a slight newly formed bone was observed in group A; however, more bone area was found in group B and group C. In group D, a larger area of newly formed bone was found not only in the periphery but also in the centre of the space. At week 8, newly formed bone area increased in all four groups.
Jiang [18]	BMSCs transduced with BMP-2 produced higher levels of BMP-2 during the entire culture period as compared with LacZ and untransduced MSCs using ELISA	Upregulation of collagen type I in MSCs transduced with BMP-2. Runx2 showed moderate upregulation. Osteopontin showed sustained marked upregulation. Osteocalcin showed a steep increase. Osteogenic markers in LacZ transduced bMSCs remained at basal levels.		A larger defined radio-opaque new bone formation and mineralization was observed in BMP-2- transduced bMSCs group when compared to the LacZ and untransduced groups.	Increased bone formation in BMP-2-transduced bMSCs implants, less bone formation in LacZ or untransduced bMSCs-seeded scaffolds and no obvious bone formation was found in scaffold alone defects using histological sections. Immunohistochemistry displayed intensive BMP-2 staining in both bone matrix and surrounding fibroblastic-like tissue for BMP-2-transduced bMSCs whereas in LacZ bMSCs and untransduced bMSCs groups, BMP-2 staining was present but much weaker. No obvious positive staining was detected in the scaffold alone group.
Jiang [19]		bFGF was at a highest level at day 7 in bFGF transfected MSCs and sustained at high level in the next 3 weeks. Negative signal of bFGF was detected in MSCs or MSCs transfected with EGFP.		At 8 weeks, radiodensity of distracted callus in group was higher than those in groups A and B while radiodensity in group B was higher than in group A. µCT showed that the lingual cortical bone was formed well than the buccal cortical bone in all groups.	Immunohistochemistry showed bFGF expressed in bFGF transfected MSCs while negative signals in MSCs transfected with EGFP. Histology revealed newly formed trabeculae in all groups.
Jin [20]					Expression of BMP-7 and noggin was undetectable by 10 and 35 days after surgery by immunohistochemistry. Minimal to no osteogenesis was seen in GFP and noggin groups at early time point. Defects treated with BMP-7 demonstrated cartilage and limited areas of bone in the majority of the defects. At 35 days extensive bone formation was seen in most of the defects treated by BMP-7 while minimal osteogenesis and cementogenesis and lack of fibre insertion was noted in GFP and noggin groups.

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Jin [21]		PCR showed expression of PDGF-B in PDGF-B transduced SDFs but not in cells transduced by luc or PDGF-A or cells without any adenovirus transduction.	The highest was at day 1 post-gene delivery and decreased at days 4-7. At 14-28 days postgene transfer, luciferase decreased compared to day 1.		Immunostaining was performed at days 3, 7and 14. In PDGF-B-treated group, greater numbers of positively stained cells on the surfaces of the alveolar bone and denuded tooth roots as well as the tissues surrounding the collagen matrix containing PDGF-B compared to other treatments at both days 3 and 7. At 3 days after treatment, no significant evidence of bone or cementum formation in any of the treatment groups and very few cells invaded into the adenovirus collagen implant.
Kanzaki [22]					No severe inflammations in periodontal tissue on repeated local RANKL gene transfer. Strong RANKL protein expression in the periodontium after 2 or 4 days from RANKL gene transfer. Very few RANKL protein expressions in the periodontium after 6 days from RANKL gene transfer. The number of osteoclasts was high at day 2 after RANKL gene transfer. The number of osteoclasts was reduced time dependently.
Kroczek [23]				Ssome differences between early and late period of consolidation in relation to the osteoinductive substance applied. The central distraction zone had no ossification. Induction with TGF-b revealed crystallization spots dispersed homogenously over the central distraction zone. Osteoinduction with BMP-7 showed consolidation of the central distraction zone after 1 week with a small gap in the central distraction zone. In the late consolidation period, the gap was bridged by fine bone trabeculae. Induction with BMP-2 resulted in an accelerated, dense new bone formation.	Lamination of the distracted bone areas adjacent to the osteotomy sites with longitudinally orientated columns of lamellar bone. The bone trabeculae showed osteoid deposition and early mineralization along their sides. The process of bone formation resembled more an intramembranous than chondroid ossification mode. Induction with TGF-b resulted in bone formation similar to one without induction. Positive immunostaining of BMP-2 was observed in distracted callus in all groups. Cellular elements with increased BMP-2 expression were found both in the distraction zone and in the consolidated osseous area close to the osteotomy region. A reduced BMP-2 expression was found in the central distraction zones.

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Kuboki [24]		The absence of LacZ in liver, kidney, heart, and brain in LacZ- or control group. In TMJ of LacZ-injected animals, expression of LacZ was detected and not detected in the joints of control group.			There was no observable difference between the virus-injected and the PBS-injected joints. The frontal section of the mandibular joint 1 week after LacZ injection clearly showed that articular surface-lining cells were stained blue.
Lai [25]				Radiodensity of distraction areas in group A was higher than that in groups B and C at Weeks 2 and 6 after the distraction procedure.	Bone cells in the distracted areas were stretched along in the direction of the distraction. At 2 week, the two fragments of mandibles in all groups were filled with newly formed bone trabeculae. Similar results were seen in groups B and C, but much denser and thicker bone trabecules were observed in the distracted areas in group A than in group B and group C. At 6 weeks, the distraction gaps of the mandible were full of newly generated bone in all three groups.
Lai [26]				Radiograph of a distracted mandible at 2 weeks showed that callus appeared to be greater in group A when compared with group B which was higher than group C.	Bone regeneration in distraction gaps was intramembranous ossification. At 2 weeks, the new bone trabeculae formation began bridging in the 3 groups. More thick and dense trabecules were seen in the distraction gaps in group A than group B and C. At 6 weeks, the gaps were filled with newly formed bone in all groups. At 2 weeks, immunohistochemistry of BSP showed areas of fibrous connective tissue within the gaps and were mainly detected in the cellular components of fibroblast like cells, preosteoblasts, and osteoblasts in all 3 groups. Cells in group A showed greater amount and more intense staining for BSP within the gaps than group B which is more than group C.

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Lattanzi [27]		Efficient LMP-3 expression 24 and 48 h. qPCR demonstrated that LMP-3 in transduced cells slightly increased in a time-dependent manner.		All rats treated with LMP-3 transduced SDFs showed positive X-rays at 8 and 12 weeks after surgery. No radiological evidences of bone formation could be demonstrated in three out of four animals at the earliest time point and in animals treated with scaffold alone or with non-transduced cells. 3DµCT revealed the successful repair of the defects implanted with LMP-3 cell constructs, which occurred in a time-related manner until 12 weeks after implantation. No bone formation was observed in the control group.	All rats treated with LMP-3 transduced SDFs showed positive histology at 8 and 12 weeks. No histological evidences of bone formation could be demonstrated in three out of four animals at the earliest time point and in animals treated with scaffold alone or with non-transduced cells.
Li [28]		BMP-7 was expressed in BMP-7 transfected MSCs while MSCs transfected with N1 exhibited negative signals.		Radiodensity in group A was higher than in group B and C. At 8 weeks, increasing mineralization in the implants was seen in group A than the other two groups.	Immunocytochemistry showed BMP-7 was expressed in BMP-7 transfected MSCs while N1 transfected MSCs exhibited negative signals. Immunocytochemistry of ALP and collagen I in group A was stronger than in group B. New bone formation was found in the implanted area in all three groups. At 4 weeks, the interface zone was surrounded by primitive mesenchymal cells differentiated into osteoblasts and new bone matrix was progressively deposited and became ossified. At week 4 and 8, all the parameters were significantly higher in group A than in group B than in group C. However, no significant difference in these parameters was found among three groups at week 16.
Li [29]					The percentage of new alveolar area in transfected and non-transfected BMSC were significantly higher than the control and there was also significant difference between two experimental groups. The percentage of new cementum length in two experimental groups was significantly higher than the control but there was no significant difference between two BMSCs groups.

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Li [30]		Vastatin was only found in the experimental group. There were no transcripts detectable in the control group.			Positive signals in immunostaining at day 7 while absence of signals in control group. Expression of Vastatin was the highest on day 7, decreased from day 14 to day 60. The expression was in the proliferative and chondroblast layers on day 7. On day 14, Vastatin expressed in chondrocyte and pre-hypertrophic chondrocyte layers. The expression moved to the pre-hypertrophic chondrocyte and hypertrophic chondrocyte layers on day 21. On day 30, the expression moved deeper to hypertrophic chondrocyte layer. Only minor e xpression could be found in the deep hypertrophic chondrocyte layer on day 60.
Long [31]	BMP-2 levels were significantly higher in BMP-2 transfected MSCs compared with lacZ– transfected MSCs			The distraction gaps in group B rabbits did not show ideal new bone formation at week 2 while group A and C showed partial. The distraction gap in group A and C animals showed more mature new bone formation and higher radiopacity at week 4 compared with week 2. At week 8, radiograph of group A and C were almost identical to each other. µCT showed little new bone formation in the distraction gaps of group B animals at week 2. However, in groups A and C, new bone tissue was gradually mineralized from the centre to the margin in the distraction gap. More trabecular bone was mineralized at week 4 in group C than in group A. Groups A and C looked similar at week 8.	

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Park J [32]					Immunocytochemistry of osteocalcin showed mineralization in genetically modified BMSC but rarely in control group. In both gene transfer groups, the amount of osteocalcin increased similarly. At 4 weeks endochondral bone formation occurred in the gene transfer groups and in the control; however, the amount of newly formed bone in the control was much less than in genetically modified BMSC. Treatment of defects with BMP-2-infected BMSC resulted in nearly complete bony healing within 4 weeks after the transplantation.
Park S [33]	BMP-2 expression level in the BMP2/ PDLSCs was significantly higher than in non-transduced PDLSCs. BMP-2 expression increased for 7 days and decreased until day 21.			The bone was lost at 4 months after the induction of experimental peri-implantitis in radiographs.	The bone labelling experiments demonstrated that new bone formation and re-osseointegration in the BMP2/PDLSC group occurred along the implant surface until week 8. PDLSC group showed less newly formed bone than BMP2/PDLSC group. The control group showed a limited amount of new bone formation around the peri-implantitis defects.
Rabie [34]	VEGF delivered group was higher than those two control groups from day 21 to day 60. VEGF expressed from mandibular condyle was significantly increased from day 14 and lasted during the whole time periods. On day 30, VEGF expression was more than in control group.	the expression of VEGF in condylar cartilage at day 7 and the maximum level at 21 days consistent with the result of in situ hybridization.			Immunohistochemistry confirmed increased VEGF expression in VEGF delivered condyle and positive signal in nearly all layers of condyle at day 30. VEGF expression was limited to the hypertrophic layer in control groups. The length and width of the condylar head increased significantly. The length of the condylar process significantly increased. Collagen type II was positive in chondroblast and hypertrophic layer. In control groups, collagen type II and type X positive layer decreased with age. However, after VEGF delivery, the collagen type II positive layer was significantly increased at day 21, compared to eGFP and PBS injection.

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Steinhardt [35]		High levels of BMP2 in the cells but the protein expression levels were very similar.		Almost fully regenerated defect after 8 weeks. Minimal regeneration was observed after 8 weeks in control group infected with lacZ.	Masson trichrome staining revealed formation of new bone tissue and almost complete healing of the defect implanted with MSC-hBMP2. Minimal amount of new bone tissue was evident but no complete regeneration in lacZ or no implant.
Su [36]		Increased OPG level in hOPG transfected cells compared with non-transfected cells.			Toluidine blue staining showed no bone regeneration detected at the alveolar bone control group. A small amount of new bone could be seen in the β-TCP group with some osteoid formation in the periphery and centre of β-TCP scaffold. PDLSCs/β-TCP group showed more new alveolar bone formation, with numerous small bone trabeculae interconnected with each other.
Sun [37]	High BMP-2 in MSCs transduced with BMP-2 as compared with EGFP.				BMP-2 immunocytochemistry showed high staining in BMP-2 infected MSCs than that in control and EGFP infected cells. In BMP-2-MSCs/scaffold and EGFP-MSCs/scaffold, more newly formed trabeculae were found dose to the parent bony wall and lifted membrane. At 4 weeks after implantation, newly formed bone area in the entire augmented area was larger than that at 2 weeks.
Sun [38]	High concentration of BMP-2 in the supernatant of cultured cells. There was no BMP-2 detected in uninfected cells during the entire time course.				More new bone tissue was found in the peripheral part of the grafted defects than in the central part. The central part of the grafts showed that the amount of bone in groups A and B was significantly larger than in group C. In the unfilled controls, there was more fibrous connective tissue formed in the defects after 12 weeks and no full bone healing was found.
Sun [39]		q-PCR showed the higher expression of Runx2 in Runx- transfected ADSCs than GFP transfected ADSCs and controls.		At week 9, radiograph of Groups A2 and D2 showed mature bone formation. µCT indicated the formation of new bone in Groups A2 and D2 than in the other two groups. Little new bone formation was observed in the distraction gaps of Groups B2 and C2.	The distraction gaps in specimens from Groups A2 and D2 were filled primarily with fibrous tissue and tiny trabeculae at week 3. By 6 weeks, more new bone tissue was formed with thicker and wider trabeculae.

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Table 3: Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Sun [40]					At 8 weeks, a complete osseous healing occurred and dense new periodontal ligament fibers rich in blood vessels were observed in BMP-2 group and rhBMP-2 group whereas fewer new bone occurred and sparse collagen fibers aligned irregularly were observed in the blank control group. The height of new bone and cementum were significantly greater in the two experimental group than in the blank control group.
Tan [41]				New bone formation in the two groups but the density of the newly formed bone in the bFGF-modified BMSC group was higher than that in BMSC-alone. µCT showed extensive new bone apposition in continuity with the trabecular host bone structure in the bFGF-modified BMSC transplantation group and BMSC-alone transplantation group.	Both groups exhibited periodontal regeneration, including newly formed cementum, periodontal ligament and bone. The newly formed bone and periodontal ligament in sites receiving bFGF-modified BMSC were greater than those receiving BMSC alone.
Tang [42]				Radiographs confirmed that implanted BMSCs expressing BMP-2 promoted bone formation..	BMP-2 expression was detected by immunohistochemistry in transfected cells but not in the untreated BMSCs. Bone formation was observed on the composites seeded with transfected BMSCs expressing BMP-2 and the group implanted with CHA seeded with untreated BMSCs but the negative control implants did not induce bone formation. At 4 weeks the bone defects that were treated with transfected BMSCs showed formation of mature bone matrix with a trabecular pattern at the defect margin. At week 8, the defect was nearly completely closed and the newly formed mature bone had a typical trabecular pattern.
Tang [43]					New bone formation was found at the margin of the defect treated with the BMSC modified by hBMP-2 gene transfer at 4 weeks and appeared mature 8. However, the amount of newly formed bone was much less with some adipose tissue at defect margins 8 weeks in control group.

Table 3: Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Wang [44]	Secretion of NGF from the transduced MSC which increased to day 7.				Control group had signs of nerve degeneration with few regenerating nerve fibres whereas in experimental group there were abundant regenerating nerve fibres.
Wei [45]				Six months after transplantation, bone-like tissue formation was observed in HA/TCP group with no obvious boundary between the newly regenerated tissue and bone as well as HA/TCP/ DPSC/PDLSC sheet implant formed a hard root structure and a clear PDL space was found between the implant and surrounding bony tissue. µCT demonstrated that there was no obvious hard root structure and PDL space in HA/TCP group whereas a visible root structure and PDL space-like areas in HA/TCP/DPSC/PDLSC sheet group.	PDLSCs sheet had two or three layers and uniformly spread as a two dimensional tissue structure. Immunostaining for vimentin was positive. Fibronectin and type I collagen were present in the harvested PDLSC sheet.
Wen [46]		At 7 days, the expression levels of COL-1 and RUNX2 in PDLSCS were higher than those in eGFP-PDLSCs; the expression levels of ALP and OPN eGFP-PDLSCs were similar to those in PDLSCs.			6 weeks after surgery new regenerated bone, newly formed cementum and periodontal ligament were observed in group A and B. Strong expression of GFP and OPN was observed in the newly formed bone and cementum in the experimental group.
Yang [47]	The production of proinflammatory cytokines was also significantly decreased in serum samples.	Increased TSG-6 expression in transfected iPSC-MSCs whereas low TSG-6 expression in untransfected iPSC-MSCs. Systemic administration of iPSC-MSCs and iPSC-MSCs/ TSG-6 reduced periodontal inflammation.			The infiltration of inflammatory cells in the periodontal tissues was markedly decreased in iPSC-MSCs/TSG-6 group.
Ye [48]				Higher bone density was found in the rabbit mandibular central fissures of group I 4 to 8 weeks after implantation.	Much more new bony callus in group I than in other groups.

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Table 3: Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Yu [49]				Cells transduced with MKP-1 exhibited reduced bone resorption after LPS stimulation compared with LacZ or HEPES control.	There were no significant inflammatory cells and few multinucleated osteoclasts on the alveolar bone surface in the periodontal tissues injected with PBS. In contrast, there were significantly more inflammatory cells more fibroblasts and more multinucleated osteoclasts in the periodontal tissues injected with LPS. Immunohistological staining revealed that MKP-1 was present in the periodontal tissues of rats injected with MKP-1 but undetectable in control groups of rats.
Zhang [50]	The maximum concentration of BMP7 in the culture media was detected after 6–9 days incubation and then followed by a moderate decline.	Significant differences in expression levels of OPN and BSP when HPLCs were cultured in BMP7 scaffolds.			The new bone formation of Group 2 was significantly greater than other groups at 4 and 8weeks. BMP7 group significantly increased the percentage bone defect fill in the defects compared to other groups.
Zhang [51]	HPLCs incubated in Group 3 produced higher level PDGF-B and produced higher level BMP7 in Group 2 during the entire culture period. There was no significant difference in the production of PDGF between groups 3 and 4. Similar results were noted in BMP7 secreted by Group 2 and Group 4.	Osteopontin and Type I collagen values of the PDGF-B expressing scaffolds were significantly greater than that of the control. The significant differences were observed in the mRNA expression levels of osteopontin, bone sialoprotein and Type I collagen when the HPLCs were cultured in combination scaffolds compared with BMP-7 or PDGF-B expressing scaffolds.			The new bone formation of the BMP-7 expressing scaffolds and the combination were significantly greater than that of the control at 4 and 8 weeks.
Zhang [52]	by 7 days, over fourfold significant increases in PDGF-B and BMP7 was observed. The addition of adPDGF-B significantly increased cell recruitment approximately eight times more than control scaffolds and over six times higher than BMP7 scaffolds.	In all scaffolds containing BMP7 or PDGF-B+BMP7, mRNA levels of each gene was significantly increased. The scaffolds containing adPDGF-B alone was only able to significantly upregulate mRNA levels of COL1.		Control defects demonstrated little tissue formation with regeneration of periodontal tissues. Defects filled with scaffolds alone regenerated little new periodontal tissues. Compared to scaffolds/PDGF-B. In contrast, scaffolds containing BMP7 demonstrated greater new bone formation. Scaffolds with PDGF-B and BMP7 demonstrated qualitative features similar to those of native periodontal structures.	Control defects demonstrated little tissue formation with regeneration of periodontal tissues below 20% for cementum, alveolar bone and PDL. Defects filled with scaffolds alone regenerated less new periodontal tissues. Scaffolds containing PDGF-B demonstrated new formation of PDL. In contrast, scaffolds containing BMP7 demonstrated greater new bone formation. Scaffolds with PDGF-B and BMP7 demonstrated qualitative features similar to those of native periodontal structures.

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Table 3: Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Zhao [53]		OPN and OCN from BMP-2-transduced MSCs showed only a slight increase relative to GFP-transduced MSCs. At 9 days of culture, OPN dramatically increased in BMP-2-transduced MSCs compared with GFP-transduced MSCs.		Radiopacities at the defect sites inβ-TCP alone group, untreated MSCs/β-TCP group and GFP-transduced MSCs/β-TCP group. µCT showed that bone formation was less for defects filled with untreated MSCs/β-TCP and GFP-transduced MSCs /β-TCP but still advanced when compared with the implantation of β-TCP alone. Substantial new bone formation was observed after 8 weeks in the critical size defects which received BMP-2-transduced MSCs/β-TCP construct.	mall amount of irregularly arranged woven bone tissue at the centre pores of β-TCP scaffold and fibrous connective tissue was still frequently observed. In the defects filled with implantation of BMP-2-transduced MSCs /β-TCP construct, mature newly formed bone tissue with few fibrous connective tissues infiltration was observed in the β-TCP pores at both centre and marginal area. Bone marrow also largely formed accompanied with the bony ingrowth.
Zhao [54]				BMD and BVF were significantly increased in the OPG transfection group compared to the control and mock groups.	The amount of ERR in the three groups was minimal and no significant differences among the three groups at the first two time points. By the last day of orthodontic tooth movement, the volume of ERR in all three groups was significantly increased. After 2 weeks of retention, the volume of ERR in all three groups was significantly decreased especially in OPG transfection group. In the control and mock groups, there was significantly more ERR by the last day of retention. Immunohistochemistry showed that OPG protein expression was facilitated in the periodontium when was injected in the OPG transfection group.
Zhou [55]					After 6 weeks, the height of new alveolar bone and cementum and the formation of new connective tissue were significantly greater in the experimental group than in the control groups.
Zhou [56]				New bone formation was observed in the defect. The height of the newly formed bone was more than that of the original bone crest and there was close fusion between the old and new bone. In the cell control group and scaffold control group, the height of the newly formed bone was not as good as that in the experimental group. In the negative control, there was virtually no new bone formation.	Immunohistochemistry showed that the expression of OPG protein in the BMSCs OPG group was higher than that in the control group. Significantly more tissue regeneration for the scaffolds with BMSCs OPG was noted compared with the other groups.

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Table 3: Endpoint results of the main analytical methods used for the experiments

Author	Endpoint results of the main analytical methods				
	ELISA	PCR	Bioluminescence	Radiograph (plain or µCT)	Histology/Immunohistochemistry
Zou [57]		HIF-1a mRNA and protein expression was upregulated in the target gene groups compared with the control group.		Scaffolds implanted in the correct position and tightly contacted the implant. In the HIF-1a expressing groups, new bone formation and osseointegration were superior to the GFP, CMPC and blank groups as measured by bone density and the bone contact ratio of dental implants. µCT showed that the new bone formation in the HIF and cHIF groups was greater than that in the other groups at 12 weeks.	Higher in HIF group than CMPC group, the blank group or the GFP group but less than the percentage in the cHIF group. BIC in each target gene groups was significantly higher than the control groups and no significant difference was observed between the CMPC group and the blank group. There were significant differences in bone density between the cHIF or HIF group and each control group but no significant difference was seen among the three control groups.

Table 4: Categories and grading used to assess the quality of the selected studies

Item	Description	Grade
1	**Title**	0 = inaccurate/not concise 1 = accurate and concise
2	**Abstract** Summary of the background, research objectives, including details of the species or strain of animal used, key methods, principal findings and conclusions of the study	0 = clearly inaccurate 1 = possibly accurate 2 = clearly accurate
3	**Introduction** Background-objectives, experimental approach and rationale, relevance to human biology	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
4	**Introduction** Objectives-primary and secondary	0 = not clear 1 = clear
5	**Methods** Ethical statement-nature of the review permission, relevant licenses, national and institutional guidelines for the care and use of animals	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
6	**Methods** Study design-number of experimental and control groups, any steps taken to minimize bias (i.e., allocation concealment, randomization, blinding)	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
7	**Methods** Experimental procedure-precise details (i.e., how, when, where, why)	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
8	**Methods** Experimental animals-species, strain, sex, developmental stage, weight, source of animals	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
9	**Methods** Housing and husbandry-conditions and welfare-related assessment interventions (i.e., type of cage, bedding material, number of cage companions, light/dark cycle, temperature, access to food and water)	0 = clearly insufficient 1 = possibly sufficient 2 = clearly sufficient
10	**Methods** Sample size-total number of animals used in each experimental group, details of calculation methods	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate
11	**Methods** Allocation of animals to experimental groups-randomization or matching, order in which animals were treated or assessed	0 = no 1 = yes
12	**Methods** Experimental outcomes-definition of primary and secondary outcomes	0 = no 1 = unclear/not complete 2 = yes
13	**Methods** Statistical methods-details and unit of analysis	0 = no 1 = unclear/not complete 2 = yes
14	**Results** Baseline data characteristics and health status of animals	0 = no 1 = yes
15	**Results** Number analysed-absolute numbers in each group included in each analysis, explanation for exclusion	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate
16	**Results** Outcomes and estimation-results for each analysis with a measure of precision, as standard error or confidence interval	0 = no 1 = unclear/not complete 2 = yes
17	**Results** Adverse events-details and notifications for reduction	0 = no 1 = unclear/not complete 2 = yes
18	**Discussion** Interpretation/scientific implications-study limitations including animal model, implications for the 3Rs	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate
19	**Discussion** Generalizability/translation-relevance to human biology	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate
20	**Discussion** Funding-sources, role of the funders	0 = clearly inadequate 1 = possibly adequate 2 = clearly adequate

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Table 5: Quality assessment of articles included using ARRIVE guidelines

Reference	Items																					
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	T(36)	Score
Abramson[1]	1	0	2	1	0	2	0	1	0	2	1	0	0	0	0	1	0	0	0	2	14	0.38
Alden[2]	1	1	2	1	0	1	1	1	0	2	1	1	0	0	0	2	0	2	2	2	21	0.58
Ashinoff[3]	1	1	2	1	2	2	2	2	1	2	1	2	2	1	2	2	2	1	1	2	33	0.91
Chang[4]	1	2	2	1	2	2	2	2	0	2	1	2	2	1	2	1	2	2	2	2	34	0.94
Chang[5]	1	2	2	1	2	2	2	1	0	2	1	2	2	1	1	1	0	1	1	2	28	0.77
Chang[6]	1	1	2	1	0	1	1	2	0	2	1	2	2	1	1	2	2	1	1	2	27	0.75
Chen[7]	1	1	2	1	2	1	1	1	0	2	1	2	2	1	2	1	2	1	1	2	28	0.77
Chen[8]	1	2	2	1	2	1	1	1	0	1	1	1	2	1	2	2	1	1	1	2	26	0.72
Chen[9]	1	2	1	1	2	2	2	2	1	1	1	1	0	1	1	2	1	1	1	2	26	0.72
Cirelli[10]	1	2	2	1	2	2	2	2	1	2	1	2	2	0	1	2	0	1	2	2	31	0.86
Cao[11]	1	2	1	1	2	2	2	2	0	2	1	1	2	0	2	2	1	1	1	2	28	0.77
Dai[12]	1	2	2	1	2	2	1	1	0	2	1	2	2	0	1	1	0	1	1	2	26	0.72
Dunn[13]	1	2	2	1	2	2	2	2	0	2	0	2	2	0	1	1	0	1	1	2	27	0.75
Hu[14]	1	1	2	1	2	2	2	2	0	2	1	2	2	1	1	2	2	1	1	2	31	0.86
Iglesias-Linares[15]	1	2	2	1	2	2	1	1	0	2	1	2	2	0	1	2	2	2	2	2	31	0.86
Jiang[16]	1	1	2	1	2	2	2	2	1	2	1	2	2	1	1	2	2	1	1	2	32	0.88
Jiang[17]	1	1	2	1	2	2	2	2	1	2	1	2	2	1	1	2	2	1	1	2	32	0.88
Jiang[18]	1	2	2	1	2	2	2	2	0	2	1	2	2	0	1	2	1	1	1	2	30	0.83
Jiang[19]	1	1	2	1	2	2	2	2	0	2	1	2	2	1	2	2	1	1	1	2	31	0.86
Jin[20]	1	2	2	1	2	2	2	1	0	2	1	2	2	0	1	2	1	1	1	2	29	0.80
Jin[21]	1	2	2	1	0	2	2	2	0	2	1	2	2	0	1	2	1	1	1	2	28	0.77
Kanzaki[22]	1	2	2	1	2	2	1	1	0	2	1	2	2	1	1	2	2	2	2	2	32	0.88
Kroczek[23]	1	2	2	1	2	1	2	2	0	2	0	2	2	1	1	2	1	1	1	2	29	0.80
kuboki[24]	1	1	2	1	2	2	2	1	0	2	1	2	0	0	1	1	2	1	1	2	26	0.72
Lai[25]	1	1	2	1	2	2	2	1	0	2	1	2	2	1	1	2	2	1	1	2	30	0.83
Lai[26]	1	1	2	1	2	2	2	2	0	2	1	2	2	1	1	2	2	1	1	2	31	0.86
Lattanzi[27]	1	1	2	1	2	2	2	1	0	2	1	2	0	1	2	2	1	1	1	2	28	0.77
Li[28]	1	1	2	1	2	2	1	1	0	2	1	2	2	0	1	2	1	1	1	2	27	0.75
Li[29]	1	1	2	1	2	2	1	2	0	2	1	2	2	0	1	2	1	1	1	2	28	0.77
Li[30]	1	2	2	1	1	2	1	1	0	1	1	1	2	0	0	1	1	1	1	2	22	0.61
Long[31]	1	1	2	1	2	2	2	2	0	2	1	2	2	0	1	2	2	1	1	2	30	0.83
Park J[32]	1	2	2	1	0	2	2	1	1	2	1	2	0	0	1	1	1	1	1	2	25	0.69
Park S[33]	1	2	2	1	2	2	1	1	0	2	1	2	2	0	1	2	1	2	1	2	29	0.80
Rabie[34]	1	2	2	1	2	1	1	1	0	2	1	2	2	0	1	2	1	1	1	2	27	0.75
Steinhardt[35]	1	2	2	1	2	1	2	1	0	0	0	2	2	0	1	2	1	1	2	2	25	0.69
Su[36]	1	1	2	1	2	2	2	2	1	2	1	2	2	0	1	1	1	1	1	0	27	0.75
Sun[37]	1	1	2	1	2	2	2	2	1	2	1	2	2	1	1	2	2	1	1	2	32	0.88
Sun[38]	1	1	2	1	2	2	2	1	1	2	1	2	2	1	1	1	1	1	2	0	28	0.77
Sun[39]	1	2	2	1	2	2	1	2	0	2	1	2	2	0	1	2	2	1	1	2	30	0.83
Sun[40]	1	2	0	0	0	1	1	2	0	2	0	1	2	0	0	2	1	1	0	2	18	0.5
Tan[41]	1	2	2	1	2	1	1	2	1	2	1	2	2	1	1	2	2	1	2	2	32	0.88
Tang[42]	1	1	2	1	2	2	2	2	1	2	1	2	2	0	1	1	2	1	1	2	30	0.83
Tang[43]	1	1	2	1	2	1	1	2	0	2	1	2	0	0	1	2	1	1	1	2	25	0.69
Wang[44]	1	2	2	1	2	2	2	2	0	2	1	2	2	1	1	1	1	1	1	2	30	0.83
Wei[45]	1	1	2	1	2	2	1	2	0	2	1	2	2	0	1	2	1	1	1	2	28	0.77
Wen[46]	1	1	2	1	2	2	2	1	1	2	1	2	2	0	1	2	1	1	1	2	29	0.80
Yang[47]	1	1	2	1	2	2	2	1	0	2	1	2	2	0	1	1	1	1	1	2	27	0.75
Ye[48]	1	1	2	1	2	2	2	2	1	2	1	2	2	0	1	2	1	1	1	2	30	0.83
Yu[49]	1	1	2	1	2	2	1	1	1	2	1	2	2	0	1	1	1	1	1	2	27	0.75
Zhang[50]	1	0	2	1	2	1	1	1	0	2	1	2	2	1	1	2	1	1	1	2	26	0.72
Zhang[51]	1	0	2	1	2	1	1	1	0	2	1	2	2	1	1	1	1	1	1	2	25	0.69
Zhang[52]	1	1	2	1	2	2	2	2	1	2	1	2	2	0	1	1	1	1	1	2	28	0.77
Zhao[53]	1	1	2	1	2	2	2	2	0	2	1	2	2	0	1	2	1	1	1	2	29	0.80
Zhao[54]	1	1	2	1	2	2	1	2	1	2	0	2	2	1	1	2	2	1	2	2	31	0.86
Zhou[55]	1	1	2	1	2	2	2	1	0	2	1	2	2	0	1	2	1	1	1	2	28	0.77
Zhou[56]	1	1	2	1	2	2	1	2	0	2	1	2	2	1	2	2	1	1	1	0	27	0.75
Zou[57]	1	2	2	1	2	2	2	2	0	2	1	2	2	0	1	2	1	1	1	2	30	0.83
Category score (quality obtained)	57	76	108	56	100	100	89	88	16	110	52	105	100	25	61	96	68	60	63	110		
Max category score (quality expected)	57	114	114	57	114	114	114	114	114	114	57	114	114	57	114	114	114	114	114	114		

T: Total score for all the 20 items (36 points), Score: score of the items/the total score.

Supplementary Table 1: Excluded articles with reason

AUTHOR	ARTICLE	REASON FOR EXCLUSION
Aghaloo	Aghaloo, T., Jiang, X., Soo, C., Zhang, Z., Zhang, X., Hu, J., . . . Zhang, X. (2007). A study of the role of nell-1 gene modified goat bone marrow stromal cells in promoting new bone formation. Mol Ther, 15(10), 1872-1880.	Ectopic bone
Aslan	Aslan, H., Zilberman, Y., Arbeli, V., Sheyn, D., Matan, Y., Liebergall, M., Gazit, Z. (2006). Nucleofection-based ex vivo nonviral gene delivery to human stem cells as a platform for tissue regeneration. Tissue Eng, 12(4), 877-889.	Ectopic bone
Baum	Baum, B. J., Goldsmith, C. M., Hoque, A. T., Wellner, R. B., Baccaglini, L., Ding, C., O'Connell, B. C. (2000). Salivary glands as a model for craniofacial applications of gene transfer. Int J Oral Maxillofac Surg, 29(3), 163-166.	Review
Blessmann	Blessmann, M., Al-Dam, A., Hanken, H., Assaf, A. T., Riecke, B., Klatt, J., Grobe, A. (2013). Amplification of the PPFIA1 gene region on 11q13 in oral squamous cell carcinomas (OSCC). J Craniomaxillofac Surg, 41(8), 845-849.	Cancer
Breitbart	Breitbart, A. S., Grande, D. A., Mason, J. M., Barcia, M., James, T., & Grant, R. T. (1999). Gene-enhanced tissue engineering: applications for bone healing using cultured periosteal cells transduced retrovirally with the BMP-7 gene. Ann Plast Surg, 42(5), 488-495.	Bone other than OMF
Cai	Cai, J., Zhang, Y., Liu, P., Chen, S., Wu, X., Sun, Y., Pei, D. (2013). Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells. Cell Regen (Lond), 2(1), 6.	Ectopic bone
Chan	Chan, K. K., Glenny, A. M., Weldon, J. C., Furness, S., Worthington, H. V., & Wakeford, H. (2015). Interventions for the treatment of oral and oropharyngeal cancers: targeted therapy and immunotherapy. Cochrane Database Syst Rev, 12, CD010341.	Cancer
Chang	Chang, S. C., Wei, F. C., Chuang, H., Chen, Y. R., Chen, J. K., Lee, K. C., Lou, J. (2003). Ex vivo gene therapy in autologous critical-size craniofacial bone regeneration. Plast Reconstr Surg, 112(7), 1841-1850.	Bone other than OMF
Chen	Chen, L., & Hu, G. F. (2010). Angiogenin-mediated ribosomal RNA transcription as a molecular target for treatment of head and neck squamous cell carcinoma. Oral Oncol, 46(9), 648-653.	Review
Chen	Chen, R., Chiba, M., Mori, S., Fukumoto, M., & Kodama, T. (2009). Periodontal gene transfer by ultrasound and nano/microbubbles. J Dent Res, 88(11), 1008-1013.	Review
Chuang	Chuang, C. K., Sung, L. Y., Hwang, S. M., Lo, W. H., Chen, H. C., & Hu, Y. C. (2007). Baculovirus as a new gene delivery vector for stem cell engineering and bone tissue engineering. Gene Ther, 14(19), 1417-1424.	Ectopic bone
Dai	Dai, J., & Rabie, A. B. (2007). Recombinant adeno-associated virus vector hybrids efficiently target different skeletal cells. Front Biosci, 12, 4280-4287.	In vitro studies
Dai	Dai, J., & Rabie, A. B. (2007). VEGF: an essential mediator of both angiogenesis and endochondral ossification. J Dent Res, 86(10), 937-950.	Review
Dai	Dai, J., Rabie, A. B., Hagg, U., & Xu, R. (2004). Alternative gene therapy strategies for the repair of craniofacial bone defects. Curr Gene Ther, 4(4), 469-485.	Review
Dai	Dai, J., Wang, X., & Shen, G. (2011). Cotransplantation of autologous bone marrow stromal cells and chondrocytes as a novel therapy for reconstruction of condylar cartilage. Med Hypotheses, 77(1), 132-133. doi: 10.1016/j.mehy.2011.03.045	Review
Dehari	Dehari, H., Ito, Y., Nakamura, T., Kobune, M., Sasaki, K., Yonekura, N., Hamada, H. (2003). Enhanced antitumor effect of RGD fiber-modified adenovirus for gene therapy of oral cancer. Cancer Gene Ther, 10(1), 75-85.	In vitro studies
Du	Du, J., Zhou, L., Chen, X., Yan, S., Ke, M., Lu, X., . . . Xiang, A. P. (2012). IFN-gamma-primed human bone marrow mesenchymal stem cells induce tumor cell apoptosis in vitro via tumor necrosis factor-related apoptosis-inducing ligand. Int J Biochem Cell Biol, 44(8), 1305-1314.	Ectopic bone
Evans	Evans, C. (2014). Using genes to facilitate the endogenous repair and regeneration of orthopaedic tissues. Int Orthop, 38(9), 1761-1769.	Review
Fan	Fan, Y. X., Gu, C. H., Zhang, Y. L., Zhong, B. S., Wang, L. Z., Zhou, Z. R., . . . Wang, F. (2013). Oct4 and Sox2 overexpression improves the proliferation and differentiation of bone mesenchymal stem cells in Xiaomeishan porcine. Genet Mol Res, 12(4), 6067-6079.	In vitro studies
Fang	Fang, L., Hu, Q., Hua, Z., Li, S., & Dong, W. (2008). Growth inhibition of a tongue squamous cell carcinoma cell line (Tca8113) in vitro and in vivo via siRNA-mediated down-regulation of skp2. Int J Oral Maxillofac Surg, 37(9), 847-852.	Ectopic bone
Ferreira	Ferreira, J. R., Hirsch, M. L., Zhang, L., Park, Y., Samulski, R. J., Hu, W. S., & Ko, C. C. (2013). Three-dimensional multipotent progenitor cell aggregates for expansion, osteogenic differentiation and 'in vivo' tracing with AAV vector serotype 6. Gene Ther, 20(2), 158-168.	Bone other than OMF
Fujioka	Fujioka, K., Kishida, T., Ejima, A., Yamamoto, K., Fujii, W., Murakami, K., Mazda, O. (2015). Inhibition of osteoclastogenesis by osteoblast-like cells genetically engineered to produce interleukin-10. Biochem Biophys Res Commun, 456(3), 785-791.	Ectopic bone
Gao	Gao, Q., Tong, W., Luria, J. S., Wang, Z., Nussenbaum, B., & Krebsbach, P. H. (2010). Effects of bone morphogenetic protein-2 on proliferation and angiogenesis in oral squamous cell carcinoma. Int J Oral Maxillofac Surg, 39(3), 266-271.	Ectopic bone
Gao	Gao, X., Usas, A., Tang, Y., Lu, A., Tan, J., Schneppendahl, J., Huard, J. (2014). A comparison of bone regeneration with human mesenchymal stem cells and muscle-derived stem cells and the critical role of BMP. Biomaterials, 35(25), 6859-6870.	Bone other than OMF
Ge	Ge, W., Shi, L., Zhou, Y., Liu, Y., Ma, G. E., Jiang, Y., Feng, H. (2011). Inhibition of osteogenic differentiation of human adipose-derived stromal cells by retinoblastoma binding protein 2 repression of RUNX2-activated transcription. Stem Cells, 29(7), 1112-1125.	Ectopic bone

Supplementary Table 1: Excluded articles with reason

AUTHOR	ARTICLE	REASON FOR EXCLUSION
Harada	Harada, K., Supriatno, Kawaguchi, S., Onoue, T., Kawashima, Y., Yoshida, H., & Sato, M. (2005). High antitumor activity using intratumoral injection of plasmid DNA with mutant-type p27Kip1 gene following in vivo electroporation. Oncol Rep, 13(2), 201-206.	Cancer
Hattori	Hattori, H., Mizutani, H., & Ueda, M. (2002). [Sonic hedgehog]. Clin Calcium, 12(2), 233-237.	Review
Heikinheimo	Heikinheimo, K., Kurppa, K. J., & Elenius, K. (2015). Novel targets for the treatment of ameloblastoma. J Dent Res, 94(2), 237-240.	Review
Helmrich	Helmrich, U., Di Maggio, N., Guven, S., Groppa, E., Melly, L., Largo, R. D., Banfi, A. (2013). Osteogenic graft vascularization and bone resorption by VEGF-expressing human mesenchymal progenitors. Biomaterials, 34(21), 5025-5035.	Ectopic bone
Herberg	Herberg, S., Kondrikova, G., Hussein, K. A., Johnson, M. H., Elsalanty, M. E., Shi, X., Hill, W. D. (2015). Mesenchymal stem cell expression of stromal cell-derived factor-1beta augments bone formation in a model of local regenerative therapy. J Orthop Res, 33(2), 174-184.	Bone other than OMF
Hino	Hino, S., Kawamata, H., Omotehara, F., Uchida, D., Miwa, Y., Begum, N. M., Fujimori, T. (2002). Cytoplasmic TSC-22 (transforming growth factor-beta-stimulated clone-22) markedly enhances the radiation sensitivity of salivary gland cancer cells. Biochem Biophys Res Commun, 292(4), 957-963.	In vitro studies
Huang,	Huang, C., Tang, M., Yehling, E., & Zhang, X. (2014). Overexpressing sonic hedgehog peptide restores periosteal bone formation in a murine bone allograft transplantation model. Mol Ther, 22(2), 430-439.	Bone other than OMF
Huang	Huang, G., Zheng, Q., Sun, J., Guo, C., Yang, J., Chen, R., . . . Wang, J. (2008). Stabilization of cellular properties and differentiation mutilpotential of human mesenchymal stem cells transduced with hTERT gene in a long-term culture. J Cell Biochem, 103(4), 1256-1269.	Ectopic bone
Kim	Kim, N. H., Cha, Y. H., Kim, H. S., Lee, S. E., Huh, J. K., Kim, J. K., Yook, J. I. (2014). A platform technique for growth factor delivery with novel mode of action. Biomaterials, 35(37), 9888-9896.	Bone other than OMF
Kitano	Kitano, H., Mamiya, A., Kokubun, S., & Hidai, C. (2012). Efficient nonviral gene therapy with FasL and Del1 fragments in mice. J Gene Med, 14(11), 642-650.	Ectopic bone
Koc	Koc, A., Finkenzeller, G., Elcin, A. E., Stark, G. B., & Elcin, Y. M. (2014). Evaluation of adenoviral vascular endothelial growth factor-activated chitosan/hydroxyapatite scaffold for engineering vascularized bone tissue using human osteoblasts: In vitro and in vivo studies. J Biomater Appl, 29(5), 748-760.	Bone other than OMF
Kurihara	Kurihara, Y., Watanabe, Y., Onimatsu, H., Kojima, T., Shirota, T., Hatori, M., Fujiwara, T. (2009). Telomerase-specific virotheranostics for human head and neck cancer. Clin Cancer Res, 15(7), 2335-2343.	Cancer
Kyrkanides	Kyrkanides, S., Kambylafkas, P., Miller, J. H., Tallents, R. H., & Puzas, J. E. (2007). The cranial base in craniofacial development: a gene therapy study. J Dent Res, 86(10), 956-961.	In vitro studies
Laurencin	Laurencin, C. T., Attawia, M. A., Lu, L. Q., Borden, M. D., Lu, H. H., Gorum, W. J., & Lieberman, J. R. (2001). Poly(lactide-co-glycolide)/hydroxyapatite delivery of BMP-2-producing cells: a regional gene therapy approach to bone regeneration. Biomaterials, 22(11), 1271-1277.	Bone other than OMF
Lee	Lee, S. Y., Park, H. R., Rhee, J., Park, Y. M., & Kim, S. H. (2013). Therapeutic effect of oncolytic adenovirus expressing relaxin in radioresistant oral squamous cell carcinoma. Oncol Res, 20(9), 419-425.	In vitro studies
Li	Li, C., Shi, F., Yang, D., Wang, J., Jian, X., & Jiang, C. (2012). [Natural killer and cytotoxic T lymphocyte-mediated cytotoxicity enhanced by genetic overexpression of MHC class I chain-related protein A in oral squamous cell carcinoma: an experimental study in vivo]. Hua Xi Kou Qiang Yi Xue Za Zhi, 30(1), 32-35.	In vitro studies
Li	Li, M., Li, Z., Li, J., Jin, L., Jin, C., Han, C., Sun, F. (2015). Enhanced antitumor effect of cisplatin in human oral squamous cell carcinoma cells by tumor suppressor GRIM19. Mol Med Rep, 12(6), 8185-8192. doi: 10.3892/mmr.2015.4423	Cancer
Li,	Li, S., Yang, X., Wang, P., & Ran, X. (2013). The effects of GLUT1 on the survival of head and neck squamous cell carcinoma. Cell Physiol Biochem, 32(3), 624-634.	Ectopic bone
Li	Li, Y., Li, L. J., Wang, L. J., Zhang, Z., Gao, N., Liang, C. Y., . . . Han, B. (2014). Selective intra-arterial infusion of rAd-p53 with chemotherapy for advanced oral cancer: a randomized clinical trial. BMC Med, 12, 16.	Cancer
Li	Li, Y., Tian, W., & Wang, D. (2003). [An experimental study on gene transfection of human interleukin-1 receptor antagonist gene into chondrocytes of temporomandibular joint]. Hua Xi Kou Qiang Yi Xue Za Zhi, 21(1), 19-21.	In vitro studies
Liang	Liang, Q. X., Liang, Y. C., Xu, Z. Y., Chen, W. L., Xie, H. L., & Zhang, B. (2014). RECK overexpression reduces invasive ability in ameloblastoma cells. J Oral Pathol Med, 43(8), 613-618.	In vitro studies
Liu	Liu, J., Xu, L., Li, Y., & Ma, J. (2011). Temporally controlled multiple-gene delivery in scaffolds: A promising strategy to enhance bone regeneration. Med Hypotheses, 76(2), 173-175.	Review
Liu	Liu, S., Chen, P., Hu, M., Tao, Y., Chen, L., Liu, H., . . . Gao, G. (2013). Randomized, controlled phase II study of post-surgery radiotherapy combined with recombinant adenoviral human p53 gene therapy in treatment of oral cancer. Cancer Gene Ther, 20(6), 375-378.	Cancer
Liu	Liu, X., Huang, H., Wang, J., Wang, C., Wang, M., Zhang, B., & Pan, C. (2011). Dendrimers-delivered short hairpin RNA targeting hTERT inhibits oral cancer cell growth in vitro and in vivo. Biochem Pharmacol, 82(1), 17-23.	Ectopic bone

Supplementary Table 1: Excluded articles with reason

AUTHOR	ARTICLE	REASON FOR EXCLUSION
Lutz	Lutz, R., Park, J., Felszeghy, E., Wiltfang, J., Nkenke, E., & Schlegel, K. A. (2008). Bone regeneration after topical BMP-2-gene delivery in circumferential peri-implant bone defects. Clin Oral Implants Res, 19(6), 590-599.	Bone other than OMF
Ma	Ma, D., & Mao, T. (2012). [Cell-based approaches to promote bone regeneration in distraction osteogenesis]. Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi, 26(12), 1512-1515.	No gene therapy
Matsumoto	Matsumoto, G., Kushibiki, T., Kinoshita, Y., Lee, U., Omi, Y., Kubota, E., & Tabata, Y. (2006). Cationized gelatin delivery of a plasmid DNA expressing small interference RNA for VEGF inhibits murine squamous cell carcinoma. Cancer Sci, 97(4), 313-321.	Ectopic bone
Matsumoto	Matsumoto, G., Ohmi, Y., & Shindo, J. (2001). Angiostatin gene therapy inhibits the growth of murine squamous cell carcinoma in vivo. Oral Oncol, 37(4), 369-378.	Ectopic bone
Matsumoto	Matsumoto, G., Omi, Y., Lee, U., Kubota, E., & Tabata, Y. (2011). NK4 gene therapy combined with cisplatin inhibits tumour growth and metastasis of squamous cell carcinoma. Anticancer Res, 31(1), 105-111.	Ectopic bone
Matsumoto	Matsumoto, G., Sasakuri, K., Tsukinoki, K., Ohmi, Y., Lee, U., & Shindo, J. (2002). Growth of human squamous cell carcinoma xenografts in mice is inhibited by local angiostatin gene therapy. Oral Oncol, 38(6), 543-548.	Ectopic bone
Matsumoto	Matsumoto, G., Yajima, N., Saito, H., Nakagami, H., Omi, Y., Lee, U., & Kaneda, Y. (2010). Cold shock domain protein A (CSDA) overexpression inhibits tumor growth and lymph node metastasis in a mouse model of squamous cell carcinoma. Clin Exp Metastasis, 27(7), 539-547.	Ectopic bone
Meshii	Meshii, N., Takahashi, G., Okunaga, S., Hamada, M., Iwai, S., Takasu, A., Yura, Y. (2013). Enhancement of systemic tumor immunity for squamous cell carcinoma cells by an oncolytic herpes simplex virus. Cancer Gene Ther, 20(9), 493-498.	Cancer
Mizuno	Mizuno, H., Emi, N., Abe, A., Takahashi, I., Kojima, T., Saito, H., . . . Ueda, M. (1999). Successful culture and sustainability in vivo of gene-modified human oral mucosal epithelium. Hum Gene Ther, 10(5), 825-830.	Ectopic bone
Musgrave	Musgrave, D. S., Bosch, P., Lee, J. Y., Pelinkovic, D., Ghivizzani, S. C., Whalen, J., . . . Huard, J. (2000). Ex vivo gene therapy to produce bone using different cell types. Clin Orthop Relat Res(378), 290-305.	Ectopic bone
Naito	Naito, S., Obayashi, S., Sumi, T., Iwai, S., Nakazawa, M., Ikuta, K., & Yura, Y. (2006). Enhancement of antitumor activity of herpes simplex virus gamma(1)34.5-deficient mutant for oral squamous cell carcinoma cells by hexamethylene bisacetamide. Cancer Gene Ther, 13(8), 780-791.	Cancer
Nakase	Nakase, M., Inui, M., Okumura, K., Kamei, T., Nakamura, S., & Tagawa, T. (2005). p53 gene therapy of human osteosarcoma using a transferrin-modified cationic liposome. Mol Cancer Ther, 4(4), 625-631.	Ectopic bone
Nishikawa	Nishikawa, M., Hayashi, Y., Yamamoto, N., Fukui, T., Fukuhara, H., Mitsudo, K., Yoshida, J. (2003). Cell death of human oral squamous cell carcinoma cell line induced by herpes simplex virus thymidine kinase gene and ganciclovir. Nagoya J Med Sci, 66(3-4), 129-137.	Ectopic bone
Okumura	Okumura, K., Nakase, M., Nakamura, S., Kamei, T., Inui, M., & Tagawa, T. (2007). Bax gene therapy for human osteosarcoma using cationic liposomes in vivo. Oncol Rep, 17(4), 769-773.	Ectopic bone
O'Malley	O'Malley, B. W., Jr., Li, D., Buckner, A., Duan, L., Woo, S. L., & Pardoll, D. M. (1999). Limitations of adenovirus-mediated interleukin-2 gene therapy for oral cancer. Laryngoscope, 109(3), 389-395.	Cancer
Omotehara	Omotehara, F., Uchida, D., Hino, S., Begum, N. M., Yoshida, H., Sato, M., & Kawamata, H. (2000). In vivo enhancement of chemosensitivity of human salivary gland cancer cells by overexpression of TGF-beta stimulated clone-22. Oncol Rep, 7(4), 737-740.	Cancer
Otani	Otani, K., Yamahara, K., Ohnishi, S., Obata, H., Kitamura, S., & Nagaya, N. (2009). Nonviral delivery of siRNA into mesenchymal stem cells by a combination of ultrasound and microbubbles. J Control Release, 133(2), 146-153.	In vitro studies
Pan	Pan, C. B., Huang, H. Z., Wang, J. G., Hou, J. S., & Li, H. G. (2004). [The inhibitory effect of human endostatin gene on tumor growth of tongue squamous cell carcinoma]. Zhonghua Kou Qiang Yi Xue Za Zhi, 39(4), 273-276.	Cancer
Park	Park, J., Lutz, R., Felszeghy, E., Wiltfang, J., Nkenke, E., Neukam, F. W., & Schlegel, K. A. (2007). The effect on bone regeneration of a liposomal vector to deliver BMP-2 gene to bone grafts in peri-implant bone defects. Biomaterials, 28(17), 2772-2782.	Bone other than OMF
Ren	Ren, M. L., Peng, W., Yang, Z. L., Sun, X. J., Zhang, S. C., Wang, Z. G., & Zhang, B. (2012). Allogeneic adipose-derived stem cells with low immunogenicity constructing tissue-engineered bone for repairing bone defects in pigs. Cell Transplant, 21(12), 2711-2721.	Bone other than OMF
Sato	Sato, D., Kurihara, Y., Kondo, S., Shirota, T., Urata, Y., Fujiwara, T., & Shintani, S. (2013). Antitumor effects of telomerase-specific replication-selective oncolytic viruses for adenoid cystic carcinoma cell lines. Oncol Rep, 30(6), 2659-2664.	Cancer
Sun	Sun, C. X., He, R. G., Cheung, L. K., Zhang, Z. Y., Chen, W. T., Liu, X. K., Chen, S. S. (2002). The biological behaviour of human adenoid cystic carcinoma cells transduced with interleukin-2-gene. Int J Oral Maxillofac Surg, 31(6), 650-656.	Cancer
Takaoka	Takaoka, H., Takahashi, G., Ogawa, F., Imai, T., Iwai, S., & Yura, Y. (2011). A novel fusogenic herpes simplex virus for oncolytic virotherapy of squamous cell carcinoma. Virol J, 8, 294.	Cancer
Talwar	Talwar, R., Di Silvio, L., Hughes, F. J., & King, G. N. (2001). Effects of carrier release kinetics on bone morphogenetic protein-2-induced periodontal regeneration in vivo. J Clin Periodontol, 28(4), 340-347.	Bone other than OMF

Supplementary Table 1: Excluded articles with reason

AUTHOR	ARTICLE	REASON FOR EXCLUSION
Tsuda	Tsuda, H., Wada, T., Ito, Y., Uchida, H., Dehari, H., Nakamura, K., Hamada, H. (2003). Efficient BMP2 gene transfer and bone formation of mesenchymal stem cells by a fiber-mutant adenoviral vector. Mol Ther, 7(3), 354-365.	Ectopic bone
Tsuda	Tsuda, H., Wada, T., Yamashita, T., & Hamada, H. (2005). Enhanced osteoinduction by mesenchymal stem cells transfected with a fiber-mutant adenoviral BMP2 gene. J Gene Med, 7(10), 1322-1334.	Ectopic bone
Van Damme	Van Damme, A., Thorrez, L., Ma, L., Vandenburgh, H., Eyckmans, J., Dell'Accio, F., Chuah, M. K. (2006). Efficient lentiviral transduction and improved engraftment of human bone marrow mesenchymal cells. Stem Cells, 24(4), 896-907.	Ectopic bone
Wang	Wang, A., Huang, H., & Li, S. (2003). Therapeutic effect of AdCMVCD/5-FC system and metabolism of 5-FC in the treatment of human tongue squamous cell carcinoma. Chin Med J (Engl), 116(2), 248-252.	Cancer
Westberg	Westberg, S., Sadeghi, A., Svensson, E., Segall, T., Dimopoulou, M., Korsgren, O., . . . von Euler, H. (2013). Treatment efficacy and immune stimulation by AdCD40L gene therapy of spontaneous canine malignant melanoma. J Immunother, 36(6), 350-358.	Cancer
Xiao	Xiao, R., Yu, G., Jia, H., & Cai, Z. (2000). The expression kinetics of myogenin in facial muscle denervation. Chin J Dent Res, 3(1), 7-11.	Muscle
Xie	Xie, C., Xue, M., Wang, Q., Schwarz, E. M., O'Keefe, R. J., & Zhang, X. (2008). Tamoxifen-inducible CreER-mediated gene targeting in periosteum via bone-graft transplantation. J Bone Joint Surg Am, 90 Suppl 1, 9-13.	Bone other than OMF
Xu	Xu, J. H., Pan, C. B., Huang, H. Z., Zhang, B., Wang, J. G., & Zhang, L. T. (2007). [Silencing of survivin gene enhances chemosensitivity of human tongue cancer cell line Tca8113 to cisplatin]. Zhonghua Kou Qiang Yi Xue Za Zhi, 42(5), 280-283.	Cancer
Xu	Xu, Q., Liu, X., Cai, Y., Yu, Y., & Chen, W. (2010). RNAi-mediated ADAM9 gene silencing inhibits metastasis of adenoid cystic carcinoma cells. Tumour Biol, 31(3), 217-224.	Cancer
Yan	Yan, M. N., Dai, K. R., Tang, T. T., Zhu, Z. A., & Lou, J. R. (2010). Reconstruction of peri-implant bone defects using impacted bone allograft and BMP-2 gene-modified bone marrow stromal cells. J Biomed Mater Res A, 93(1), 304-313.	Bone other than OMF
Yang	Yang, L., Zhang, Y., Dong, R., Peng, L., Liu, X., Wang, Y., & Cheng, X. (2010). Effects of adenoviral-mediated coexpression of bone morphogenetic protein-7 and insulin-like growth factor-1 on human periodontal ligament cells. J Periodontal Res, 45(4), 532-540.	Ectopic bone
Yoo	Yoo, G. H., Moon, J., Leblanc, M., Lonardo, F., Urba, S., Kim, H., Wolf, G. (2009). A phase 2 trial of surgery with perioperative INGN 201 (Ad5CMV-p53) gene therapy followed by chemoradiotherapy for advanced, resectable squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and larynx: report of the Southwest Oncology Group. Arch Otolaryngol Head Neck Surg, 135(9), 869-874.	Cancer
Yu	Yu, D., Wang, A., Huang, H., & Chen, Y. (2008). PEG-PBLG nanoparticle-mediated HSV-TK/GCV gene therapy for oral squamous cell carcinoma. Nanomedicine (Lond), 3(6), 813-821.	Cancer
Yu	Yu, D. S., Huang, H. Z., Hu, X. W., Liu, X. Q., Tang, H. K., & Wang, A. X. (2006). [Radiation-inducible promoters-mediated cdglytk gene in the treatment of buccal carcinoma in golden hamster]. Zhonghua Kou Qiang Yi Xue Za Zhi, 41(9), 549-552.	Cancer
Zhang	Zhang, T., Hamada, K., Hyodo, M., Itoh, H., Tani, K., Goda, H., Hamakawa, H. (2011). Gene therapy for oral squamous cell carcinoma with IAI.3B promoter-driven oncolytic adenovirus-infected carrier cells. Oncol Rep, 25(3), 795-802.	Cancer
Zhou	Zhou, H., Fei, W., Bai, Y., Zhu, S., Luo, E., Chen, K., & Hu, J. (2012). RNA interference-mediated downregulation of hypoxia-inducible factor-1alpha inhibits angiogenesis and survival of oral squamous cell carcinoma in vitro and in vivo. Eur J Cancer Prev, 21(3), 289-299.	Cancer
Zhou	Zhou, X., Zhang, Z., Yang, X., Chen, W., & Zhang, P. (2009). Inhibition of cyclin D1 expression by cyclin D1 shRNAs in human oral squamous cell carcinoma cells is associated with increased cisplatin chemosensitivity. Int J Cancer, 124(2), 483-489.	Cancer
Zou	Zou, L., Luo, Y., Chen, M., Wang, G., Ding, M., Petersen, C. C., Bunger, C. (2013). A simple method for deriving functional MSCs and applied for osteogenesis in 3D scaffolds. Sci Rep, 3, 2243.	Ectopic bone

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Systematic Review
Clinical Pathology

Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review

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R. Fliefel, M. Tröltzsch, J. Kühnisch, M. Ehrenfeld, S. Otto: Treatment strategies and outcomes of bisphosphonate-related osteonecrosis of the jaw (BRONJ) with characterization of patients: a systematic review. Int. J. Oral Maxillofac. Surg. 2015; 44: 568–585.

Abstract. The aim of this systematic review was to answer the question: What are the treatments available for bisphosphonate-related osteonecrosis of the jaws (BRONJ) and their outcomes? A literature search of PubMed, Cochrane Library, and Web of Science databases was conducted in accordance with the PRISMA statement, search phrases were ('jaw osteonecrosis' OR 'bisphosphonate-related osteonecrosis' OR 'bisphosphonate osteonecrosis') AND ('treatment' OR 'outcomes'). Ninety-seven articles published between 2003 and February 2014 were reviewed. The studies reported 4879 cases of BRONJ. The mean age of the patients was 66.5 ± 4.7 years. The male to female ratio was 1:2. The mean duration of bisphosphonate (BP) administration was 38.2 ± 15.7 months. The quality of the publications was good, with some moderate and poor. Minimally invasive surgical treatment was the treatment most used. Medical treatment was also used. Adjunctive treatments included laser, growth factors, hyperbaric oxygen and ozone. The articles provided a broad range of outcome variables to assess the treatment of BRONJ and the outcomes of each treatment. Considerable heterogeneity was found regarding study design, sample size, and treatment modalities. Clinical trials with larger samples are required to provide sufficient information for each treatment modality to predict the outcomes of each treatment.

Key words: bisphosphonates BRONJ; management; outcomes risk factors; systematic review.

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Bisphosphonates (BPs) are a class of drugs used across a wide range of disciplines including endocrinology, oncology, orthopaedics, and dentistry.[1,2] They are commonly prescribed for bone diseases such as osteoporosis, Paget's disease of bone, hypercalcaemia of malignancy, osteolytic bone metastases, and osteolytic lesions of multiple myeloma.[1,3,4] Their use has resulted in a statistically significant reduction in skeletal complications, including pathological fractures, spinal cord compression, and hypercalcaemia of malignant disease, and has reduced the need for subsequent radiotherapy or surgery to bone.[5–7]

BPs are synthetic analogues of the naturally occurring pyrophosphate molecule. They are broadly classified on the basis of whether or not they contain a nitrogen atom, with nitrogen-containing bisphosphonates (N-BPs) being more potent than non-N-BPs.[8] They differ from one another in the substitution of the active side chains on their phosphorous–carbon phosphorous structural backbone.

The mechanism of action of BPs is the inhibition of bone resorption by suppressing osteoclast activation and inducing osteoclast apoptosis.[9,10] The efficacy of BPs has been established in several studies.[11–14] However, their use may have side effects.[15] Bisphosphonate-related osteonecrosis of the jaw (BRONJ) has been characterized as a main side effect of BP therapy.[16–18] The first descriptions of BRONJ were reported in 2003.[19–21] Since then, numerous reports on the development of osteonecrosis of the jaw in patients treated with BPs have been published.[22–34]

BRONJ lesions may remain silent until the occurrence of a triggering event, such as an invasive dental procedure, infection, or mechanical trauma to the jawbone, as well as the concomitant use of immunosuppressive and chemotherapy drugs.[35–37] According to a recent position paper from the American Association of Oral and Maxillofacial Surgeons (AAOMS), risk factors for the development of BRONJ can be grouped into drug-related, local, demographic and systematic, genetic, and preventive.[38] The clinical manifestations of BRONJ vary from necrotic bone exposure (ranging from a few millimetres in size to larger areas, which can be asymptomatic for weeks, months, or years[39]), simple swellings of the soft tissues, and abscesses, to more complex cases presenting with fistulas and diffuse pain.[40]

There are two major theories regarding the pathophysiology of BRONJ. One is the osteoclast-based, 'inside-out' theory, in which inhibition of osteoclastic activity and marked suppression of bone turnover, together with the spread of physiological micro-damage and possibly local infection, leads to bone death within the jaw, with subsequent exposure. As such, the bone exposure would be a late event. The second, 'outside-in' theory suggests a break in the oral mucosa leads to ingress of bacteria and local infection, which, coupled with poor bone remodelling, leads to bone death. BRONJ may result from a combination of these two mechanisms, and hypovascularity may also play an important role.[41,42] Although there have been reports relating no obvious co-morbidity factors, it is reasonable to believe that co-factors play a relevant role in the development of these lesions.[23,43]

The management of BRONJ has centred on efforts to eliminate or reduce the severity of symptoms, to slow or prevent the progression of disease, and to eradicate diseased bone.[44] There is currently no gold standard for the treatment of BRONJ. Several treatment options have been described in relation to the AAOMS staging of BRONJ.[45] No agreement on a surgical versus non-surgical approach to therapy has been reached in the treatment of BRONJ.[46–49] Some recommendations focus on prevention and a conservative approach.[19,25,27,50]

Treatment strategies include the administration of antibiotics, oral antibacterial mouth rinse, cessation of BPs if possible, pain control, surgical debridement or resection for long-term palliation of infection and pain,[23,50] sequential removal of sequestrum (extensive involvement may necessitate a large area of debridement to include a segmental mandibulectomy and partial maxillectomy[23]), mandibular reconstruction with the fibula flap,[51] and covering the exposed areas with tissue flaps.[19] Hyperbaric oxygen (HBO) therapy, fluorescence-guided bone resection, and low-intensity laser therapy have also been studied as therapeutic tools.[48,52–54]

Other treatment modalities that increase bone wound healing using growth and differentiation factors are being studied,[55,56] as well as transplantation of intra-lesional autologous bone marrow stem cells.[57] More recently, teriparatide (N-terminal 34-amino acid recombinant human parathyroid hormone) has been reported for the medical treatment of BRONJ.[58] Pentoxifylline and α-tocopherol in addition to antimicrobial therapy has been shown to decrease the area of bone exposure and symptoms in BRONJ patients.[59] The use of ozone in combination with antibiotics and surgery for patients with exposed bone lesions has also been the subject of a clinical investigation and found to resolve pain, secretions, and halitosis.[60]

The main objective of this study was to conduct a systematic review of the literature to determine the treatment strategies available for BRONJ, describing the outcome variables measured for each treatment modality and the success of the treatment expressed by the outcome.

Materials and methods

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were applied for this review.[61] PRISMA comprises a 27-item checklist and a four-phase flow diagram that relates to the title, abstract, introduction, methods, results, and discussion sections of articles, and funding. They were developed based on recommendations regarding what should be included in an accurate and complete report of systematic reviews and meta-analyses.

The systematic search covered the time period 2003 (the year of the initial description of BRONJ) to 28 February 2014. All publications identified in the literature search were retrieved from online journals and selected on the basis of the inclusion criteria.

Inclusion and exclusion criteria

The following inclusion criteria were applied: (1) academic publications; the review included any published studies (cross-sectional surveys, cohort, and case–control studies), clinical trials, case series, and retrospective studies; (2) publication in the English language confirming the diagnosis of BRONJ in accordance with the AAOMS or American Society of Bone and Mineral Research (ASBMR) definitions; (3) studies on humans; (4) participants of any age and gender with a clinical diagnosis of BRONJ; (5) any form of treatment; (6) outcomes variables should be mentioned in the publication; (7) outcome of the treatment.

The following exclusion criteria were applied: (1) single case reports of BRONJ; (2) experimental laboratory studies; (3) case series with fewer than five patients; (4) literature reviews, letters, editorials, doctoral theses, and abstracts.

Disease definition

The disease definition, as proposed by AAOMS and ASBMR, includes the persistence of exposed necrotic bone in the

oral cavity for 8 weeks, despite adequate treatment, in a patient with current or a previous history of BP use, without local evidence of malignancy, and no prior radiotherapy to the affected region.[24,40,62,63]

A clinical staging system has been proposed to classify patients with established BRONJ, with appropriate treatment for each stage (Table 1).[24,40,63,64]

Electronic database search

Three databases – PubMed, the Cochrane Library, and Web of Science – were electronically searched. The heading sequence ('jaw osteonecrosis' OR 'bisphosphonate-related osteonecrosis' OR 'bisphosphonate osteonecrosis') AND ('treatment' OR 'outcomes') was searched as text word. The results of the database searches were combined and duplicate articles were excluded. All references were gathered and screened for eligibility.

In the first round search, abstracts were reviewed and all articles containing the key words were retained. Articles that were not in English were excluded. Complete versions were then obtained for all articles that that met the inclusion criteria.

In the second round search and evaluation, a manual search was done of the reference lists of all the articles retained after the first round for appropriate studies relevant to the review topic. A search of the unpublished literature was not performed. Literature reviews and systematic reviews were also considered, with the objective of identifying cases already reported. All of the articles were read in full for final selection.

In the third round search, each of the publications was critically appraised for assessment of validity, and the following data were extracted from the accepted articles and recorded in a standardized spread sheet: reference and year, study design, number of patients in the study, mean age of patients, gender of patients, location of the lesions, primary cause of the BRONJ, types of BP used, route of administration of the BP used, range of duration of use of BP triggering factors, co-morbidities, treatment methods, outcome variables measured, follow-up period, and outcomes of the different treatments.

Statistical analysis

The duration of BP exposure was defined as the time in months from the date of first BP infusion to the last recorded infusion.

A qualitative data analysis was performed with the aim of summarizing the results of the studies included. The mean age of patients with osteonecrosis of the jaw and the ratio of male to female patients were calculated to determine whether any particular stratum had a greater predisposition to develop osteonecrosis of the jaw than another. The existence of potential risk factors for osteonecrosis of the jaw was examined: the mean dose and range, the duration of treatment, and the proportions of patients receiving immunosuppressant therapy (e.g., corticosteroids), with other comorbidities, and with a history of dental trauma, infection, or surgical procedures.

The quality of accepted publications was assessed based on a modification of the ASBMR,[65] by the reporting of 12 parameters for all patients diagnosed with BRONJ: age, sex, primary cause of the disease, name of the bisphosphonate used, duration or treatment, mode of administration, affected site, medical history (concomitant medications, comorbidities), triggering factors, treatment, outcome variable measured, and treatment outcome. The quality of each publication was classified as good (10–12 variables reported), moderate (5–9 variables reported), or poor (1–4 variables reported).

Results

The results of the literature search are presented in a flow chart, showing study selection according to the PRISMA statement (2009)[61] (Fig. 1). The initial search strategy yielded 1355 titles/abstracts from the databases analyzed: 1085 from PubMed, 235 from Web of Science, and 35 from the Cochrane Library; five additional articles were identified through a

Table 1. Staging of and treatment strategies for bisphosphonate-related osteonecrosis of the jaw (BRONJ) according to the American Association of Oral and Maxillofacial Surgeons (AAOMS).[38,64]

BRONJ stage	Clinical conditions	Treatment strategies
At risk	No apparent necrotic bone in patients who have been treated with either oral or IV bisphosphonates	No treatment indicated Patient education
Stage 0	No clinical evidence of necrotic bone, but non-specific clinical findings and symptoms	Systemic management, including the use of pain medication and antibiotics
Stage 1	Exposed and necrotic bone in asymptomatic patients without evidence of infection	Oral anti-bacterial mouth rinse Clinical follow-up on a quarterly basis Patient education and review of indications for continued BP use
Stage 2	Exposed and necrotic bone associated with infection as evidenced by pain and erythema in the region of exposed bone, with or without purulent drainage	Symptomatic treatment with oral antibiotics Oral anti-bacterial mouth rinse Pain control Superficial debridement to relieve soft tissue irritation
Stage 3	Exposed necrotic bone in patients with pain and erythema and one or more of the following: exposed and necrotic bone extending beyond the region of alveolar bone, such as the inferior border and ramus in the mandible, or maxillary sinus or zygoma in the maxilla, resulting in pathological fracture, extraoral fistula, or oral–antral/oral–nasal communication, or osteolysis extending to the inferior border of the mandible or to the maxillary sinus floor	Oral anti-bacterial mouth rinse Antibiotic therapy and pain control Debridement/surgical resection for prolonged relief of pain and infection

IV, intravenous; BP, bisphosphonate.

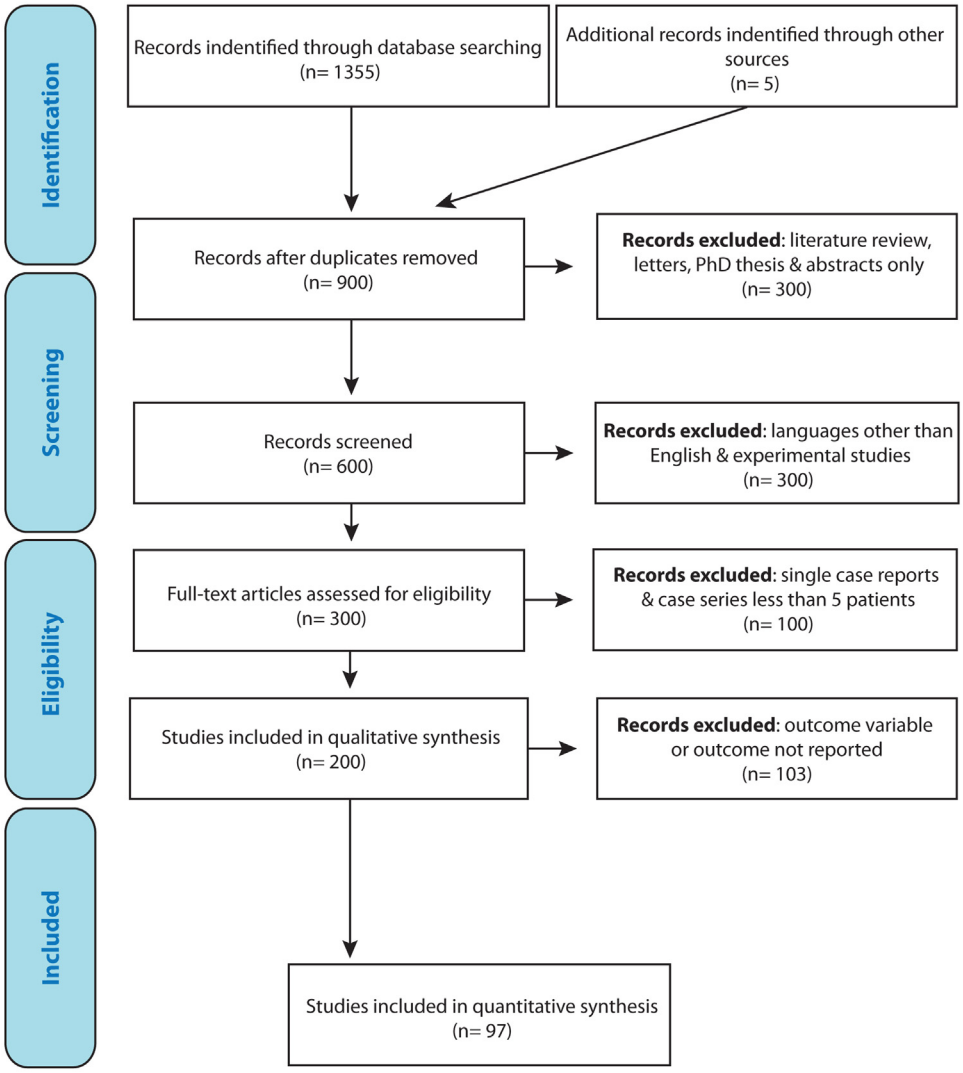

Fig. 1. Flow chart of the search strategy and study selection used in this systematic review.

hand search of the relevant reference lists, bringing the number of accepted articles to 1360. After title and abstract screening and/or paper analyses, 300 potentially relevant articles were identified for article retrieval and full-text review. Two hundred papers were included in the qualitative synthesis and finally 103 papers were excluded after a preliminary review, due to non-compliance with the inclusion/exclusion criteria, lack of outcome, not being related to the proposed research question, or being irrelevant (Fig. 1).

The remaining 97 articles were included in the final review. These articles described 4879 cases of BRONJ. With regard to the quality of the publications, 79 were classified as good (81.4%), 16 as moderate (16.5%), and two publications as poor (2.1%). Of the 97 accepted publications, 35 (36.1%) were case series,[27,49,51,52,59,66–95] 3 (3.1%) were clinical trials,[96–98] 18 (18.6%) were prospective studies,[99–116] 37 (38.1%) were retrospective studies,[17,35,44,117–150] and 4 (4.1%) were clinical reports[151–154] (Table 2).

Age and gender

A total of 4879 patients were identified and treated in the 97 publications; the mean age of these patients was 66.5 ± 4.7 years. In the 4481 cases in which the sex distribution was reported, 1471 were male patients (32.8%) and 3010 were female patients (67.2%), showing a female predilection with a female to male ratio of 2:1 among all reported cases (Table 3).

BRONJ characteristics

Eighty-nine publications described the site of BRONJ in 4627 patients receiving BPs; the site was not reported for only eight publications.[59,87,94,95,97,98,111,154] BRONJ lesions were located most commonly in the mandible (3011 patients; 65.1%), followed by the maxilla (1320 patients; 28.5%) or both jaws (296 patients; 6.4%) (Table 3).

Primary cause of disease

BP therapy was started in 4602 cases for the following indications: multiple myeloma (1434 cases; 31.2%), breast cancer (1359 cases; 29.5%), osteoporosis (903 cases; 19.6%), prostate cancer (442 cases; 9.6%), metastasis (116 cases; 2.5%), and other cancers including lung, renal, and bladder carcinoma (348 cases; 7.6%). Most patients (60.7%) had multiple myeloma or metastatic breast cancer (Table 3).

Table 2. Summary of the publications included in the systematic review: study design, total number of patients, mean patient age, duration of administration of bisphosphonates, quality of publications, and treatment modalities.

Reference	Study design	Number of patients	Age, years	Duration of BP administration, months	Quality of publication	Treatment
Thumbigere-Math 2009[117]	Retrospective	26	64	45.8	Good	Medical and minimally invasive surgery
Thumbigere-Math 2012[118]	Retrospective	18	60	44.3	Good	
Anavi-Lev 2013[95]	CS	52	70.7	40	Good	
Holzinger 2013[101]	Prospective	88	NR	NR	Moderate	
Saussez 2009[125]	Retrospective	34	62	34.5	Good	
Montebugnoli 2007[49]	CS	16	61.2	17.9	Good	
Dannemann 2006[69]	CS	14	65	NR	Good	
Beninati 2013[102]	Prospective	51	68	41	Good	
Alons 2009[126]	Retrospective	7	66.9	55.2	Good	
Lazarovici 2009[72]	CS	101	63.5	48.5	Good	
Junquera 2009[73]	CS	21	65.1	25	Good	
Stanton 2009[128]	Retrospective	33	64.5	NR	Good	
Estilo 2008[130]	Retrospective	28	NR	34.1	Good	
Dimitrakopoulos 2006[131]	Retrospective	11	61	6	Good	
Fortuna 2012[104]	Prospective	26	68.4	23.3	Good	
Abu-Id 2008[134]	Retrospective	78	65.6	12	Good	
Pozzi 2007[135]	Retrospective	35	70	36	Good	
Williamson 2010[107]	Prospective	40	64	NR	Good	
Longobardi 2007[76]	CS	18	55	42.3	Good	
Wutzl 2012[110]	Prospective	58	68.3	35.5	Good	
O'Ryan 2012[144]	Retrospective	30	77	52.8	Good	
Scoletta 2010[113]	Prospective	37	68	25.5	Good	
Nomura 2013[89]	CS	13	71.2	29.6	Good	
Jabbour 2012[90]	CS	14	69	37.5	Good	
Mücke 2011[100]	Prospective	108	68.5	NR	Moderate	Medical, minimally invasive and major surgery
Mortensen 2007[68]	CS	7	66	NR	Good	
O'Ryan 2009[129]	Retrospective	59	61.4	NR	Good	
Elad 2006[152]	CR	57	62.7	NR	Good	
Stockmann 2010[106]	Prospective	50	69.5	31	Good	
Ibrahim 2008[17]	Retrospective	8	66.5	14.6	Good	
Kim 2012[150]	Retrospective	21	64.3	30	Good	
Yarom 2007[127]	Retrospective	11	69.7	49.2	Good	
Hong 2010[139]	Retrospective	24	72.1	43.1	Good	
Lerman 2013[44]	Retrospective	120	63	36	Good	
Maurer 2011[138]	Retrospective	21	69	47.4	Good	
Hansen 2013[154]	CR	37	NR	NR	Poor	
Hoefert 2011[122]	Retrospective	47	66.1	41.9	Good	Medical
Marx 2005[27]	CS	119	NR	NR	Good	
Van den Wyngaert 2009[153]	CR	33	58	27	Good	
Moretti 2011[108]	Prospective	34	69.0	39	Good	
Alsehimy 2014[109]	Prospective	96	66.5	NR	Good	
Lazarovici 2010[86]	CS	27	70	NR	Good	
Nicolatou-Galitis 2011[114]	Prospective	63	63.6	37.1	Good	
Epstein 2010[59]	CS	6	75	74.6	Good	
Vescovi 2011[123]	Retrospective	567	67.2	NR	Good	Minimally invasive surgery
Graziani 2012[132]	Retrospective	347	67	23	Good	
Mercer 2013[133]	Retrospective	91	69.8	60	Good	
Kos 2010[136]	Retrospective	18	67.0	34.9	Good	
Wutzl 2006[78]	CS	17	64.8	32	Good	
Ferlito 2012[94]	CS	94	66	24	Moderate	
Schubert 2012[116]	Prospective	258	NR	NR	Moderate	

Table 2 (*Continued*)

Reference	Study design	Number of patients	Age, years	Duration of BP administration, months	Quality of publication	Treatment
Rugani 2010[66]	CS	5	75.4	36	Good	Laser
Romeo 2011[67]	CS	12	62	NR	Moderate	
Angiero 2009[120]	Retrospective	49	69.7	14.8	Good	
Stübinger 2009[151]	CR	8	59.1	53	Good	
Vescovi 2014[121]	Retrospective	63	NR	NR	Moderate	
Vescovi 2012[70]	CS	151	66.6	48.2	Good	
Vescovi 2007[74]	CS	19	71	NR	Moderate	
Manfredi 2011[137]	Retrospective	25	70.4	55.9	Good	
Atalay 2011[143]	Retrospective	20	55.4	32.4	Good	
Scoletta 2010[111]	Prospective	20	71.3	42.9	Good	
Rugani 2013[81]	CS	12	63.9	NR	Moderate	
Vescovi 2010[146]	Retrospective	91	67	NR	Moderate	
Vescovi 2008[91]	CS	28	70.3	NR	Moderate	
Martins 2012[119]	Retrospective	22	58.09	24.68	Good	
Curi 2011[71]	CS	25	60.7	NR	Good	Growth factor (PRP or BMP2)
Mozzati 2012[140]	Retrospective	32	69.7	37	Good	
Bocanegra-Perez 2012[112]	Prospective	8	66.3	1	Good	
Coviello 2012[93]	CS	7	75.57	66	Good	
Cicciu 2012[83]	CS	20	NR	NR	Poor	
Ripamonti 2011[96]	RCT	10	65	NR	Moderate	Ozone
Agrillo 2007[75]	CS	58	64	NR	Moderate	
Ripamonti 2012[98]	RCT	24	62.5	NR	Good	
Agrillo 2012[35]	Retrospective	131	60	NR	Good	
Boonyapakorn 2008[105]	Prospective	22	61.1	NR	Good	Discontinuation of BP
Urade 2011[141]	Retrospective	263	68.1	NR	Good	
Park 2010[80]	CS	5	72.6	79.2	Good	
Watters 2013[88]	CS	109	64	NR	Good	
Wilde 2011[145]	Retrospective	24	NR	NR	Good	
Chiu 2010[77]	CS	12	69.7	67.2	Good	Hyperbaric oxygen
Freiberger 2012[97]	RCT	22	66.1	NR	Moderate	
Freiberger 2007[52]	CS	16	NR	18	Moderate	
Kwon 2012[82]	CS	6	77.5	55.2	Good	Teriparatide
Narvaez 2013[87]	CS	7	72	55.2	Good	
Kim 2014[149]	Retrospective	15	77.1	45.6	Good	
Pautke 2011[99]	Prospective	15	63.2	44.4	Good	Guided debridement
Fleisher 2008[79]	CS	10	NR	NR	Moderate	
Seth 2010[124]	Retrospective	11	61.3	NR	Good	Major surgery
Carlson 2009[103]	Prospective	82	NR	NR	Moderate	
Badros 2006[142]	Retrospective	22	61	NR	Good	
Bedogni 2011[115]	Prospective	30	66	NR	Good	
Jacobsen 2012[147]	Retrospective	110	67	NR	Good	
Voss 2012[148]	Retrospective	21	68.5	40.1	Good	
Hanasono 2013[92]	CS	13	66.6	NR	Good	
Nocini 2009[51]	CS	7	61	NR	Good	
Lemound 2012[85]	CS	20	68	34.8	Good	
Blus 2013[84]	CS	8	71.3	32	Good	
Total		4879	66.5 ± 4.7	38.2 ± 15.7		

BP, bisphosphonate; CS, case series; RCT, randomized clinical trial; CR, clinical report; NR, not reported; PRP, platelet-rich plasma; BMP2, bone morphogenetic protein 2.

Characteristics of bisphosphonate treatment

The bisphosphonate prescribed was specified for 4118 patients with BRONJ. Overall, 2427 (58.9%) patients received zoledronate, 571 (13.9%) patients received pamidronate, 523 (12.7%) patients received alendronate, 128 (3.1%) patients received ibandronate, and 469 (11.4%) patients received a combination of BPs (Table 3).

BP treatment was principally intravenous (IV) in 3245 patients (83.2%), while 656 patients (16.8%) received oral BPs (Table 3).

Duration of treatment

There was variability in the duration of BP therapy, which ranged from 1 to 79.2 months, with a mean duration of 38.2 ± 15.7 months.

Triggering factors and comorbidities

The most important triggering factors for the development of BRONJ were described for 3198 cases in the articles included. Tooth extraction was the principal cause (1974 patients; 61.7%), followed by

Table 3. Characteristics of patients diagnosed with BRONJ.

Characteristics	Details	Number	Percentage (%)
Gender	Male	1471	32.8
	Female	3010	67.2
Location	Maxilla	1320	28.5
	Mandible	3011	65.1
	Both	296	6.4
Primary cause of the disease	Multiple myeloma	1434	31.2
	Breast cancer	1359	29.5
	Osteoporosis	903	19.6
	Prostate cancer	442	9.6
	Other cancers	348	7.6
	Metastasis	116	2.5
Type of BP administered	Zoledronate	2427	58.9
	Pamidronate	571	13.9
	Alendronate	523	12.7
	Ibandronate	128	3.1
	Combination	469	11.4
Route of administration of BP	IV	3245	83.2
	Oral	656	16.8
Triggering factors	Extraction	1974	61.7
	Dental implant	123	3.9
	Dental surgery	230	7.2
	Periodontal disease	159	5.0
	Prosthetic trauma	237	7.4
	Spontaneous	475	14.8
Comorbidities	Diabetes	298	11.2
	Corticosteroids	658	24.6
	Hypertension	225	8.4
	Thrombosis	108	4.0
	Smoking	215	8.0
	Chemotherapy	1062	39.7
	None	108	4.1

BRONJ, bisphosphonate-related osteonecrosis of the jaws; BP, bisphosphonate; IV, intravenous.

trauma from manipulation of dental implants (123 cases; 3.9%). A history of dental surgery was reported for 230 patients (7.2%), periodontal diseases in 159 patients (5.0%), and prosthesis-induced trauma in 237 patients (7.4%). A large proportion of BRONJ lesions appeared spontaneously (475 patients; 14.8%) (Table 3).

With regard to concomitant diseases and medications, 2674 patients had comorbidities. Diabetes mellitus was observed in 298 patients (11.2%) and hypertension in 225 patients (8.4%); 1062 (39.7%) patients were under chemotherapy, 215 (8.0%) patients were smokers, 108 (4.0%) patients had thrombocoagulopathies, 658 (24.6%) were taking corticosteroids, and 108 (4.1%) were free from any concomitant diseases. The incidence of BRONJ was associated with chemotherapy (39.7%) of the patients compared to corticosteroid therapy (24.6%) (Table 3).

Management of osteonecrosis of the jaw

Regarding the management of the BRONJ lesions, the studies reported discontinuation of BP administration (5.1%) in addition to treatment by medical therapy (50%) or minimally invasive surgical therapy (45.9%); 22.4% of patients underwent major surgical procedures, such as segmental resection of the jaw bones.

Various adjunctive treatments such as hyperbaric oxygen (HBO) therapy, laser therapy, ozone therapy, teriparatide, fluorescence-guided debridement, treatment with growth factors (platelet-rich plasma (PRP) or bone morphogenetic protein 2 (BMP2)), and ultrasonic therapy were also mentioned.

Medical treatment of BRONJ was reported in 49 publications[17,27,44,49,59,68,69,72,73,76,80,86,88–90,95,100–102,104–110,113,114,117,118,122,125–131,134,135,138,139,141,144,145,150,152–154] and minimally invasive surgical treatment in 44 publications.[17,27,44,49,68,69,72,73,76,78,89,90,94,100–102,104–107,110,113,116–118,123,125–136,139,141,144,150,152,154] Major surgical intervention was reported in 22 publications,[17,44,68,92,100,103,106,115,124,127,129,137–139,141,142,145,147,148,150,152,154] including the use of surgical flaps in two publications[51,85] and ultrasonic therapy in one.[84] Laser therapy was reported in 14 publications,[66,67,70,74,81,91,111,119–121,137,143,146,151] ozone therapy in four publications,[35,75,96,98] PRP in five publications,[71,93,112,119,140] BMP2 in one,[83] HBO in three publications,[52,77,97] and teriparatide in three publications.[82,87,149] Fluorescence or tetracycline guided debridement was reported in two publications.[79,99]

Seven hundred and fifteen patients were treated by medical and minimally invasive surgical treatment, 422 patients were treated by medical, minimally invasive and major surgical treatment, 286 patients were treated by medical treatment only, 767 patients were treated by minimally invasive surgical treatment, 252 patients were treated by major surgical treatment, 25 patients were treated by guided debridement, 322 patients were treated with laser therapy, 92 patients were treated with growth factors, 161 patients were treated with ozone therapy, 361 patients stopped BP treatment in addition to other treatment modalities, 45 patients were treated with HBO, and 27 patients were treated with teriparatide (Table 4).

Follow-up and treatment outcomes

After the initial BRONJ treatment, follow-up periods, which were reported in only 80 publications, ranged from 4 weeks to 50 months, with a mean of 12.9 ± 9.9 months.

Outcome measures

A total of seven outcome variables were used in the studies. The most frequently measured outcome was mucosal healing (47 publications; 48.5%), followed by bone exposure (30 publications; 30.9%), pain (31 publications; 31.9%), changes in signs and symptoms (28 publications; 28.9%), improvement in stage (14 publications; 14.4%), reduction in lesion size and number (12 publications; 12.4%), and finally infection control (seven publications; 7.2%).

The outcome of the treatment was classified as (1) complete healing, defined as complete regrowth of the oral mucosa over previously exposed bone; (2) partial healing, defined as either a decrease in lesion size (largest linear dimension) or the number of lesions, and/or cessation of pain or signs of infection; (3) stable disease, defined as no improvement in clinical signs or symptoms; (4) progressive disease, defined as an increase in the size or number of lesions, or increased pain and severity of infection; (5) regressive disease, defined as a decrease in the size or number

Table 4. Outcome of each treatment modality.

Treatment	Outcome, number of patients (%)							Total number of patients (%)
	Complete healing	Partial healing	Stable lesion	Progressive lesion	Regressive lesion	Recurrent lesion	Non-healing lesion	
Medical and minimally invasive surgery	278 (38.9)	125 (17.5)	94 (13.1)	52 (7.3)	64 (9.0)	5 (0.7)	97 (13.6)	715 (20.6)
Medical, minimally invasive and major surgery	169 (40.0)	105 (24.9)	34 (8.1)	19 (4.5)	5 (1.2)	47 (11.1)	43 (10.2)	422 (12.1)
Medical treatment	129 (45.1)	52 (18.2)	23 (8.0)	8 (2.8)	52 (18.2)	20 (7.0)	2 (0.7)	286 (8.2)
Minimally invasive surgery	301 (39.2)	0 (0)	152 (19.8)	61 (8.0)	231 (30.1)	0 (0)	22 (2.9)	767 (22.1)
Major surgery	207 (82.1)	11 (4.4)	8 (3.2)	5 (2.0)	0 (0)	11 (4.4)	10 (4.0)	252 (7.3)
Guided debridement	12 (48)	10 (40)	0 (0)	1 (4)	0 (0)	0 (0)	2 (8)	25 (0.7)
Laser therapy	146 (45.3)	18 (5.6)	81 (25.2)	5 (1.6)	33 (10.2)	2 (0.6)	37 (11.5)	322 (9.3)
Growth factors	75 (81.5)	2 (2.2)	6 (6.5)	0 (0)	8 (8.7)	1 (1.1)	0 (0)	92 (2.6)
Ozone therapy	93 (57.8)	27 (16.8)	5 (3.1)	0 (0)	28 (17.4)	0 (0)	8 (5.0)	161 (4.6)
Discontinuation of bisphosphonates	127 (35.2)	27 (7.5)	142 (39.3)	50 (13.9)	3 (0.8)	5 (1.4)	7 (1.9)	361 (10.4)
Hyperbaric oxygen	12 (26.7)	8 (17.8)	2 (4.4)	6 (13.3)	17 (37.8)	0 (0)	0 (0)	45 (1.3)
Teriparatide	22 (81.5)	5 (18.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	27 (0.8)
Total	1571 (45.2)	390 (11.2)	547 (15.7)	207 (6.0)	441 (12.7)	91 (2.6)	228 (6.6)	3475

of lesions, or decreased pain and severity of infection; and (6) negligible or no healing when there was no sign of improvement.

The outcomes of the treatment modalities of the BRONJ were assessed in 3475 patients. Outcomes of the different treatment modalities were compared (Table 4).

Medical and minimally invasive surgical treatment

Seven hundred and fifteen patients were treated by medical and conservative surgical treatment; 278 patients (38.9%) showed complete healing, 125 (17.5%) showed partial healing, 94 (13.1%) had stable lesions, 52 (7.3%) had progressive lesions, 64 (9.0%) had regressive lesions, only five (0.7%) had recurrent lesions, and 97 patients (13.6%) had lesions with negligible or no healing.

Medical, minimally invasive and major surgical treatment

Four hundred and twenty-two patients were treated by medical, conservative and surgical treatment; 169 patients (40.0%) showed complete healing, 105 (24.9%) showed partial healing, 34 (8.1%) had stable lesions, 19 (4.5%) had progressive lesions, five (1.2%) had regressive lesions, 47 (11.1%) had recurrent lesions, and 43 patients (10.2%) had lesions with negligible or no healing.

Medical treatment only

Two hundred and eighty-six patients were treated medically; 129 patients (45.1%) showed complete healing, 52 (18.2%) showed partial healing, 23 (8.0%) had stable lesions, eight (2.8%) had progressive lesions, 52 (18.2%) had regressive lesions, 20 (7.0%) had recurrent lesions, and two patients (0.7%) had lesions with negligible or no healing.

Minimally invasive surgical treatment

Seven hundred and seventy-six patients were treated with conservative surgery; 301 patients (39.2%) showed complete healing, no patients (0%) showed partial healing, 152 (19.8%) had stable lesions, 61 (8.0%) had progressive lesions, 231 (30.1%) had regressive lesions, no patients (0%) had recurrent lesions, and 22 patients (2.9%) had lesions with negligible or no healing.

Major surgical treatment

Two hundred and fifty-two patients were treated with major surgery; 207 patients (82.1%) showed complete healing, 11 (4.4%) showed partial healing, eight (3.2%) had stable lesions, five (2.0%) had progressive lesions, no patients (0%) had regressive lesions, 11 (4.4%) had recurrent lesions, and 10 patients (4.0%) had lesions with negligible or no healing.

Guided debridement

Twenty-five patients were treated with guided debridement; 12 patients (48%) showed complete healing, 10 (40%) showed partial healing, no patients (0%) had stable lesions, one (4%) had progressive lesions, no patients (0%) had regressive lesions, no patients (0%) had recurrent lesions, and two patients (8%) had lesions with negligible or no healing.

Laser treatment

Three hundred and twenty-two patients were treated with laser therapy; 146 patients (45.3%) showed complete healing, 18 (5.6%) showed partial healing, 81 (25.2%) had stable lesions, five (1.6%) had progressive lesions, 33 (10.2%) had regressive lesions, two (0.6%) had recurrent lesions, and 37 patients (11.5%) had lesions with negligible or no healing.

Growth factor (PRP and BMP2) treatment

Ninety-two patients were treated with growth factors; 75 patients (81.5%) showed complete healing, two (2.2%) showed partial healing, six (6.5%) had stable lesions, no patients (0%) had progressive lesions, eight (8.7%) had regressive lesions, one (1.1%) had recurrent lesions, and no patients (0%) had lesions with negligible or no healing.

Ozone treatment

One hundred and sixty-one patients were treated with ozone therapy; 93 patients (57.8%) showed complete healing, 27 (16.8%) showed partial healing, five (3.1%) had stable lesions, no patients (0%) had progressive lesions, 28 (17.4%) had regressive lesions, no patients (0%) had recurrent lesions, and eight patients (5.0%) had lesions with negligible or no healing.

Discontinuation of BP treatment in addition to other treatment modalities

Three hundred and sixty-one patients stopped BP treatment; 127 patients (35.2%) showed complete healing, 27 (7.5%) showed partial healing, 142 (39.3%) had stable lesions, 50 (13.9%) had progressive lesions, three (0.8%) had regressive lesions, five (1.4%) had recurrent lesions, and seven patients (1.9%) had lesions with negligible or no healing.

Hyperbaric oxygen treatment

Forty-five patients were treated with HBO; 12 patients (26.7%) showed complete healing, eight (17.8%) showed partial healing, two (4.4%) had stable lesions, six (13.3%) had progressive lesions, 17 (37.8%) had regressive lesions, no patients (0%) had recurrent lesions, and no (0%) patients had lesions with negligible or no healing.

Teriparatide treatment

Twenty-seven patients were treated with teriparatide; 22 patients (81.5%) showed complete healing, five (18.5%) showed partial healing, no patients (0%) had stable lesions, no patients (0%) had progressive lesions, no patients (0%) had regressive lesions, no patients (0%) had recurrent lesions, and no patients (0%) had lesions with negligible or no healing.

The treatment of BRONJ and the outcome variables measured with the mean follow-up of each treatment are summarized in Table 5.

Discussion

The aim of this systematic review was to summarize the literature concerning patients receiving BPs, the treatments of BRONJ, and the outcomes of these treatments. There was high clinical heterogeneity among the studies included, which is unsurprising given the differing

Table 5. Summary of treatment modalities and the outcome variables measured with the mean follow-up of each treatment.

Treatment	Outcome variables measured	Follow-up, months
Medical and minimally invasive	Improved signs and symptoms, decrease in lesion size and number, elimination of pain, reduction in soft and hard tissue inflammation, no bone exposure or bone exposure less than 1–2 mm, no suppuration, improvement of stage, persistence of fistula, cessation of pus and extraoral manifestations, mucosal coverage, radiographic success (cessation of bony destruction), presence or recurrence of infection, BRONJ at stage 0	11.1 ± 6.6
Medical, minimally invasive and major surgery	Closure of oro-antral fistula, stage improvement, healing of the lesion, extension of exposed bone areas, bone exposure, decrease in pain, healing of mucosa, improved signs and symptoms, asymptomatic lesions, patients free from symptoms, recurrence of BRONJ, recurrence of sinusitis	11.6 ± 5.2
Medical	No fistula, reduction of exposed bone, reduction in pain, closure of the mucosal defect, persistence of exposed bone or progressive necrosis, reduction in size of the lesion, size of necrotic lesions, resolution of BRONJ manifestations, cessation of pus or purulent secretion, mucosal inflammation, improvement in signs and symptoms	16.4 ± 5.2
Minimally invasive surgery	Improvement of the stage (transition to a less severe stage), deterioration of wound healing, recurrence rate of wound dehiscence, closure of lesion, pain reduction, complete healing of soft tissue, signs of inflammation, exposed bone, no symptoms of infection for a minimum of 3 months	6.4 ± 3.6
Laser	Effectiveness of surgical laser application, pain reduction, infection control, mucosal healing, no signs and symptoms, healing evaluated radiographically, complete removal of visible necrotic bone, absence of new exposed bone near surgical area, no signs of infection, stage improvement, size of the lesion, oedema, visual analogue score of pain, presence of pus, fistulas and halitosis, bone exposure	10.7 ± 9.7
Growth factor (PRP or BMP2)	Intact and healed mucosa, no exposed necrotic bone, no sign of infection or fistula, absence of pain, no radiographic signs of residual infection or evidence of bone sequestration, bleeding	18.2 ± 18.3
Ozone	Spontaneous expulsion or sequestrum of necrotic bone to be removed surgically, healed and re-epithelialized mucosa, presence or absence of oral mucosa redness around the lesion area, petechiae or bleeding, pain intensity, diminishing of symptoms	9.9 ± 5.5
Discontinuation of BP	Healing of the mucosa, pain relief, bleeding, stage improvement, resolution of symptoms, presence or absence of exposed necrotic bone, radiographic evidence of BRONJ, no fistulas, absence of swelling	27.8 ± 29.2
Hyperbaric oxygen (HBO)	Clinical evidence of symptom relief, pain reduction, absence of sequestrum, oral lesion size and number, regrowth of oral mucosa over exposed bone	20 ± 5.7
Teriparatide	Change in biochemical markers (osteocalcin and C terminal telopeptide cross-link type I collagen), clinical and radiographic healing, improvement of BRONJ stage	4.5 ± 2.1
Major surgery	Osseous union judged clinically and radiographically, without signs of residual infection, or exposed bone at the time of evaluation, postoperative complication, infection, recurrence of BRONJ, oral pain, exposed bone, mucosal healing, percentage of flap survival, percentage of complications at the donor and recipient site, symptom-free	18 ± 5.2
Guided debridement	Closure of mucosa, exposed bone, symptom-free	1.5 ± 0.7

BRONJ, bisphosphonate-related osteonecrosis of the jaws; PRP, platelet-rich plasma; BMP2, bone morphogenetic protein 2; BP, bisphosphonate.

interventions used and the considerable variations in techniques applied and combinations or delivery of interventions. Differences in the search periods may explain the higher prevalence of BRONJ in the present review.

There are some limitations with respect to the search strategy. It is possible that eligible studies were missed despite the extended search. Also, the grey literature was excluded because basic information such as authorship, publication date, and the publishing body cannot be discerned with certainty. This review did not include searches of EMBASE, SCOPUS, or abstracts from dental, maxillofacial, and surgical conferences, which may also have contributed to an underestimation of the number of reported BRONJ cases. The continuously increasing numbers of BRONJ cases since its first appearance is apparent in this systematic review.

The occurrence of BRONJ appears to be related to the cumulative dose, the duration of treatment, and the type of BP used,[62,155–158] with a positive correlation for higher doses, longer durations of therapy, and nitrogen-containing BPs.

Earlier studies reported that the type of BP may play a role in the development of BRONJ, particularly the nitrogen-containing BPs like pamidronate and zoledronate, with a higher risk for zoledronate followed by pamidronate.[23,27,40,155,156,159–163] The cumulative hazard of developing BRONJ is significantly greater with zoledronate treatment than with pamidronate or pamidronate plus zoledronate[159,161] due to the more potent inhibitory effect on the bone turnover rate and the stronger anti-resorptive activity of zoledronate compared to pamidronate. Zoledronate is 10–100 times more potent than pamidronate.[164] Consistent with these studies, we noted that most patients in the publications had received zoledronate only (58.9%) or pamidronate only (13.9%), or zoledronate plus pamidronate (11.4%).

The mean duration of BP treatment was 38.2 ± 15.7 months; this is a crucial factor for the development of BRONJ. It has been suggested that the development of BRONJ requires a long period of exposure.[23] As reported in the literature, the risk of developing BRONJ is related to the duration of therapy and the risk appears to be higher after 3 years of treatment in association with clinical risk factors.[48] Recently, Lo et al.[165] reported a higher prevalence of BRONJ (0.21%) in patients treated with these drugs for more than 4 years, in comparison with those treated for less than 2.5 years.

Current data suggest that IV BPs are much more frequently associated with BRONJ than oral BPs.[48,159,166] This has led to the development of different management strategies for patients on oral and IV BPs. This is in accordance with our search, which showed that 83.2% of BRONJ lesions developed following IV BP use. The results confirm data from other studies indicating that the prevalence of BRONJ is much lower in patients on oral BPs than in patients treated with IV BPs.[167]

A greater incidence of BRONJ has been reported in patients with malignancies, particularly those with multiple myeloma and breast cancer.[155,159–161] Our results agree with these reports – BRONJ was more frequently noted in patients with multiple myeloma and breast cancer compared with the prostate cancer, lung cancer, renal cell carcinoma, and the other neoplasms group.

With regard to a history of invasive dental treatment, 61.7% of the patients had undergone a dental extraction before the development of BRONJ. This finding is consistent with the review by Badros et al., which reported a significant association between the occurrence of BRONJ and age and a history of dental extraction in patients with multiple myeloma treated with IV BPs.[142] In agreement with published reports, tooth extraction in this review was associated with the development of BRONJ.[18,27,156,168–170] According to the publications in this systematic review, BRONJ was spontaneous in 14.8% of cases. Our findings correspond to those of the authors reporting a higher percentage of so-called spontaneous cases, varying from 14.1% to 60%.[18,21,23,30,40,48,70,88,105,118,123,130,144,171–176] This may be due to the fact that it is difficult to establish the initiating factor in some patients.

Correlations between the occurrence of BRONJ and specific co-medications such as corticosteroids or chemotherapy have been discussed.[41,167,177,178] These treatments may also increase the vulnerability of the oral mucosa and reduce its nutritive supply.[27,41,174] Of the patients, 39.7% were under chemotherapy. Moreover 24.6% used corticosteroids. In fact, corticosteroids and some other chemotherapy medications possess an anti-angiogenic effect by inhibiting vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF).[27,41,174,179]

There is considerable discussion in the literature regarding whether ageing plays a significant role in the development of BRONJ. Some studies have found no statistically significant correlation between ageing and BRONJ,[180,181] whereas others have included advanced age as a BRONJ co-factor[80,159,182]; this could be related to the physiological effects of ageing, including inflammatory issues,[183] immune dysfunction,[184] a reduction in blood flow and remodelling ability,[185,186] and increased oxidative stress.[187] In fact, these features are all implicated in the pathogenesis of BRONJ and could explain why the disease is not reported in young patients, even those with other associated risk factors.[188]

Some authors have reported a positive correlation between gender and BRONJ.[80] It has been speculated that oestrogen therapy may play a role in this correlation, since hormonal replacement has been associated with an increased risk of BRONJ .[189] However, there is controversy regarding gender as a BRONJ co-factor. Some studies have found no statistically significant correlation between gender and BRONJ.[159,182] The large proportion of female patients reported in some studies[1,43,48,80,127,137,190–195] could represent a coincidence, since women take oral BPs more frequently than males, especially because rheumatoid arthritis and osteoporosis are more common in women.[196] In accordance with other series reported in the literature,[172] the present review found a high prevalence of BRONJ among women (67.2%).

BRONJ affects the mandible more often than the maxilla. The ratio of mandible to maxilla involvement found was 2:1. This could be attributed to the decreased vascularity of the mandible and to the existing local conditions. This distribution is similar to that reported by other authors .[113,128,197] Only the mandible and maxilla appear to be susceptible, highlighting their unique nature compared with other parts of the skeleton. The jaws are the only bones in the human body that are in frequent contact with the outside world and are subject to repeated micro-trauma through the presence of teeth and the forces of mastication; moreover the turnover of alveolar bone is 10-fold greater than that in the long bones.[45] BRONJ occurred more often in the mandible (59%) than in the maxilla (27%), as also reported by Marx et al.[27] A possible explanation for osteonecrosis, especially in the mandible, might be the anti-angiogenic effect of BPs[198–201] and anatomical and physiological features of the mandibular bone that may increase the risk of an osteonecrotic pathology.[202] This action would result in direct induction of avascular necrosis of tissue repair and may interrupt the intraosseous circulation and blood flow in the jaw.[30] Furthermore, BPs can

also inhibit endothelial cell function[201] and increase the rate of apoptosis,[199] leading to a decrease in capillary tube formation.[203]

The management of BRONJ remains a controversial topic. Several treatment protocols have been proposed, but there is no general consensus with regard to many crucial questions, such as whether or not performing surgery is beneficial.[67] Some authors have reported that the discontinuation of BPs for a variable period (1–6 months) before and after interventions favours the surgical outcome,[110,204] emphasizing a possible anti-angiogenic effect on the soft tissues around the necrosis; the removal of this effect may have a role in healing. There may also be psychological aspects. Patients may be stressed by the idea of taking drugs that could have an adverse effect on the bones.

Our results show that minimally invasive surgical treatment was the most commonly used method for the management of BRONJ; 767 patients were managed using sequestrectomy, curettage, debridement, or smoothening of bone. These results are in agreement with those of Alons et al.[126] who treated seven patients with sequestrectomy and curettage of the defect with a minimum of periosteal deflection. Mitsimponas et al.[205] reported a complete success rate of 53% in a patient group using different surgical procedures, including bone smoothing, incision and drainage, ulcer excision, and closure after debridement. Eckert et al.[206] demonstrated a 58% success rate in 24 operated patients. The surgical procedures included resection of the necrotic bone and stable soft tissue closure. Millesi et al.[207] treated 55 patients with sequestrectomy, debridement, or partial resection, with or without osteosynthesis after 6 months, and found an overall complete success in 50%.

Carlson and Basile[103] reported high cure rates and improved stages of disease after surgery. They stated that performing a segmental resection of the mandible and partial maxillectomies with the intention of achieving vital bone margins are of crucial importance in the management of BRONJ. According to Otto et al.,[208] surgery may be the only curative treatment in refractory disease. In these studies the authors favour radical surgery. The observation of the efficacy of resection for BRONJ has recently been reported in the dental literature.[115,134]

Medical treatment is favoured in the AAOMS Position Paper, whose authors state that surgery should be deferred as long as possible.[45] Van den Wyngaert et al.[153] and Scoletta et al.[113] have stated that the medical treatment of BRONJ leads to mucosal healing in 50% of cases. However, the healing rate of BRONJ lesions in the group studied was also significantly associated with the stage of BRONJ at presentation, with lower healing rates observed for high stages.[153] Gomez Font et al., in their BRONJ update, recommended a long-term antibiotic regime and chlorhexidine 3 or 4 times a day. Aggressive surgical therapies were not considered; moreover, inadequate healing with a lack of mucosal closing was confirmed.[209]

The application of growth factors is also considered a treatment option because of improving the soft and hard tissue healing. Acting like chemotactic agents, they stimulate angiogenesis, migration, proliferation, and the differentiation of stem cells from the surrounding mesenchymal tissues into bone-forming cells in the area of injury.[142,210] A new therapy for BRONJ based on the application of recombinant human BMP2 (rhBMP2) has been discussed and shows how growth factor application leads to an increase in soft tissue healing.[83] Some studies have reported the treatment of refractory cases of BRONJ with bone resection followed by topical application of PRP[55,56,211] in which the PRP is an autologous concentration of human platelets and a source of different protein growth factors. Protein growth factors such as platelet-derived growth factor, transforming growth factor beta (TGF-β), VEGF, and epidermal growth factor beta are polypeptides released from the platelets when they are activated and can induce paracrine effects on stimulated cells.[212–214].

Recurrent BRONJ lesions have been managed successfully by use of a surgical laser. Stübinger et al.[151] and Vescovi et al.[146] used an Er:YAG laser for bony debridement. The use of ozone is also effective on avascular necrosis-related pathologies and acts by stimulating and/or preserving the endogenous antioxidant system and by blocking the xanthine/xanthine oxidase pathway, active in free radical synthesis[215–217]; it also acts by activating the blood circulation, increasing the number of red blood cells and the haemoglobin concentration,[218] enhancing diapedesis and phagocytosis, and stimulating the mononuclear phagocytic system.[218–220]

The proposed rationale behind the beneficial effects of HBO therapy in BRONJ is increased wound healing, a reduction of oedema and inflammation, stem cell mobilization, and moderation of the suppression of bone turnover by BPs.[221] Recent studies have revealed that HBO therapy also generates reactive oxygen species (ROS) and reactive nitrogen species (RNS), which affect the signalling process critical to wound healing.[221,222] HBO therapy may also improve inflammation and infection around necrotic tissues by increasing blood vessels, the oxygen concentration, and antibiotic levels in patients with BRONJ.[221,222]

Teriparatide, a recombinant human parathyroid hormone, is an osteo-anabolic agent that has stimulatory effects on osteoblasts and subsequently osteoclasts, and increases bone turnover by promoting bone formation with positive balancing in bone metabolism.[223,224] Teriparatide regulates bone resorption by increasing osteoclastic activity.[225] Therefore, teriparatide is known to have rapid and strong stimulatory effects on bone remodelling, even in the face of previous exposure to BPs.[226–230] The use of teriparatide on refractory BRONJ lesions was first described by Harper and Fung,[231] who observed soft tissue healing in a patient administered teriparatide for 3 months. Additionally, in a case study, Ohbayashi et al.[232] demonstrated bone regeneration 6 months after teriparatide therapy in a refractory BRONJ patient. Ma et al.[230] showed that teriparatide reverses the inhibitory effects of anti-resorptive drugs such as BPs *in vivo*. The BPs suppress osteoclastic activity by inducing apoptosis of these cells and cause them to detach from the bone surface.[233]

Fluorescence-guided bone resection was introduced as an adjunctive treatment in the surgical therapy of BRONJ to determine the extent of the surgical debridement.[53,234]

It is difficult to compare the outcomes of the different BRONJ therapies for two mutually non-exclusive reasons: First, the definition of therapy success has not been universally defined, and in particular studies favouring medical therapy regimens often consider maintaining the status as success. Second, only a few studies have, to date, compared the therapy outcomes of medical and surgical treatment in a controlled clinical manner.[49]

The key factors for successful treatment have not yet been identified clearly. There are several aspects that are likely to influence the success of surgery and could cause progression of the disease.[66] Comparing different studies regarding therapeutic success in BRONJ is made difficult by the different definitions of success.[122]

Ruggiero and Drew[235] considered preservation of quality of life by controlling pain, managing infection, and preventing

the development of new areas of necrosis as a treatment goal. Taking this into consideration, the relief of symptoms may very well be a 'success' for the oncology patient.[126] Vescovi et al.[236] defined 'clinical success' as a positive result (e.g., transition from a higher stage to a lower stage, complete mucosal healing), or a minimum time span of 3 months without clinical symptoms. With regard to the definition of BRONJ,[167] 'clinical success' should principally include the absence of pain and other symptoms of oral infection, a lack of oral or cutaneous fistulas, and an intact mucosal covering over formerly exposed bone.[54]

Comparisons between the outcomes of different therapies are also complicated by the inclusion of patients taking different BPs and doing so in an uncontrolled clinical manner.[71]

The treatment outcome is considered a success when oral mucosal healing is maintained without bone exposure or infection and there is acceptable radiographic healing for a 12-month period after surgery. Therefore, following patients for at least 1 year postoperatively may be indicated to identify the possibility of recurrence of disease[71]; this is in accordance with the results of our study, which showed a mean follow-up period of 12.9 ± 9.9 months.

Data on treatment outcomes of osteonecrosis of the jaw in the literature are vague and scarce. Marx et al.[27] reported that 90% of patients were free of pain under continuous antibiotic treatment, but they did not specify the type of response (complete response, partial response, or non-response). Mavrokokki et al.[156] reported that 70% of patients were classified as ongoing cases and that 30% had been resolved, but there were no details regarding partial response and non-response. Abu-Id et al.[134] recently published the results of a multicentre study from Germany, Austria, and Switzerland based on questionnaires applied to 78 BRONJ patients. They reported that 60% of their 78 patients were treated with minor invasive surgical procedures or medical treatment with local disinfectants and antibiotics. The remaining patients were treated radically by means of bone resection up to viable bone. Thirty-eight percent of the patients treated medically were classified as responsive, as were 86% of the patients treated radically.

Mucosal coverage is the main goal of BRONJ treatment in order to prevent secondary infection. The management of BRONJ remains controversial, and there is no definitive standard of care for this disease. Non-surgical, conservative, and minimally invasive treatment regimens for BRONJ are considered useful to control the disease, leading to predictable good results in cases of lower stages of BRONJ. Further research is indicated particularly for higher stage BRONJ (refractory stage 3 lesions). BRONJ may also be approached with new adjunctive treatments such as ozone therapy, HBO, or growth factors in order to ensure an optimal patient treatment protocol. The application of adjunctive treatments remains an opinion-based approach rather than an evidence-based one. Controlled studies or clinical trials should be performed to evaluate these adjunctive treatments for BRONJ patients.

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References

1. Conte-Neto N, Bastos AS, Spolidorio LC, Marcantonio RAC, Marcantonio E. Oral bisphosphonate-related osteonecrosis of the jaws in rheumatoid arthritis patients: a critical discussion and two case reports. *Head Face Med* 2011;**7**:7.
2. Patel S, Choyee S, Uyanne J, Nguyen AL, Lee P, Sedghizadeh PP, et al. Non-exposed bisphosphonate-related osteonecrosis of the jaw: a critical assessment of current definition, staging, and treatment guidelines. *Oral Dis* 2012;**18**:625–32.
3. Arantes HP, Silva AG, Lazaretti-Castro M. Bisphosphonates in the treatment of metabolic bone diseases. *Arq Bras Endocrinol Metabol* 2010;**54**:206–12.
4. Griz L, Caldas G, Bandeira C, Assuncao V, Bandeira F. Paget's disease of bone. *Arq Bras Endocrinol Metabol* 2006;**50**:814–22.
5. Lipton A, Theriault RL, Hortobagyi GN, Simeone J, Knight RD, Mellars K, et al. Pamidronate prevents skeletal complications and is effective palliative treatment in women with breast carcinoma and osteolytic bone metastases—long term follow-up of two randomized, placebo-controlled trials. *Cancer* 2000;**88**:1082–90.
6. Berenson JR, Rosen LS, Howell A, Porter L, Coleman RE, Morley W, et al. Zoledronic acid reduces skeletal-related events in patients with osteolytic metastases. *Cancer* 2001;**91**:1191–200.
7. Saad F. Clinical benefit of zoledronic acid for the prevention of skeletal complications in advanced prostate cancer. *Clin Prostate Cancer* 2005;**4**:31–7.
8. Rogers MJ, Crockett JC, Coxon FP, Monkkonen J. Biochemical and molecular mechanisms of action of bisphosphonates. *Bone* 2011;**49**:34–41.
9. Hellstein JW, Marek CL. Bisphosphonate osteochemonecrosis (bis-phossy jaw): is this phossy jaw of the 21st century? *J Oral Maxillofac Surg* 2005;**63**:682–9.
10. Russell RG, Watts NB, Ebetino FH, Rogers MJ. Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. *Osteoporos Int* 2008;**19**:733–59.
11. Hollick RJ, Reid DM. Role of bisphosphonates in the management of postmenopausal osteoporosis: an update on recent safety anxieties. *Menopause Int* 2011;**17**:66–72.
12. Basso U, Maruzzo M, Roma A, Camozzi V, Luisetto G, Lumachi F. Malignant hypercalcemia. *Curr Med Chem* 2011;**18**:3462–7.
13. Hadji P. Clinical considerations for the use of antiresorptive agents in the treatment of metastatic bone disease. *Crit Rev Oncol Hematol* 2011;**80**:301–13.
14. Jin Y, An X, Cai YC, Cao Y, Cai XY, Xia Q, et al. Zoledronic acid combined with chemotherapy bring survival benefits to patients with bone metastases from nasopharyngeal carcinoma. *J Cancer Res Clin Oncol* 2011;**137**:1545–51.
15. Pichardo SE, van Merkesteyn JP. Bisphosphonate related osteonecrosis of the jaws: spontaneous or dental origin? *Oral Surg Oral Med Oral Pathol Oral Radiol* 2013;**116**:287–92.
16. Wysowski DK. Reports of esophageal cancer with oral bisphosphonate use. *N Engl J Med* 2009;**360**:89–90.
17. Ibrahim T, Barbanti F, Giorgio-Marrano G, Mercatali L, Ronconi S, Vicini C, et al. Osteonecrosis of the jaw in patients with bone metastases treated with bisphosphonates: a retrospective study. *Oncologist* 2008;**13**:330–6.
18. Bagan JV, Jimenez Y, Murillo J, Hernandez S, Poveda R, Sanchis JM, et al. Jaw osteonecrosis associated with bisphosphonates: multiple exposed areas and its relationship to teeth extractions. Study of 20 cases. *Oral Oncol* 2006;**42**:327–9.
19. Marx RE. Pamidronate (Aredia) and zoledronate (Zometa) induced avascular necrosis

of the jaws: a growing epidemic. *J Oral Maxillofac Surg* 2003;**61**:1115–7.
20. Migliorati CA. Bisphosphonates and oral cavity avascular bone necrosis. *J Clin Oncol* 2003;**21**:4253–4.
21. Wang J, Goodger NM, Pogrel MA. Osteonecrosis of the jaws associated with cancer chemotherapy. *J Oral Maxillofac Surg* 2003;**61**:1104–7.
22. Rosenberg TJ, Ruggiero S. Osteonecrosis of the jaws associated with the use of bisphosphonates. *J Oral Maxillofac Surg* 2003;**61**(8 Suppl):60.
23. Ruggiero SL, Mehrotra B, Rosenberg TJ, Engroff SL. Osteonecrosis of the jaws associated with the use of bisphosphonates: a review of 63 cases. *J Oral Maxillofac Surg* 2004;**62**:527–34.
24. Ruggiero SL, Fantasia J, Carlson E. Bisphosphonate-related osteonecrosis of the jaw: background and guidelines for diagnosis, staging and management. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2006;**102**:433–41.
25. Greenberg MS. Intravenous bisphosphonates and osteonecrosis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2004;**98**:259–60.
26. Gibbs SDJ, O'Grady J, Seymour JF, Prince HM. Bisphosphonate-induced osteonecrosis of the jaw requires early detection and intervention. *Med J Aust* 2005;**183**:549–50.
27. Marx RE, Sawatari Y, Fortin M, Broumand V. Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. *J Oral Maxillofac Surg* 2005; **63**:1567–75.
28. Mehrotra B, Ruggiero SL. Bisphosphonate related osteonecrosis (BRON) of the jaw: single institutional update. *ASH Annu Meet Abstr* 2005;**106**:291.
29. Melo MD, Obeid G. Osteonecrosis of the jaws in patients with a history of receiving bisphosphonate therapy: strategies for prevention and early recognition. *J Am Dent Assoc* 2005;**136**:1675–81.
30. Migliorati CA, Schubert MM, Peterson DE, Seneda LM. Bisphosphonate-associated osteonecrosis of mandibular and maxillary bone: an emerging oral complication of supportive cancer therapy. *Cancer* 2005; **104**:83–93.
31. Purcell PM, Boyd IW. Bisphosphonates and osteonecrosis of the jaw. *Med J Aust* 2005;**182**:417–8.
32. Vannucchi AM, Ficarra G, Antonioli E, Bosi A. Osteonecrosis of the jaw associated with zoledronate therapy in a patient with multiple myeloma. *Br J Haematol* 2005; **128**:738.
33. Bilezikian JP. Osteonecrosis of the jaw—do bisphosphonates pose a risk? *N Engl J Med* 2006;**355**:2278–81.
34. Van Poznak C, Ward BB. Osteonecrosis of the jaw. *Curr Opin Orthop* 2006;**17**: 462–8.
35. Agrillo A, Filiaci F, Ramieri V, Riccardi E, Quarato D, Rinna C, et al. Bisphosphonate-related osteonecrosis of the jaw (BRONJ): 5 year experience in the treatment of 131 cases with ozone therapy. *Eur Rev Med Pharmacol Sci* 2012;**16**: 1741–7.
36. Silverman SL, Landesberg R. Osteonecrosis of the jaw and the role of bisphosphonates: a critical review. *Am J Med* 2009;**122**(2 Suppl):S33–45.
37. Bagan J, Scully C, Sabater V, Jimenez Y. Osteonecrosis of the jaws in patients treated with intravenous bisphosphonates (BRONJ): a concise update. *Oral Oncol* 2009;**45**:551–4.
38. Ruggiero SL, Dodson TB, Fantasia J, Goodday R, Aghaloo T, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons Position Paper on medication-related osteonecrosis of the jaw—2014 update. *J Oral Maxillofac Surg* 2014;**72**:1938–56.
39. Brozoski MA, Traina AA, Deboni MC, Marques MM, Naclério-Homem MdG. Osteonecrose maxilar associada ao uso de bisfosfonatos. *Rev Bras Reumatol* 2012;**52**: 265–70.
40. American Association of Oral and Maxillofacial Surgeons. Position Paper on bisphosphonate-related osteonecrosis of the jaws. *J Oral Maxillofac Surg* 2007;**65**: 369–76.
41. Migliorati CA, Siegel MA, Elting LS. Bisphosphonate-associated osteonecrosis: a long-term complication of bisphosphonate treatment. *Lancet Oncol* 2006;**7**:508–14.
42. Reid IR. Osteonecrosis of the jaw: who gets it, and why? *Bone* 2009;**44**:4–10.
43. Marunick M, Miller R, Gordon S. Adverse oral sequelae to bisphosphonate administration. *J Mich Dent Assoc* 2005;**87**:44–9.
44. Lerman MA, Xie W, Treister NS, Richardson PG, Weller EA, Woo SB. Conservative management of bisphosphonate-related osteonecrosis of the jaws: staging and treatment outcomes. *Oral Oncol* 2013;**49**: 977–83.
45. Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons Position Paper on bisphosphonate-related osteonecrosis of the jaw—2009 update. *Aust Endod J* 2009;**35**:119–30.
46. Magopoulos C, Karakinaris G, Telioudis Z, Vahtsevanos K, Dimitrakopoulos I, Antoniadis K, et al. Osteonecrosis of the jaws due to bisphosphonate use. A review of 60 cases and treatment proposals. *Am J Otolaryngol* 2007;**28**:158–63.
47. Bedogni A, Saia G, Ragazzo M, Bettini G, Capelli P, D'Alessandro E, et al. Bisphosphonate-associated osteonecrosis can hide jaw metastases. *Bone* 2007;**41**:942–5.
48. Marx RE, Cillo JE, Ulloa JJ. Oral bisphosphonate-induced osteonecrosis: risk factors, prediction of risk using serum CTX testing, prevention, and treatment. *J Oral Maxillofac Surg* 2007;**65**:2397–410.
49. Montebugnoli L, Felicetti L, Gissi DB, Pizzigallo A, Pelliccioni GA, Marchetti C. Biphosphonate-associated osteonecrosis can be controlled by nonsurgical management. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2007;**104**:473–7.
50. Vogel F, Scholz H, Al-Nawas B, Elies W, Kresken M, Lode H, et al. Rational use of oral antibiotics. Findings of an expert commission of the Paul Ehrlich Society for Chemotherapy. *Med Monatsschr Pharm* 2002;**25**:193–204.
51. Nocini PF, Saia G, Bettini G, Ragazzo M, Blandamura S, Chiarini L, et al. Vascularized fibula flap reconstruction of the mandible in bisphosphonate-related osteonecrosis. *Eur J Surg Oncol* 2009;**35**:373–9.
52. Freiberger JJ, Padilla-Burgos R, Chhoeu AH, Kraft KH, Boneta O, Moon RE, et al. Hyperbaric oxygen treatment and bisphosphonate-induced osteonecrosis of the jaw: a case series. *J Oral Maxillofac Surg* 2007;**65**:1321–7.
53. Pautke C, Bauer F, Tischer T, Kreutzer K, Weitz J, Kesting M, et al. Fluorescence-guided bone resection in bisphosphonate-associated osteonecrosis of the jaws. *J Oral Maxillofac Surg* 2009;**67**:471–6.
54. Vescovi P, Merigo E, Meleti M, Manfredi M. Bisphosphonate-associated osteonecrosis (BON) of the jaws: a possible treatment? *J Oral Maxillofac Surg* 2006;**64**:1460–2.
55. Curi MM, Cossolin GS, Koga DH, Araujo SR, Feher O, dos Santos MO, et al. Treatment of avascular osteonecrosis of the mandible in cancer patients with a history of bisphosphonate therapy by combining bone resection and autologous platelet-rich plasma: report of 3 cases. *J Oral Maxillofac Surg* 2007;**65**:349–55.
56. Adornato MC, Morcos I, Rozanski J. The treatment of bisphosphonate-associated osteonecrosis of the jaws with bone resection and autologous platelet-derived growth factors. *J Am Dent Assoc* 2007; **138**:971–7.
57. Cella L, Oppici A, Arbasi M, Moretto M, Piepoli M, Vallisa D, et al. Autologous bone marrow stem cell intralesional transplantation repairing bisphosphonate related osteonecrosis of the jaw. *Head Face Med* 2011;**7**:16.
58. Bashutski JD, Eber RM, Kinney JS, Benavides E, Maitra S, Braun TM, et al. Teriparatide and osseous regeneration in the oral cavity. *N Engl J Med* 2010;**363**: 2396–405.
59. Epstein MS, Wicknick FW, Epstein JB, Berenson JR, Gorsky M. Management of bisphosphonate-associated osteonecrosis: pentoxifylline and tocopherol in addition to antimicrobial therapy. An initial case series. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2010;**110**:593–6.

60. Petrucci MT, Gallucci C, Agrillo A, Mustazza MC, Foa R. Role of ozone therapy in the treatment of osteonecrosis of the jaws in multiple myeloma patients. *Haematologica* 2007;**92**:1289–90.
61. Moher D, Liberati A, Tetzlaff J, Altman DG, PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *J Clin Epidemiol* 2009;**62**:1006–12.
62. Burr DB. Summary of ASBMR Task Force on ONJ. *J Musculoskelet Neuronal Interact* 2007;**7**:354–5.
63. Ruggiero SL, Mehrotra B. Bisphosphonate-related osteonecrosis of the jaw: diagnosis, prevention, and management. *Annu Rev Med* 2009;**60**:85–96.
64. Ruggiero SL. Bisphosphonate-related osteonecrosis of the jaw: an overview. *Ann N Y Acad Sci* 2011;**1218**:38–46.
65. Khosla S, Burr D, Cauley J, Dempster DW, Ebeling PR, Felsenberg D, et al. Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research. *J Bone Miner Res* 2007;**22**:1479–91.
66. Rugani P, Acham S, Truschnegg A, Obermayer-Pietsch B, Jakse N. Bisphosphonate-associated osteonecrosis of the jaws: surgical treatment with ErCrYSGG-laser. Case report. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2010;**110**:E1–6.
67. Romeo U, Galanakis A, Marias C, Vecchio AD, Tenore G, Palaia G, et al. Observation of pain control in patients with bisphosphonate-induced osteonecrosis using low level laser therapy: preliminary results. *Photomed Laser Surg* 2011;**29**:447–52.
68. Mortensen M, Lawson W, Montazem A. Osteonecrosis of the jaw associated with bisphosphonate use: presentation of seven cases and literature review. *Laryngoscope* 2007;**117**:30–4.
69. Dannemann C, Zwahlen R, Gratz KW. Clinical experiences with bisphosphonate induced osteochemonecrosis of the jaws. *Swiss Med Wkly* 2006;**136**:504–9.
70. Vescovi P, Merigo E, Meleti M, Manfredi M, Guidotti R, Nammour S. Bisphosphonates-related osteonecrosis of the jaws: a concise review of the literature and a report of a single-centre experience with 151 patients. *J Oral Pathol Med* 2012;**41**:214–21.
71. Curi MM, Cossolin GSI, Koga DH, Zardetto C, Christianini S, Feher O, et al. Bisphosphonate-related osteonecrosis of the jaws—an initial case series report of treatment combining partial bone resection and autologous platelet-rich plasma. *J Oral Maxillofac Surg* 2011;**69**:2465–72.
72. Lazarovici TS, Yahalom R, Taicher S, Elad S, Hardan I, Yarom N. Bisphosphonate-related osteonecrosis of the jaws: a single-center study of 101 patients. *J Oral Maxillofac Surg* 2009;**67**:850–5.
73. Junquera L, Gallego L, Cuesta P, Pelaz A, de Vicente JC. Clinical experiences with bisphosphonate-associated osteonecrosis of the jaws: analysis of 21 cases. *Am J Otolaryngol* 2009;**30**:390–5.
74. Vescovi P, Merigo E, Meleti M, Fornaini C, Nammour S, Manfredi M. Nd:YAG laser biostimulation of bisphosphonate-associated necrosis of the jawbone with and without surgical treatment. *Br J Oral Maxillofac Surg* 2007;**45**:628–32.
75. Agrillo A, Ungari C, Filiaci F, Priore P, Iannetti G. Ozone therapy in the treatment of avascular bisphosphonate-related jaw osteonecrosis. *J Craniofac Surg* 2007;**18**:1071–5.
76. Longobardi G, Boniello R, Gasparini G, Pagano I, Pelo S. Surgical therapy for osteonecrotic lesions of the jaws in patients in therapy with bisphosphonates. *J Craniofac Surg* 2007;**18**:1012–7.
77. Chiu CT, Chiang WF, Chuang CY, Chang SW. Resolution of oral bisphosphonate and steroid-related osteonecrosis of the jaw—a serial case analysis. *J Oral Maxillofac Surg* 2010;**68**:1055–63.
78. Wutzl A, Eisenmenger G, Hoffmann M, Czerny C, Moser D, Pietschmann P, et al. Osteonecrosis of the jaws and bisphosphonate treatment in cancer patients. *Wien Klin Wochenschr* 2006;**118**:473–8.
79. Fleisher KE, Doty S, Kottal S, Phelan J, Norman RG, Glickman RS. Tetracycline-guided debridement and cone beam computed tomography for the treatment of bisphosphonate-related osteonecrosis of the jaw: a technical note. *J Oral Maxillofac Surg* 2008;**66**:2646–53.
80. Park W, Kim NK, Kim MY, Rhee YM, Kim HJ. Osteonecrosis of the jaw induced by oral administration of bisphosphonates in Asian population: five cases. *Osteoporos Int* 2010;**21**:527–33.
81. Rugani P, Truschnegg A, Acham S, Kirnbauer B, Jakse N. Use of photodynamic therapy in treatment of bisphosphonate-related osteonecrosis of the jaws: literature review and case series. *J Anal Bioanal Tech* 2013;**S1**:006.
82. Kwon YD, Lee DW, Choi BJ, Lee JW, Kim DY. Short-term teriparatide therapy as an adjunctive modality for bisphosphonate-related osteonecrosis of the jaws. *Osteoporos Int* 2012;**23**:2721–5.
83. Cicciu M, Herford AS, Juodzbalys G, Stoffella E. Recombinant human bone morphogenetic protein type 2 application for a possible treatment of bisphosphonates-related osteonecrosis of the jaw. *J Craniofac Surg* 2012;**23**:784–8.
84. Blus C, Szmukler-Moncler S, Giannelli G, Denotti G, Orru G. Use of ultrasonic bone surgery (Piezosurgery) to surgically treat bisphosphonate-related osteonecrosis of the jaws (BRONJ). A case series report with at least 1 year of follow-up. *Open Dent J* 2013;**7**:94–101.
85. Lemound J, Eckardt A, Kokemuller H, von See C, Voss PJ, Tavassol F, et al. Bisphosphonate-associated osteonecrosis of the mandible: reliable soft tissue reconstruction using a local myofascial flap. *Clin Oral Investig* 2012;**16**:1143–52.
86. Lazarovici TS, Yahalom R, Taicher S, Schwartz-Arad D, Peleg O, Yarom N. Bisphosphonate-related osteonecrosis of the jaw associated with dental implants. *J Oral Maxillofac Surg* 2010;**68**:790–6.
87. Narvaez J, Narvaez JA, Gomez-Vaquero C, Nolla JM. Lack of response to teriparatide therapy for bisphosphonate-associated osteonecrosis of the jaw. *Osteoporos Int* 2013;**24**:731–3.
88. Watters AL, Hansen HJ, Williams T, Chou JF, Riedel E, Halpern J, et al. Intravenous bisphosphonate-related osteonecrosis of the jaw: long-term follow-up of 109 patients. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2013;**115**:192–200.
89. Nomura T, Shibahara T, Uchiyama T, Yamamoto N, Shibui T, Yakushiji T, et al. Bisphosphonate-related osteonecrosis of jaw (BRONJ) in Japanese population: a case series of 13 patients at our clinic. *Bull Tokyo Dent Coll* 2013;**54**:117–25.
90. Jabbour Z, El-Hakim M, Mesbah-Ardakani P, Henderson JE, Albuquerque Jr R. The outcomes of conservative and surgical treatment of stage 2 bisphosphonate-related osteonecrosis of the jaws: a case series. *Int J Oral Maxillofac Surg* 2012;**41**:1404–9.
91. Vescovi P, Merigo E, Manfredi M, Meleti M, Fornaini C, Bonanini M, et al. Nd:YAG laser biostimulation in the treatment of bisphosphonate-associated osteonecrosis of the jaw: clinical experience in 28 cases. *Photomed Laser Surg* 2008;**26**:37–46.
92. Hanasono MM, Militsakh ON, Richmon JD, Rosenthal EL, Wax MK. Mandibulectomy and free flap reconstruction for bisphosphonate-related osteonecrosis of the jaws. *JAMA Otolaryngol Head Neck Surg* 2013;**139**:1135–42.
93. Coviello V, Peluso F, Dehkhargani SZ, Verdugo F, Raffaelli L, Manicone PF, et al. Platelet-rich plasma improves wound healing in multiple myeloma bisphosphonate-associated osteonecrosis of the jaw patients. *J Biol Regul Homeost Agents* 2012;**26**:151–5.
94. Ferlito S, Puzzo S, Palermo F, Verzi P. Treatment of bisphosphonate-related osteonecrosis of the jaws: presentation of a protocol and an observational longitudinal study of an Italian series of cases. *Br J Oral Maxillofac Surg* 2012;**50**:425–9.
95. Anavi-Lev K, Anavi Y, Chaushu G, Alon DM, Gal G, Kaplan I. Bisphosphonate related osteonecrosis of the jaws: clinicopathological investigation and histomorphometric analysis. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2013;**115**:660–6.

96. Ripamonti CI, Cislaghi E, Mariani L, Maniezzo M. Efficacy and safety of medical ozone (O(3)) delivered in oil suspension applications for the treatment of osteonecrosis of the jaw in patients with bone metastases treated with bisphosphonates: preliminary results of a phase I–II study. *Oral Oncol* 2011;**47**:185–90.
97. Freiberger JJ, Padilla-Burgos R, McGraw T, Suliman HB, Kraft KH, Stolp BW, et al. What is the role of hyperbaric oxygen in the management of bisphosphonate-related osteonecrosis of the jaw: a randomized controlled trial of hyperbaric oxygen as an adjunct to surgery and antibiotics. *J Oral Maxillofac Surg* 2012;**70**:1573–83.
98. Ripamonti CI, Maniezzo M, Boldini S, Pessi MA, Mariani L, Cislaghi E. Efficacy and tolerability of medical ozone gas insufflations in patients with osteonecrosis of the jaw treated with bisphosphonates—preliminary data. *J Bone Oncol* 2012;**1**:81–7.
99. Pautke C, Bauer F, Otto S, Tischer T, Steiner T, Weitz J, et al. Fluorescence-guided bone resection in bisphosphonate-related osteonecrosis of the jaws: first clinical results of a prospective pilot study. *J Oral Maxillofac Surg* 2011;**69**:84–91.
100. Mücke T, Koschinski J, Deppe H, Wagenpfeil S, Pautke C, Mitchell DA, et al. Outcome of treatment and parameters influencing recurrence in patients with bisphosphonate-related osteonecrosis of the jaws. *J Cancer Res Clin Oncol* 2011;**137**:907–13.
101. Holzinger D, Seemann R, Klug C, Ewers R, Millesi G, Baumann A, et al. Long-term success of surgery in bisphosphonate-related osteonecrosis of the jaws (BRONJs). *Oral Oncol* 2013;**49**:66–70.
102. Beninati F, Pruneti R, Ficarra G. Bisphosphonate-related osteonecrosis of the jaws (BRONJ). *Med Oral Patol Oral Cir Bucal* 2013;**18**:e752–8.
103. Carlson ER, Basile JD. The role of surgical resection in the management of bisphosphonate-related osteonecrosis of the jaws. *J Oral Maxillofac Surg* 2009;**67**(5 Suppl):85–95.
104. Fortuna G, Ruoppo E, Pollio A, Aria M, Adamo D, Leuci S, et al. Multiple myeloma vs. breast cancer patients with bisphosphonates-related osteonecrosis of the jaws: a comparative analysis of response to treatment and predictors of outcome. *J Oral Pathol Med* 2012;**41**:222–8.
105. Boonyapakorn T, Schirmer I, Reichart PA, Sturm I, Massenkeil G. Bisphosphonate-induced osteonecrosis of the jaws: prospective study of 80 patients with multiple myeloma and other malignancies. *Oral Oncol* 2008;**44**:857–69.
106. Stockmann P, Vairaktaris E, Wehrhan F, Seiss M, Schwarz S, Spriewald B, et al. Osteotomy and primary wound closure in bisphosphonate-associated osteonecrosis of the jaw: a prospective clinical study with 12 months follow-up. *Support Care Cancer* 2010;**18**:449–60.
107. Williamson RA. Surgical management of bisphosphonate induced osteonecrosis of the jaws. *Int J Oral Maxillofac Surg* 2010;**39**:251–5.
108. Moretti F, Pelliccioni GA, Montebugnoli L, Marchetti C. A prospective clinical trial for assessing the efficacy of a minimally invasive protocol in patients with bisphosphonate-associated osteonecrosis of the jaws. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011;**112**:777–82.
109. Alsehimy MM. Efficacy of a nonsurgical treatment regimen in patients with bisphosphonate-related osteonecrosis of the jaws in Saudi Arabia. *SAGE Open Med* 2014:2. http://dx.doi.org/10.1177/2050312114522995.
110. Wutzl A, Pohl S, Sulzbacher I, Seemann R, Lauer G, Ewers R, et al. Factors influencing surgical treatment of bisphosphonate-related osteonecrosis of the jaws. *Head Neck* 2012;**34**:194–200.
111. Scoletta M, Arduino PG, Reggio L, Dalmasso P, Mozzati M. Effect of low-level laser irradiation on bisphosphonate-induced osteonecrosis of the jaws: preliminary results of a prospective study. *Photomed Laser Surg* 2010;**28**:179–84.
112. Bocanegra-Perez S, Vicente-Barrero M, Knezevic M, Castellano-Navarro JM, Rodriguez-Bocanegra E, Rodriguez-Millares J, et al. Use of platelet-rich plasma in the treatment of bisphosphonate-related osteonecrosis of the jaw. *Int J Oral Maxillofac Surg* 2012;**41**:1410–5.
113. Scoletta M, Arduino PG, Dalmasso P, Broccoletti R, Mozzati M. Treatment outcomes in patients with bisphosphonate-related osteonecrosis of the jaws: a prospective study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2010;**110**:46–53.
114. Nicolatou-Galitis O, Papadopoulou E, Sarri T, Boziari P, Karayianni A, Kyrtsonis MC, et al. Osteonecrosis of the jaw in oncology patients treated with bisphosphonates: prospective experience of a dental oncology referral center. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011;**112**:195–202.
115. Bedogni A, Saia G, Bettini G, Tronchet A, Totola A, Bedogni G, et al. Long-term outcomes of surgical resection of the jaws in cancer patients with bisphosphonate-related osteonecrosis. *Oral Oncol* 2011;**47**:420–4.
116. Schubert M, Klatte I, Linek W, Muller B, Doring K, Eckelt U, et al. The Saxon bisphosphonate register—therapy and prevention of bisphosphonate-related osteonecrosis of the jaws. *Oral Oncol* 2012;**48**:349–54.
117. Thumbigere-Math V, Sabino MC, Gopalakrishnan R, Huckabay S, Dudek AZ, Basu S, et al. Bisphosphonate-related osteonecrosis of the jaw: clinical features, risk factors, management, and treatment outcomes of 26 patients. *J Oral Maxillofac Surg* 2009;**67**:1904–13.
118. Thumbigere-Math V, Tu L, Huckabay S, Dudek AZ, Lunos S, Basi DL, et al. A retrospective study evaluating frequency and risk factors of osteonecrosis of the jaw in 576 cancer patients receiving intravenous bisphosphonates. *Am J Clin Oncol* 2012;**35**:386–92.
119. Martins MA, Martins MD, Lascala CA, Curi MM, Migliorati CA, Tenis CA, et al. Association of laser phototherapy with PRP improves healing of bisphosphonate-related osteonecrosis of the jaws in cancer patients: a preliminary study. *Oral Oncol* 2012;**48**:79–84.
120. Angiero F, Sannino C, Borloni R, Crippa R, Benedicenti S, Romanos GE. Osteonecrosis of the jaws caused by bisphosphonates: evaluation of a new therapeutic approach using the Er:YAG laser. *Lasers Med Sci* 2009;**24**:849–56.
121. Vescovi P, Merigo E, Meleti M, Manfredi M, Fornaini C, Nammour S, et al. Conservative surgical management of stage I bisphosphonate-related osteonecrosis of the jaw. *Int J Dent 2014* 2014:1076–90.
122. Hoefert S, Eufinger H. Relevance of a prolonged preoperative antibiotic regime in the treatment of bisphosphonate-related osteonecrosis of the jaw. *J Oral Maxillofac Surg* 2011;**69**:362–80.
123. Vescovi P, Campisi G, Fusco V, Mergoni G, Manfredi M, Merigo E, et al. Surgery-triggered and non surgery-triggered bisphosphonate-related osteonecrosis of the jaws (BRONJ): a retrospective analysis of 567 cases in an Italian multicenter study. *Oral Oncol* 2011;**47**:191–4.
124. Seth R, Futran ND, Alam DS, Knott PD. Outcomes of vascularized bone graft reconstruction of the mandible in bisphosphonate-related osteonecrosis of the jaws. *Laryngoscope* 2010;**120**:2165–71.
125. Saussez S, Javadian R, Hupin C, Magremanne M, Chantrain G, Loeb I, et al. Bisphosphonate-related osteonecrosis of the jaw and its associated risk factors: a Belgian case series. *Laryngoscope* 2009;**119**:323–9.
126. Alons K, Kuijpers SC, de Jong E, van Merkesteyn JP. Treating low- and medium-potency bisphosphonate-related osteonecrosis of the jaws with a protocol for the treatment of chronic suppurative osteomyelitis: report of 7 cases. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2009;**107**:e1–7.
127. Yarom N, Yahalom R, Shoshani Y, Hamed W, Regev E, Elad S. Osteonecrosis of the jaw induced by orally administered bisphosphonates: incidence, clinical features, predisposing factors and treatment outcome. *Osteoporos Int* 2007;**18**:1363–70.
128. Stanton DC, Balasanian E. Outcome of surgical management of bisphosphonate-related osteonecrosis of the jaws: review

of 33 surgical cases. *J Oral Maxillofac Surg* 2009;**67**:943–50.
129. O'Ryan FS, Khoury S, Liao W, Han MM, Hui RL, Baer D, et al. Intravenous bisphosphonate-related osteonecrosis of the jaw: bone scintigraphy as an early indicator. *J Oral Maxillofac Surg* 2009;**67**:1363–72.
130. Estilo CL, Van Poznak CH, Wiliams T, Bohle GC, Lwin PT, Zhou Q, et al. Osteonecrosis of the maxilla and mandible in patients with advanced cancer treated with bisphosphonate therapy. *Oncologist* 2008;**13**:911–20.
131. Dimitrakopoulos I, Magopoulos C, Karakasis D. Bisphosphonate-induced avascular osteonecrosis of the jaws: a clinical report of 11 cases. *Int J Oral Maxillofac Surg* 2006;**35**:588–93.
132. Graziani F, Vescovi P, Campisi G, Favia G, Gabriele M, Gaeta GM, et al. Resective surgical approach shows a high performance in the management of advanced cases of bisphosphonate-related osteonecrosis of the jaws: a retrospective survey of 347 cases. *J Oral Maxillofac Surg* 2012;**70**:2501–7.
133. Mercer E, Norton T, Woo S, Treister N, Dodson TB, Solomon DH. Ninety-one osteoporosis patients affected with bisphosphonate-related osteonecrosis of the jaw: a case series. *Calcif Tissue Int* 2013;**93**:241–8.
134. Abu-Id MH, Warnke PH, Gottschalk J, Springer I, Wiltfang J, Acil Y, et al. 'Bisphossy jaws'—high and low risk factors for bisphosphonate-induced osteonecrosis of the jaw. *J Craniomaxillofac Surg* 2008;**36**:95–103.
135. Pozzi S, Marcheselli R, Sacchi S, Baldini L, Angrilli F, Pennese E, et al. Bisphosphonate-associated osteonecrosis of the jaw: a review of 35 cases and an evaluation of its frequency in multiple myeloma patients. *Leuk Lymphoma* 2007;**48**:56–64.
136. Kos M, Brusco D, Kuebler J, Engelke W. Clinical comparison of patients with osteonecrosis of the jaws, with and without a history of bisphosphonates administration. *Int J Oral Maxillofac Surg* 2010;**39**:1097–102.
137. Manfredi M, Merigo E, Guidotti R, Meleti M, Vescovi P. Bisphosphonate-related osteonecrosis of the jaws: a case series of 25 patients affected by osteoporosis. *Int J Oral Maxillofac Surg* 2011;**40**:277–84.
138. Maurer P, Sandulescu T, Kriwalsky MS, Rashad A, Hollstein S, Stricker I, et al. Bisphosphonate-related osteonecrosis of the maxilla and sinusitis maxillaris. *Int J Oral Maxillofac Surg* 2011;**40**:285–91.
139. Hong JW, Nam W, Cha IH, Chung SW, Choi HS, Kim KM, et al. Oral bisphosphonate-related osteonecrosis of the jaw: the first report in Asia. *Osteoporos Int* 2010;**21**:847–53.
140. Mozzati M, Gallesio G, Arata V, Pol R, Scoletta M. Platelet-rich therapies in the treatment of intravenous bisphosphonate-related osteonecrosis of the jaw: a report of 32 cases. *Oral Oncol* 2012;**48**:469–74.
141. Urade M, Tanaka N, Furusawa K, Shimada J, Shibata T, Kirita T, et al. Nationwide survey for bisphosphonate-related osteonecrosis of the jaws in Japan. *J Oral Maxillofac Surg* 2011;**69**:e364–71.
142. Badros A, Weikel D, Salama A, Goloubeva O, Schneider A, Rapoport A, et al. Osteonecrosis of the jaw in multiple myeloma patients: clinical features and risk factors. *J Clin Oncol* 2006;**24**:945–52.
143. Atalay B, Yalcin S, Emes Y, Aktas I, Aybar B, Issever H, et al. Bisphosphonate-related osteonecrosis: laser-assisted surgical treatment or conventional surgery? *Lasers Med Sci* 2011;**26**:815–23.
144. O'Ryan FS, Lo JC. Bisphosphonate-related osteonecrosis of the jaw in patients with oral bisphosphonate exposure: clinical course and outcomes. *J Oral Maxillofac Surg* 2012;**70**:1844–53.
145. Wilde F, Heufelder M, Winter K, Hendricks J, Frerich B, Schramm A, et al. The role of surgical therapy in the management of intravenous bisphosphonates-related osteonecrosis of the jaw. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2011;**111**:153–63.
146. Vescovi P, Manfredi M, Merigo E, Meleti M, Fornaini C, Rocca JP, et al. Surgical approach with Er:YAG laser on osteonecrosis of the jaws (ONJ) in patients under bisphosphonate therapy (BPT). *Lasers Med Sci* 2010;**25**:101–13.
147. Jacobsen C, Metzler P, Obwegeser JA, Zemann W, Graetz KW. Osteopathology of the jaw associated with bone resorption inhibitors: what have we learned in the last 8 years? *Swiss Med Wkly* 2012;**142**:w13605.
148. Voss PJ, Oshero JJ, Kovalova-Muller A, Merino EAV, Sauerbier S, Al-Jamali J, et al. Surgical treatment of bisphosphonate-associated osteonecrosis of the jaw: technical report and follow up of 21 patients. *J Craniomaxillofac Surg* 2012;**40**:719–25.
149. Kim KM, Park W, Oh SY, Kim HJ, Nam W, Lim SK, et al. Distinctive role of 6-month teriparatide treatment on intractable bisphosphonate-related osteonecrosis of the jaw. *Osteoporos Int* 2014;**25**:1625–32.
150. Kim SK, Kwon TG. Clinical investigation of bisphosphonate-related osteonecrosis of the jaws in patients with malignant tumors. *J Korean Assoc Oral Maxillofac Surg* 2012;**38**:152–9.
151. Stübinger S, Dissmann JP, Pinho NC, Saldamli B, Seitz O, Sader R. A preliminary report about treatment of bisphosphonate related osteonecrosis of the jaw with Er:YAG laser ablation. *Lasers Surg Med* 2009;**41**:26–30.
152. Elad S, Yarom N, Hamed W, Ayalon S, Yahalom R, Regev E. Osteomyelitis and necrosis of the jaw in patients treated with bisphosphonates: a comparative study focused on multiple myeloma. *Clin Lab Haematol* 2006;**28**:393–8.
153. Van den Wyngaert T, Claeys T, Huizing MT, Vermorken JB, Fossion E. Initial experience with conservative treatment in cancer patients with osteonecrosis of the jaw (ONJ) and predictors of outcome. *Ann Oncol* 2009;**20**:331–6.
154. Hansen PJ, Knitschke M, Draenert FG, Irle S, Neff A. Incidence of bisphosphonate-related osteonecrosis of the jaws (BRONJ) in patients taking bisphosphonates for osteoporosis treatment—a grossly underestimated risk? *Clin Oral Investig* 2013;**17**:1829–37.
155. Woo SB, Hellstein JW, Kalmar JR. Narrative [corrected] review: Bisphosphonates and osteonecrosis of the jaws. *Ann Intern Med* 2006;**144**:753–61.
156. Mavrokokki T, Cheng A, Stein B, Goss A. Nature and frequency of bisphosphonate-associated osteonecrosis of the jaws in Australia. *J Oral Maxillofac Surg* 2007;**65**:415–23.
157. Dannemann C, Gratz KW, Riener MO, Zwahlen RA. Jaw osteonecrosis related to bisphosphonate therapy: a severe secondary disorder. *Bone* 2007;**40**:828–34.
158. Olutayo J, Agbaje JO, Jacobs R, Verhaeghe V, Velde FV, Vinckier F. Bisphosphonate-related osteonecrosis of the jaw bone: radiological pattern and the potential role of CBCT in early diagnosis. *J Oral Maxillofac Res* 2010;**1**:e3.
159. Bamias A, Kastritis E, Bamia C, Moulopoulos LA, Melakopoulos I, Bozas G, et al. Osteonecrosis of the jaw in cancer after treatment with bisphosphonates: incidence and risk factors. *J Clin Oncol* 2005;**23**:8580–7.
160. Dunstan CR, Felsenberg D, Seibel MJ. Therapy insight: the risks and benefits of bisphosphonates for the treatment of tumor-induced bone disease. *Nat Clin Pract Oncol* 2007;**4**:42–55.
161. Hoff AO, Toth BB, Altundag K, Johnson MM, Warneke CL, Hu M, et al. Frequency and risk factors associated with osteonecrosis of the jaw in cancer patients treated with intravenous bisphosphonates. *J Bone Miner Res* 2008;**23**:826–36.
162. Dimopoulos MA, Kastritis E, Anagnostopoulos A, Melakopoulos I, Gika D, Moulopoulos LA, et al. Osteonecrosis of the jaw in patients with multiple myeloma treated with bisphosphonates: evidence of increased risk after treatment with zoledronic acid. *Haematologica* 2006;**91**:968–71.
163. Dimopoulos MA, Kastritis E, Moulopoulos LA, Melakopoulos I, Anagnostopoulos A, Gika D, et al. The incidence of osteonecrosis of the jaw (ONJ) in patients with

multiple myeloma who receive biphosphonates depends on the type of biphosphonate. *Blood* 2005;**106**:637.
164. Fleisch H. Bisphosphonates: mechanisms of action. *Endocr Rev* 1998;**19**:80–100.
165. Lo JC, O'Ryan FS, Gordon NP, Yang J, Hui RL, Martin D, et al. Prevalence of osteonecrosis of the jaw in patients with oral bisphosphonate exposure. *J Oral Maxillofac Surg* 2010;**68**:243–53.
166. Osteonecrosis of the jaw and bisphosphonates. *N Engl J Med* 2005;**353**:99–102.
167. Ruggiero SL, Dodson TB, Assael LA, Landesberg R, Marx RE, Mehrotra B, et al. American Association of Oral and Maxillofacial Surgeons Position Paper on bisphosphonate-related osteonecrosis of the jaws—2009 update. *J Oral Maxillofac Surg* 2009;**67**(5 Suppl):2–12.
168. Cafro AM, Barbarano LA, Andriani A, D'Avanzo G, Nichelatti M, Gaglioti D, et al. Osteonecrosis of the jaw associated with chronic bisphosphonates therapy: an Italian experience. *ASH Annu Meet Abstr* 2005;**106**:5152.
169. King AE, Umland EM. Osteonecrosis of the jaw in patients receiving intravenous or oral bisphosphonates. *Pharmacotherapy* 2008;**28**:667–77.
170. Cavanna L, Bertè R, Arcari A, Mordenti P, Pagani R, Vallisa D. Osteonecrosis of the jaw. A newly emerging site-specific osseous pathology in patients with cancer treated with bisphosphonates. Report of five cases and review of the literature. *Eur J Intern Med* 2007;**18**:417–22.
171. Badros A, Terpos E, Katodritou E, Goloubeva O, Kastritis E, Verrou E, et al. Natural history of osteonecrosis of the jaw in patients with multiple myeloma. *J Clin Oncol* 2008;**26**:5904–9.
172. Filleul O, Crompot E, Saussez S. Bisphosphonate-induced osteonecrosis of the jaw: a review of 2,400 patient cases. *J Cancer Res Clin Oncol* 2010;**136**:1117–24.
173. Lugassy G, Shaham R, Nemets A, Ben-Dor D, Nahlieli O. Severe osteomyelitis of the jaw in long-term survivors of multiple myeloma: a new clinical entity. *Am J Med* 2004;**117**:440–1.
174. Pires FR, Miranda A, Cardoso ES, Cardoso AS, Fregnani ER, Pereira CM, et al. Oral avascular bone necrosis associated with chemotherapy and biphosphonate therapy. *Oral Dis* 2005;**11**:365–9.
175. Saad F, Brown JE, Van Poznak C, Ibrahim T, Stemmer SM, Stopeck AT, et al. Incidence, risk factors, and outcomes of osteonecrosis of the jaw: integrated analysis from three blinded active-controlled phase III trials in cancer patients with bone metastases. *Ann Oncol* 2012;**23**:1341–7.
176. Then C, Horauf N, Otto S, Pautke C, von Tresckow E, Rohnisch T, et al. Incidence and risk factors of bisphosphonate-related osteonecrosis of the jaw in multiple myeloma patients having undergone autologous stem cell transplantation. *Onkologie* 2012;**35**:658–64.
177. Migliorati CA, Epstein JB, Abt E, Berenson JR. Osteonecrosis of the jaw and bisphosphonates in cancer: a narrative review. *Nat Rev Endocrinol* 2011;**7**:34–42.
178. Hoff AO, Toth B, Hu M, Hortobagyi GN, Gagel RF. Epidemiology and risk factors for osteonecrosis of the jaw in cancer patients. *Ann N Y Acad Sci* 2011;**1218**:47–54.
179. Ziebart T, Pabst A, Klein MO, Kammerer P, Gauss L, Brullmann D, et al. Bisphosphonates: restrictions for vasculogenesis and angiogenesis: inhibition of cell function of endothelial progenitor cells and mature endothelial cells in vitro. *Clin Oral Investig* 2011;**15**:105–11.
180. Baqain ZH, Sawair FA, Tamimi Z, Bsoul N, Al Edwan G, Almasad JK, et al. Osteonecrosis of jaws related to intravenous bisphosphonates: the experience of a Jordanian teaching hospital. *Ann R Coll Surg Engl* 2010;**92**:489–94.
181. Vahtsevanos K, Kyrgidis A, Verrou E, Katodritou E, Triaridis S, Andreadis CG, et al. Longitudinal cohort study of risk factors in cancer patients of bisphosphonate-related osteonecrosis of the jaw. *J Clin Oncol* 2009;**27**:5356–62.
182. Hoff AO, Toth BB, Altundag K, Guarneri V, Adamus A, Nooka AK, et al. Osteonecrosis of the jaw in patients receiving intravenous bisphosphonate therapy. *J Clin Oncol* 2006;**24**:475s.
183. Schiffrin EJ, Morley JE, Donnet-Hughes A, Guigoz Y. The inflammatory status of the elderly: the intestinal contribution. *Mutat Res* 2010;**690**:50–6.
184. Johnson TE. Recent results: biomarkers of aging. *Exp Gerontol* 2006;**41**:1243–6.
185. Misawa Y, Kageyama T, Moriyama K, Kurihara S, Yagasaki H, Deguchi T, et al. Effect of age on alveolar bone turnover adjacent to maxillary molar roots in male rats: a histomorphometric study. *Arch Oral Biol* 2007;**52**:44–50.
186. Semba I, Funakoshi K, Kitano M. Histomorphometric analysis of age changes in the human inferior alveolar artery. *Arch Oral Biol* 2001;**46**:13–21.
187. Kulikov VY, Fridman YM, Fomin AN. Role of oxidative stress in mechanisms of premature aging in shift labor workers. *Alaska Med* 2007;**49**(2 Suppl):81–4.
188. Maines E, Monti E, Doro F, Morandi G, Cavarzere P, Antoniazzi F. Children and adolescents treated with neridronate for osteogenesis imperfecta show no evidence of any osteonecrosis of the jaw. *J Bone Miner Metab* 2012;**30**:434–8.
189. Dental management of patients receiving oral bisphosphonate therapy: expert panel recommendations. *J Am Dent Assoc* 2006;**137**:1144–50.
190. Malden NJ, Pai AY. Oral bisphosphonate associated osteonecrosis of the jaws: three case reports. *Br Dent J* 2007;**203**:93–7.
191. Khamaisi M, Regev E, Yarom N, Avni B, Leitersdorf E, Raz I, et al. Possible association between diabetes and bisphosphonate-related jaw osteonecrosis. *J Clin Endocrinol Metab* 2007;**92**:1172–5.
192. Mehanna P, Goddard R. Bisphosphonate associated osteonecrosis: an unusual case. *Aust Dent J* 2010;**55**:311–3.
193. Favia G, Pilolli GP, Maiorano E. Osteonecrosis of the jaw correlated to bisphosphonate therapy in non-oncologic patients: clinicopathological features of 24 patients. *J Rheumatol* 2009;**36**:2780–7.
194. Sedghizadeh PP, Stanley K, Caligiuri M, Hofkes S, Lowry B, Shuler CF. Oral bisphosphonate use and the prevalence of osteonecrosis of the jaw: an institutional inquiry. *J Am Dent Assoc* 2009;**140**:61–6.
195. Shin EY, Kwon YH, Herr Y, Shin SI, Chung JH. Implant failure associated with oral bisphosphonate-related osteonecrosis of the jaw. *J Periodontal Implant Sci* 2010;**40**:90–5.
196. Markenson JA. Worldwide trends in the socioeconomic impact and long-term prognosis of rheumatoid-arthritis. *Semin Arthritis Rheum* 1991;**21**:4–12.
197. Bedogni A, Blandamura S, Lokmic Z, Palumbo C, Ragazzo M, Ferrari F, et al. Bisphosphonate-associated jawbone osteonecrosis: a correlation between imaging techniques and histopathology. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2008;**105**:358–64.
198. Green JR. Bisphosphonates: preclinical review. *Oncologist* 2004;**9**(Suppl 4):3–13.
199. Milner RJ, Farese J, Henry CJ, Selting K, Fan TM, de Lorimier LP. Bisphosphonates and cancer. *J Vet Intern Med* 2004;**18**:597–604.
200. Vincenzi B, Santini D, Dicuonzo G, Battistoni F, Gavasci M, La Cesa A, et al. Zoledronic acid-related angiogenesis modifications and survival in advanced breast cancer patients. *J Interferon Cytokine Res* 2005;**25**:144–51.
201. Wood J, Bonjean K, Ruetz S, Bellahcene A, Devy L, Foidart JM, et al. Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. *J Pharmacol Exp Ther* 2002;**302**:1055–61.
202. Marx RE. Osteoradionecrosis: a new concept of its pathophysiology. *J Oral Maxillofac Surg* 1983;**41**:283–8.
203. Chuah C, Barnes DJ, Kwok M, Corbin A, Deininger MW, Druker BJ, et al. Zoledronate inhibits proliferation and induces apoptosis of imatinib-resistant chronic myeloid leukaemia cells. *Leukemia* 2005;**19**:1896–904.
204. Gronqvist A, Wistrom J, Axner O, Monsen TJ. Bactericidal effect of pulsed 1,064 nm Nd:YAG laser light on *Staphylococcus epidermidis* is of photothermal origin: an in vitro study. *Lasers Surg Med* 2000;**27**:336–40.

205. Mitsimponas K, Sereti M, Semergidis T. Bisphosphonate associated osteonecrosis: role of surgery. *J Craniomaxillofac Surg* 2008;**36**:S33.
206. Eckert AW, Maurer P, Meyer L, Kriwalsky MS, Rohrberg R, Schneider D, et al. Bisphosphonate-related jaw necrosis—severe complication in maxillofacial surgery. *Cancer Treat Rev* 2007;**33**:58–63.
207. Millesi G, Wutzl A, Biedermann E, Karschigijew G, Ewers R. Bisphosphonate induced osteonecrosis of the JAWS (BIONJ): six months follow up. *Int J Oral Maxillofac Surg* 2007;**36**:1043.
208. Otto S, Hafner S, Grotz KA. The role of inferior alveolar nerve involvement in bisphosphonate-related osteonecrosis of the jaw. *J Oral Maxillofac Surg* 2009;**67**: 589–92.
209. Gomez Font R, Martinez Garcia ML, Olmos Martinez JM. Osteochemonecrosis of the jaws due to bisphosphonate treatments. Update. *Med Oral Patol Oral Cir Bucal* 2008;**13**:E318–24.
210. Nase JB, Suzuki JB. Osteonecrosis of the jaw and oral bisphosphonate treatment. *J Am Dent Assoc* 2006;**137**:1115–9. [quiz 69–70].
211. Lee CY, David T, Nishime M. Use of platelet-rich plasma in the management of oral biphosphonate-associated osteonecrosis of the jaw: a report of 2 cases. *J Oral Implantol* 2007;**33**:371–82.
212. Arora NS, Ramanayake T, Ren YF, Romanos GE. Platelet-rich plasma: a literature review. *Implant Dent* 2009;**18**:303–10.
213. Lopez-Vidriero E, Goulding KA, Simon DA, Sanchez M, Johnson DH. The use of platelet-rich plasma in arthroscopy and sports medicine: optimizing the healing environment. *Arthroscopy* 2010;**26**:269–78.
214. Plachokova AS, Nikolidakis D, Mulder J, Jansen JA, Creugers NH. Effect of platelet-rich plasma on bone regeneration in dentistry: a systematic review. *Clin Oral Implants Res* 2008;**19**:539–45.
215. Bocci V. Does ozone therapy normalize the cellular redox balance? Implications for therapy of human immunodeficiency virus infection and several other diseases. *Med Hypotheses* 1996;**46**:150–4.
216. Hernandez F, Menendez S, Wong R. Decrease of blood cholesterol and stimulation of antioxidative response in cardiopathy patients treated with endovenous ozone therapy. *Free Radic Biol Med* 1995;**19**: 115–9.
217. Csonka C, Pataki T, Kovacs P, Muller SL, Schroeter ML, Tosaki A, et al. Effects of oxidative stress on the expression of antioxidative defense enzymes in spontaneously hypertensive rat hearts. *Free Radic Biol Med* 2000;**29**:612–9.
218. Bocci V. Ozone as Janus: this controversial gas can be either toxic or medically useful. *Mediators Inflamm* 2004;**13**:3–11.
219. Reth M. Hydrogen peroxide as second messenger in lymphocyte activation. *Nat Immunol* 2002;**3**:1129–34.
220. Babior BM, Takeuchi C, Ruedi J, Gutierrez A, Wentworth Jr P. Investigating antibody-catalyzed ozone generation by human neutrophils. *Proc Natl Acad Sci U S A* 2003;**100**:3031–4.
221. Freiberger JJ. Utility of hyperbaric oxygen in treatment of bisphosphonate-related osteonecrosis of the jaws. *J Oral Maxillofac Surg* 2009;**67**(5 Suppl):96–106.
222. Boykin Jr JV, Baylis C. Hyperbaric oxygen therapy mediates increased nitric oxide production associated with wound healing: a preliminary study. *Adv Skin Wound Care* 2007;**20**:382–8.
223. Rubin MR, Bilezikian JP. The anabolic effects of parathyroid hormone therapy. *Clin Geriatr Med* 2003;**19**:415–32.
224. Quattrocchi E, Kourlas H. Teriparatide: a review. *Clin Ther* 2004;**26**:841–54.
225. Pleiner-Duxneuner J, Zwettler E, Paschalis E, Roschger P, Nell-Duxneuner V, Klaushofer K. Treatment of osteoporosis with parathyroid hormone and teriparatide. *Calcif Tissue Int* 2009;**84**:159–70.
226. McClung MR, San Martin J, Miller PD, Civitelli R, Bandeira F, Omizo M, et al. Opposite bone remodeling effects of teriparatide and alendronate in increasing bone mass. *Arch Intern Med* 2005;**165**:1762–8.
227. Chen P, Satterwhite JH, Licata AA, Lewiecki EM, Sipos AA, Misurski DM, et al. Early changes in biochemical markers of bone formation predict BMD response to teriparatide in postmenopausal women with osteoporosis. *J Bone Miner Res* 2005;**20**: 962–70.
228. Lindsay R, Cosman F, Zhou H, Bostrom MP, Shen VW, Cruz JD, et al. A novel tetracycline labeling schedule for longitudinal evaluation of the short-term effects of anabolic therapy with a single iliac crest bone biopsy: early actions of teriparatide. *J Bone Miner Res* 2006;**21**:366–73.
229. Ettinger B, San Martin J, Crans G, Pavo I. Differential effects of teriparatide on BMD after treatment with raloxifene or alendronate. *J Bone Miner Res* 2004;**19**:745–51.
230. Ma YL, Bryant HU, Zeng Q, Schmidt A, Hoover J, Cole HW, et al. New bone formation with teriparatide [human parathyroid hormone-(1-34)] is not retarded by long-term pretreatment with alendronate, estrogen, or raloxifene in ovariectomized rats. *Endocrinology* 2003;**144**:2008–15.
231. Harper RP, Fung E. Resolution of bisphosphonate-associated osteonecrosis of the mandible: possible application for intermittent low-dose parathyroid hormone [rhPTH(1-34)]. *J Oral Maxillofac Surg* 2007;**65**:573–80.
232. Ohbayashi Y, Miyake M, Sawai F, Minami Y, Iwasaki A, Matsui Y. Adjunct teriparatide therapy with monitoring of bone turnover markers and bone scintigraphy for bisphosphonate-related osteonecrosis of the jaw. *Oral Surg Oral Med Oral Pathol Oral Radiol* 2013;**115**:e31–7.
233. Rogers MJ. From molds and macrophages to mevalonate: a decade of progress in understanding the molecular mode of action of bisphosphonates. *Calcif Tissue Int* 2004;**75**:451–61.
234. Pautke C, Bauer F, Bissinger O, Tischer T, Kreutzer K, Steiner T, et al. Tetracycline bone fluorescence: a valuable marker for osteonecrosis characterization and therapy. *J Oral Maxillofac Surg* 2010;**68**:125–9.
235. Ruggiero SL, Drew SJ. Osteonecrosis of the jaws and bisphosphonate therapy. *J Dent Res* 2007;**86**:1013–21.
236. Vescovi P, Manfredi M, Merigo E, Meleti M. Early surgical approach preferable to medical therapy for bisphosphonate-related osteonecrosis of the jaws. *J Oral Maxillofac Surg* 2008;**66**:831–2.

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New and Innovative Treatment Strategies for Medication-Related Osteonecrosis of the Jaw

10

Riham M. Fliefel and Pit J. Voss

Abstract

A large variety of treatment options have been proposed for the management of medication-related osteonecrosis of the jaw in particular for osteonecrosis of the jaw due to bisphosphonate intake. More recently, regenerative concepts using stem cells from different sources and growth factors have been introduced for the treatment of medication-related osteonecrosis of the jaws. These new and innovative concepts seem to be promising future options in the management of osteonecrosis of the jaws.

Introduction

In the current literature, treatment options for patients with established medication-related osteonecrosis of the jaw differ. While the first guidelines focused on preserving the patient's quality of life by controlling pain and secondary infection, nowadays there is a trend to a more surgical approach with the aim of complete mucosal healing of the lesions [1, 2].

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As described in the previous chapters, a large variety of treatment modalities have been reported including conservative medical management, various types of surgery, hyperbaric oxygen, and ozone and laser therapy [3–5]. In large lesions with pathological fractures, reconstruction with vascularized or nonvascularized bone has been described, but remains problematic due to poor bone healing and an obligatory graft resorption phase, donor site morbidity, and infection of foreign material. Because bisphosphonates are often administered in patients with generalized bone pathologies and the molecules not only bind to the jaws, it is not unlikely that the transferred bone will either be affected by bony metastases or also develop osteonecrosis of the jaws [6, 7].

In osteonecrotic lesions, among others, the lack of osteogenic precursors and a shortage of endothelial progenitor cells (EPCs) cause an insufficient vascular support, so that safe

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alternative therapies are needed to enhance the osteogenesis and vasculogenesis [8, 9].

While tissue engineering is the branch that brings biology, bioengineering, clinical sciences, and biotechnology together for the purpose of generating new tissues and organs and the development of biologic substitutes that can restore and maintain normal function, a variety of approaches are utilized that combine the use of morphogens, growth factors, and cytokines, with scaffolds and carriers and cells [10–12].

During the last years, the increased interest on stem cells allowed the evolution of new horizons in treatment perspectives. Stem cells are immature, undifferentiated cells that can divide and multiply for an extended period of time, differentiating into specific types of cells and tissues. They are defined as cells that self-replicate and are able to differentiate into at least two different cell types, and both criteria must be present for a cell to be called a "stem cell" [13, 14]. Embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs) represent the three different major types of stem cells [15].

During embryonic development, embryonic stem cells are derived from cells of the inner cell mass of the blastocysts. They are pluripotent and give rise to all derivatives of the three primary germ layers. The most important and potential use of ESCs is clinically in transplantation medicine, where they can be used to develop cell replacement therapies [13, 14, 16, 17]. In contrast, iPSCs refer to adult or somatic stem cells that have been genetically reprogrammed to behave like ESC [18].

ASCs are multipotent because their potential is normally limited to one or more lineages of specialized cells [16]. In addition to bone marrow, various tissues have been found to harbor mesenchymal stem cell (MSC)-like populations including adipose tissues, muscles, tendons, dental pulps, periodontal ligaments, umbilical cord blood, placenta, periosteum, liver, cartilage, synovium, synovial fluid, spleen, and thymus [19–25]. In vitro expanded bone marrow stem cells (BMMSCs) may be a rich source of osteogenic progenitor cells that are capable of promoting the repair or regeneration of skeletal defects when cultured in the presence of dexamethasone, inorganic phosphate, and vitamin C. BMMSCs can be induced to become osteoblast-like cells in vitro and form calcified nodules [26, 27].

Cell-Based Therapy in Craniofacial Tissue Engineering

The bone is the second most frequently transplanted tissue with increasing frequency. Reconstruction of craniofacial components is one of the most important and intricate objectives in stem cell-mediated regenerative medicine [28–30]. The craniofacial bone has an essential role in supporting the adjacent soft tissue, providing anchoring for dental structures and providing a stable although flexible framework for craniofacial cartilage structures. Embryologically, most craniofacial bones are derived from mesenchymal tissue through membranous ossification [31].

Facial development, including that of the teeth and oral cavity, is a classic act of interactions by stem cells of the epithelium, craniofacial mesoderm, and neural crest-derived mesenchyme [32, 33]. Cranial neural crest cells (CNC) play an important role in development of the teeth, alveolar crest, and jaw bone [34]. Thus, the biologically unique features of cranial neural crest cell-derived bone should be considered in the etiopathology of antiresorptive drug-induced osteonecrosis of the jaw.

Stem cell-based strategies are currently a promising approach in craniofacial bone tissue engineering as they supply sufficient numbers of cells that can not only form bone and associated tissues but also maintain bone as it undergoes turnover throughout life [12, 35]. Regenerative medicine for bone healing has reached the patient in the form of cell therapy approaches to treat localized bone defects or systemic diseases of the skeleton [36].

Mesenchymal stem cells (MSCs) have been isolated from a variety of mesenchymal tissues, and they can differentiate into a wide array of cell types, including osteoblasts, chondrocytes, and adipocytes. They participate in regeneration of injured tissues in different ways. On one hand, they directly differentiate into tissue-specific cells and thus substitute damaged or lost cells. On the other hand, they indirectly influence tissue regeneration

by secretion of soluble factors. Thirdly, they are able to modulate the inflammatory response. Thus, they can promote vascularization, cell proliferation, and differentiation and modulate inflammatory processes [37].

As a result of their slower growth rate and the absence of telomerase activity in vitro, mesenchymal stem cells (MSCs) are presumed to have a lower risk for tumor formation compared with embryonic stem cells (ESCs) [38]. This suggests that mesenchymal stem cells may have broader therapeutic applications compared to other adult stem cells.

Bone marrow-derived mesenchymal stem cells (BMMSCs) can be concentrated from bone marrow aspirate with different techniques. The FICOLL method (synthetic polysaccharide) and the BMAC method (bone marrow aspirate concentrate) are established methods for mononuclear cell concentration from iliac crest aspirate [28]. Percutaneous or intraoperative local administration of cell suspensions delivers progenitor or lineage-committed cells directly to the wound site.

Mesenchymal stem cells functional properties have been proved by several experimental and clinical studies using autologous BMMSC implants for healing, cell architecture repair, and recovery of local blood flow on injured and ischemic tissues for alveolar ridge augmentation and long bone defects [39–41].

Autologous bone marrow or autologous mesenchymal stem cells were successfully implanted in a number of patients to enhance fracture and osteotomy healing; fill bone defects; treat pseudarthrosis, bone cysts, and osteonecroses; or enhance spinal fusion [37]. In a randomized controlled trial, it has been shown that the new bone formation in sinus lift procedures using autologous mesenchymal stem cells in combination with bovine bone mineral is equivalent to autologous bone and bovine bone mineral [42].

Experimental and Clinical Cell-Based Therapy in Medication-Related Osteonecrosis of the Jaw

Several authors have focused on the treatment of osteonecrosis of the jaw with mesenchymal stem cells. With the ability to induce ectopic bone formation and angiogenesis, MSCs might become a promising treatment option for antiresorptive drug-induced osteonecrosis of the jaws [43].

In a mouse model, a mesenchymal stem cell-based approach to treat osteonecrosis of the jaw was tested. At 2 weeks after tooth extraction, ONJ-like wild-type mice receiving intravenous infusions with mesenchymal stem cells healed with complete soft tissue and bone regeneration at the extracted alveolar socket suggesting that cell-based immunotherapy using T regulatory cells (Tregs) or mesenchymal stem cells are promising therapeutic strategies to prevent and treat ONJ-like lesions in wild-type mice. It is discussed that cell-based therapy using systemic mesenchymal stem cell infusions can prevent or cure antiresorptive drug-induced osteonecrosis of the jaws via reestablishment of the immune balance between inhibition of T-helper-producing interleukin 17 cells (th17) and increase in Tregs [44].

In a swine model, Li et al. reported the treatment of ONJ lesions with allogenic mesenchymal stem cells and concluded to have discovered that allogenic mesenchymal stem cell-based infusions provide a safe and effective therapeutic modality for treating ONJ lesions, which sheds light on potential clinical applications for treating patients suffering from medication-related osteonecrosis of the jaws [45].

In a case report, Cella et al. published to have cured a patient with refractory osteonecrosis of the jaw, with autologous mesenchymal stem cells that were aspirated from the iliac crest and transplanted intralesionally on a gelatin sponge carrier after concentration with the FICOLL method. This procedure allowed a clinical improvement of symptoms and induced novel ossification with complete remission from a stage 3 bisphosphonate-induced osteonecrosis of the jaw [46]. In another case report, Elad et al. presented a patient with bisphosphonate-induced osteonecrosis of the jaw, where bone marrow cells were resuspended in saline and injected along the mucosal margins of two areas of exposed bone. No complications were observed with considerable reduction in the size of the alveolar bone exposures following the local infiltration of the hematopoietic stem cells. Complete healing of the lesion was achieved within a few months

of the procedure showing the great potential of hematopoietic stem cells to treat osteonecrosis of the jaws [8, 47].

In our own experience, a case series of 8 patients with refractory bisphosphonate-induced osteonecrosis of the jaws, the lesions was managed with surgical resection of necrotic bone followed by mesenchymal stem cell grafting (Fig. 10.1a–j). Marrow-derived cells were aspirated from the iliac crest and concentrated using a chair-side bone marrow concentration procedure (BMAC) to obtain mesenchymal stem cells. These MSCs were then grafted into the defect with autologous thrombin and a BioGide membrane. In all cases bony edges were rounded, and the wound was closed using a three-layer

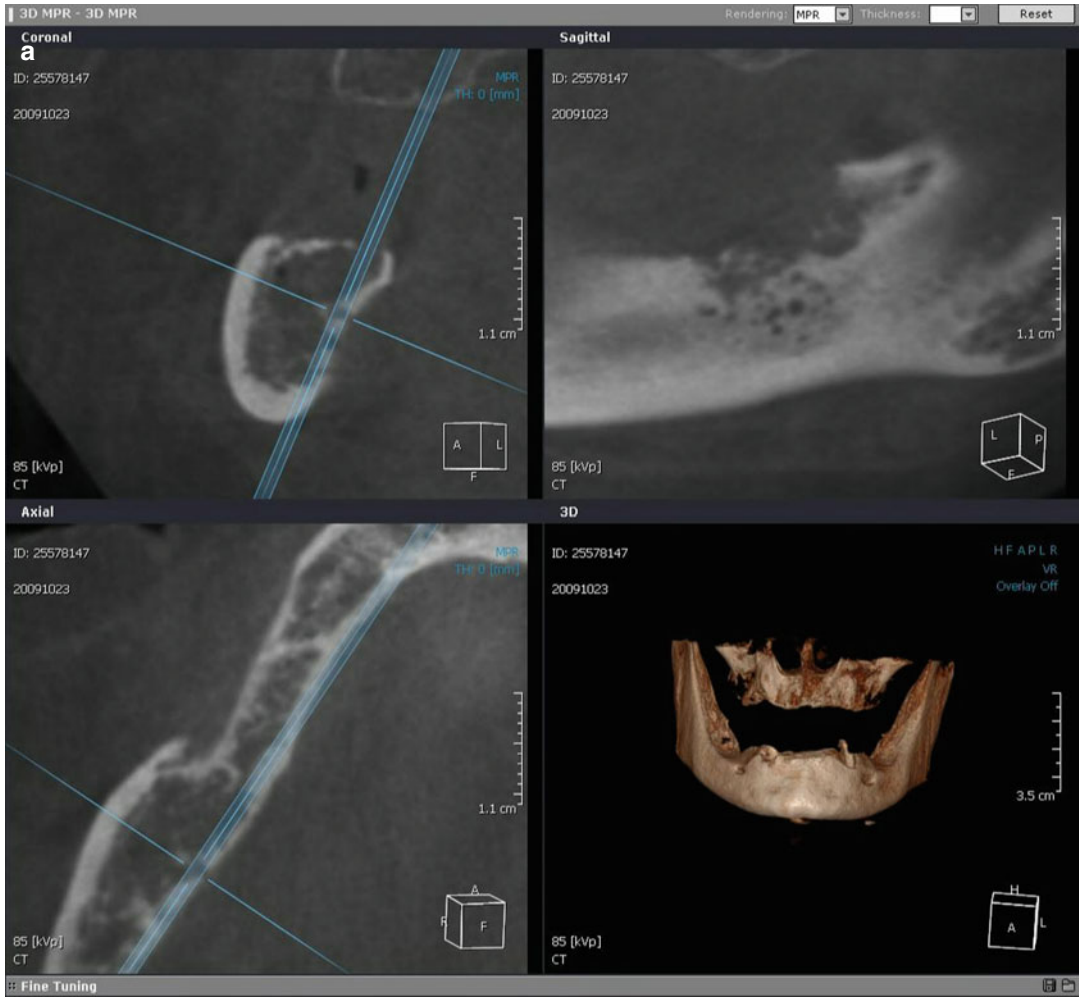

Fig. 10.1 (**a**) Preoperative cone beam CT of a 57-year-old female patient suffering from bisphosphonate-induced osteonecrosis of the jaw in the right mandible after oral bisphosphonate treatment for osteoporosis due to rheumatoid arthritis and glucocorticoid treatment. (**b**) Intraoperative exposure of the osteonecrotic lesion in the right mandible. (**c**) Exposure of the inferior alveolar nerve after complete removal of the affected bone. (**d**) Puncture of the posterior iliac crest for sampling of 50 ml bone marrow aspirate. (**e**) Transfer of the bone marrow aspirate into the SmartPReP2 centrifuge. (**f**) The suspension is centrifuged for 14 min. (**g**) Close-up of the smaller of the two chambers of the BMAC™ kit. The white line is composed of mononuclear cells including progenitor cells and mesenchymal stem cells. (**h**) BMAC is mixed with autologous thrombin and inserted under a collagen membrane. (**i**) The defect is covered with a multiple layer technique. After slitting of the vestibular periosteum, the mobile part is quilted under the lingual mucoperiosteal flap. (**j**) The wound is closed with backstitches and a running suture

Fig. 10.1 (continued)

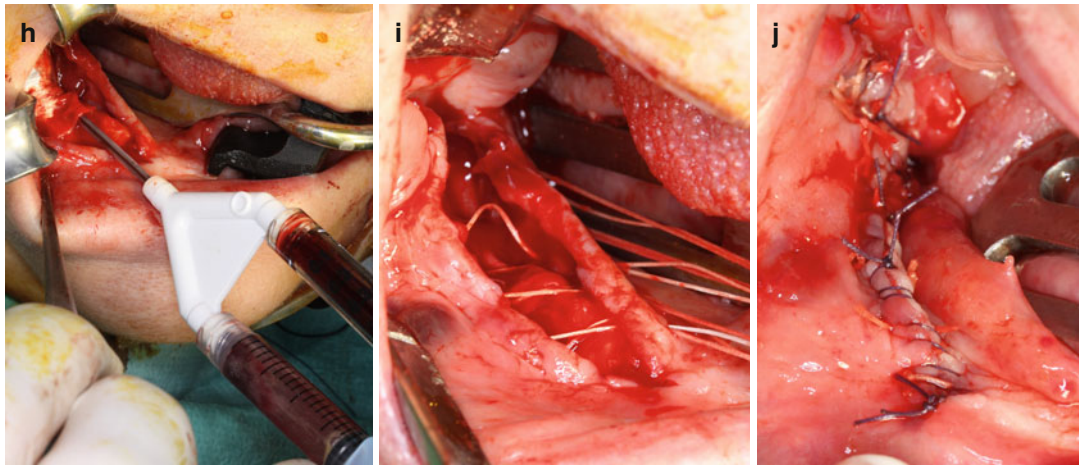

Fig. 10.1 (continued)

technique. At 12–15 months follow-up, all patients showed satisfactory healing with no signs of wound infection, dehiscence, or recurrence of osteonecrosis of the jaw. Only one patient developed significant complications, that of sepsis of unknown origin, 2 months postoperatively (unpublished own data).

Growth Factors in Treatment of Medication-Related Osteonecrosis of the Jaw

Growth factors are soluble-secreted signaling polypeptides capable of instructing specific cellular responses in a biological environment [48]. The specific cellular response triggered by growth factor signaling can result in a very wide range of cell actions, including cell survival, control over migration, differentiation, or proliferation of a specific subset of cells [49]. A variety of growth factors produced by osteogenic cells, platelets, and inflammatory cells—including bone morphogenetic proteins (BMPs), insulin-like growth factors 1 and 2, transforming growth factor-β1 (TGF-β1), platelet-derived growth factor, and fibroblast growth factor 2—are functionally involved in bone healing. The bone matrix serves as a reservoir for these growth factors [50–52].

Growth factor application to patients suffering osteonecrosis of the jaws can be considered a challenge because of improving the soft and hard tissues healing. Acting like chemotactic agents, they stimulate angiogenesis, migration, proliferation, and differentiation of stem cells from the surrounding mesenchymal tissues into bone-forming cells in an area of injury [53, 54].

The discovery of bone morphogenic proteins (BMPs) as osteoinductive factors and the subsequent development of commercially available recombinant forms of BMPs have offered the potential to replace traditional grafting techniques with de novo bone formation [55, 56]. Bone morphogenetic protein type 2 (BMP-2) application substituting the necrotic bone removal could be considered a therapeutic option for reconstruction of localized bone defects of medication-related osteonecrosis of the jaws. rhBMP-2 was applied using an absorbable collagen sponge carrier to 20 patients who underwent surgical removal of necrotic bone related to bisphosphonate therapy. The collagen was fixed to the soft tissue by an absorbable suture. The postoperative controls showed an increase in the soft tissue healing and new bone formation of the treated sites [57].

Some researchers have proposed also the use of platelet-rich plasma (PRP) in ONJ surgery based on surgical debridement and reconstruction combined with the use of platelet-rich plasma produced from the patient's autologous blood [58–68]. The rationale for the employment

of PRP in patients affected by osteonecrosis of the jaws is based on the thesis that the presence of growth factors constitutes stimulations for bone healing, which is similar to physiological healing. The growth factors in platelet-rich plasma might accelerate epithelial wound healing, decrease tissue inflammation after surgery, improve the regeneration of bone and soft tissues, and promote tissue vascularization. The additional advantages related to the use of this product are its biocompatibility and safety as an autologous product [69, 70].

In a prospective study, Scoletta et al. reported of only one wound dehiscence after extraction of 202 teeth in 63 patients under intravenous bisphosphonate treatment. After extraction, the sockets were filled with scaffold-like autologous PRP [71]. In a case series of 25 patients with osteonecrotic lesions due to bisphosphonate intake, treatment of ONJ with a combination of bone resection and platelet-rich plasma was found to be an effective therapy that should be considered an alternative treatment modality for the management of advanced ONJ cases [72].

Lee et al. also described the successful management of complications of dental implant surgery of 2 patients taking the oral form of bisphosphonates, including platelet-rich plasma and hyperbaric oxygen [60]. Several other studies reported of enhanced mucosal healing of patients with ONJ due to bisphosphonate intake treated with surgical removal of the exposed bone, platelet-rich plasma, and primary closure under antibiotic coverage [61–63, 65].

Nitrogen-containing bisphosphonates are able to inhibit pyrophosphate synthase in the mevalonate pathway. The consequently decreased synthesis of the metabolite geranylgeraniol is believed to largely account for the development of bisphosphonate-induced osteonecrosis of the jaws. In an in vitro study, Ziebart et al. demonstrated that geranylgeraniol can rescue the negative effect of bisphosphonates in human umbilical cord vein endothelial cells, fibroblasts, and osteogenic cells [73]. Geranylgeraniol could lead to new treatment strategies for bisphosphonate-induced osteonecrosis of the jaws that have to be proven in animal studies.

Conclusion

The implementation of stem cell-based concepts and the use of growth factors are promising future treatment modalities for patients suffering from medication-related osteonecrosis of the jaw.

References

1. Ruggiero SL, Drew SJ. Osteonecrosis of the jaws and bisphosphonate therapy. J Dent Res. 2007;86:1013–21.
2. Grötz KA, Piesold JU, Al-Nawas B. Bisphosphonat-assoziierte Kiefernekrose (BP-ONJ) und andere Medikamenten-assoziierte Kiefernekrosen. S3-Leitlinie AWMF-Register-Nr. 007/091, 15.04.2012. http://www.awmf.org/uploads/tx_szleitlinien/007-091l_S3_Bisphosphonat-assoziierte_Kiefernekrose_2012-04.pdf.
3. Freiberger JJ, Padilla-Burgos R, Chhoeu AH, Kraft KH, Boneta O, Moon RE, et al. Hyperbaric oxygen treatment and Bisphosphonates induced osteonecrosis of the jaw: a case series. J Oral Maxillofac Surg. 2007;65:1321–7.
4. Erkan M, Bilgi O, Mutluoglu M, Uzun G. Bisphosphonates-related osteonecrosis of the jaw in cancer patients and hyperbaric oxygen therapy. JOP. 2009;10:579–80.
5. Ficarra G, Beninati F. Bisphosphonate-related osteonecrosis of the jaws: an update on clinical, pathological and management aspects. Head Neck Pathol. 2007;1:132–40.
6. Mulliken JB, Glowacki J. Induced osteogenesis for repair and construction in the craniofacial region. Plast Reconstr Surg. 1980;65:553–60.
7. Bostrom R, Mikos A. Synthetic biodegradable polymer scaffolds. In: Atala A, Mooney DJ, Vacanti JP, Langer R, editors. Tissue engineering of bone. Boston: Birkhauser; 1997. p. 215–34.
8. Gonzálvez-García M, Rodríguez-Lozano FJ, Villanueva V, Segarra-Fenoll D, Rodríguez-González MA, Oñate-Sánchez R, et al. Mesenchymal stem cells and bisphosphonate-related osteonecrosis of the jaw: the future? Oral Dis. 2012;18:823–4.
9. Fournier P, Boissier S, Filleur S, Guglielmi J, Cabon F, Colombel M, et al. Bisphosphonates inhibit angiogenesis in vitro and testosterone-stimulated vascular regrowth in the ventral prostate in castrated rats. Cancer Res. 2002;62:6538–44.
10. Langer R, Vacanti JP. Tissue engineering. Science. 1993;260:920–6.
11. Koh CJ, Atala A. Tissue engineering, stem cells, and cloning: opportunities for regenerative medicine. J Am Soc Nephrol. 2004;15:1113–25.
12. Robey PG. Cell sources for bone regeneration: the good, the bad, and the ugly (but promising). Tissue Eng Part B Rev. 2011;17:423–30.

13. Mao JJ, Collins FM. Stem cells: sources, therapies and the dental professional. http://www.ineedce.com/courses/1486/PDF/StemCells.pdf.
14. Mao JJ. Stem cell and future of dental care. N Y State Dent J. 2008;74:20–4.
15. Leventhal A, Chen G, Negro A, Boehm M. The benefits and risks of stem cell technology. Oral Dis. 2012;18:217–22.
16. Reznick JB. Stem cells: emerging medical and dental therapies for the dental professional. http://www.stemsave.com/Docs/News/Dentaltown%20StemCell%20CE.pdf.2008.
17. Nedel F, André Dde A, de Oliveira IO, Cordeiro MM, Casagrande L, Tarquinio SB, et al. Stem cells: therapeutic potential in dentistry. J Contemp Dent Pract. 2009;10:90–6.
18. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Thomson JA, et al. Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007;318:1917–20.
19. Nakahara H, Bruder SP, Haynesworth SE, Holecek JJ, Baber MA, Goldberg VM, et al. Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum. Bone. 1990;11:181–8.
20. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci U S A. 2000;97:13625–30.
21. Romanov YA, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. Stem Cells. 2003;21:105–10.
22. Seo BM, Miura M, Gronthos S, Bartold PM, Batouli S, Brahim J, et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet. 2004;364:149–55.
23. Pountos I, Giannoudis PV. Biology of mesenchymal stem cells. Injury. 2005;36:S8–12.
24. Bi Y, Ehirchiou D, Kilts TM, Inkson CA, Embree MC, Sonoyama W, et al. Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. Nat Med. 2007;13:1219–27.
25. Zannettino AC, Paton S, Arthur A, Khor F, Itescu S, Gimble JM, et al. Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype in vitro and in vivo. J Cell Physiol. 2008;214:413–21.
26. Krebsbach PH, Robey PG. Dental and skeletal stem cells: potential cellular therapeutics for craniofacial regeneration. J Dent Educ. 2002;66:766–73.
27. Gronthos S, Graves SE, Ohta S, Simmons PJ. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. Blood. 1994;84:4164–73.
28. Warren SM, Fong KD, Chen CM, Loboa EG, Cowan CM, Lorenz HP, et al. Tools and techniques for craniofacial tissue engineering. Tissue Eng. 2003;9:187–200.
29. Cowan CM, Shi YY, Aalami OO, Chou YF, Mari C, Thomas R, et al. Adipose-derived adult stromal cells heal critical-size mouse calvarial defects. Nat Biotechnol. 2004;22:560–7.
30. Warnke PH, Springer IN, Wiltfang J, Acil Y, Eufinger H, Wehmöller M, et al. Growth and transplantation of a custom vascularised bone graft in a man. Lancet. 2004;364:766–70.
31. Schantz JT, Machens HG, Schilling AF, Teoh SH. Regenerative medicine: implications for craniofacial surgery. J Craniofac Surg. 2012;23:530–6.
32. Thesleff I. The genetic basis of tooth development and dental defects. Am J Med Genet A. 2006;140:2530–5.
33. Cordero DR, Brugmann S, Chu Y, Bajpai R, Jame M, Helms JA. Cranial neural crest cells on the move: their roles in craniofacial development. Am J Med Genet A. 2011;155A:270–9.
34. Chung IH, Yamaza T, Zhao H, Choung PH, Shi S, Chai Y. Stem cell property of postmigratory cranial neural crest cells and their utility in alveolar bone regeneration and tooth development. Stem Cells. 2009;27:866–77.
35. Sanchez-Lara PA, Warburton D. Impact of stem cells in craniofacial regenerative medicine. Front Physiol. 2012;3:188–96.
36. Horwitz EM, Gordon PL, Koo WK, Marx JC, Neel MD, McNall RY, et al. Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. Proc Natl Acad Sci U S A. 2002;99:8932–7.
37. Schmitt A, van Griensven M, Imhoff AB, Buchmann S. Application of stem cells in orthopedics. Stem Cells Int. 2012;2012:394962.
38. Rosenthal N. Prometheus's vulture and the stem-cell promise. N Engl J Med. 2003;349:267–74.
39. Sun Y, Feng Y, Zhang C. The effect of bone marrow mononuclear cells on vascularization and bone regeneration in steroid-induced osteonecrosis of the femoral head. Joint Bone Spine. 2009;76:685–90.
40. Iohara K, Nakashima M, Ito M, Ishikawa M, Nakasima A, Akamine A. Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2. J Dent Res. 2004;83:590–5.
41. Ueda M, Yamada Y, Ozawa R, Okazaki Y. Clinical case reports of injectable tissue-engineered bone for alveolar augmentation with simultaneous implant placement. Int J Periodontics Restorative Dent. 2005;25:129–37.
42. Sauerbier S, Rickert D, Gutwald R, Nagursky H, Oshima T, Xavier SP, Christmann J, Kurz P, Menne D, Vissink A, Raghoebar G, Schmelzeisen R, Wagner W, Koch FP. Bone marrow concentrate and bovine bone mineral for sinus floor augmentation: a controlled, randomized, single-blinded clinical and histological trial–per-protocol analysis. Tissue Eng Part A. 2011;17(17–18):2187–97.
43. Handschel J, Meyer U. Infection, vascularization, remodelling – are stem cell the answers for bone disease of the jaw? Head Face Med. 2011;7:5.
44. Kikuiri T, Kin I, Yamaza T, Akiyama K, Zhang Q, Li Y, et al. Cell-based immunotherapy with mesenchymal stem cells cures bisphosphonate-related osteonecrosis of the jaw – like disease in mice. J Bone Miner Res. 2010;25:1668–79.

45. Li Y, Xu J, Mao L, Liu Y, Gao R, Zheng Z, et al. Allogeneic mesenchymal stem cell therapy for bisphosphonate-related jaw osteonecrosis in Swine. Stem Cells Dev. 2013;22:2047–56.
46. Cella L, Oppici A, Arbasi M, Moretto M, Piepoli M, Vallisa D, et al. Autologous bone marrow stem cell intralesional transplantation repairing bisphosphonate related osteonecrosis of the jaw. Head Face Med. 2011;7:16–21.
47. Elad S, Czerninski R, Avgil M, Or R. Hematopoietic stem cells local transplantation for the treatment of osteonecrosis of the jaws. Support Care Cancer. 2005;13:455 (abstract).
48. Cross M, Dexter TM. Growth factors in development, transformation, and tumorigenesis. Cell. 1991; 64:271–80.
49. Lee K, Silva EA, Mooney DJ. Growth factor delivery-based tissue engineering: general approaches and a review of recent developments. J R Soc Interface. 2011;8:153–70.
50. Reddi AH. Cartilage morphogenetic proteins: role in joint development, homoeostasis, and regeneration. Ann Rheum Dis. 2003;62:73–8.
51. Tsumaki N, Tanaka K, Arikawa-Hirasawa E, Nakase T, Kimura T, Thomas JT, et al. Role of CDMP-1 in skeletal morphogenesis: promotion of mesenchymal cell recruitment and chondrocyte differentiation. J Cell Biol. 1999;144:161–73.
52. Gruber R, Mayer C, Schulz W, Graninger W, Peterlik M, Watzek G, et al. Stimulatory effects of cartilage-derived morphogenetic proteins 1 and 2 on osteogenic differentiation of bone marrow stromal cells. Cytokine. 2000;12:1630–8.
53. Nase JB, Suzuki JB. Osteonecrosis of the jaw and oral bisphosphonate treatment. J Am Dent Assoc. 2006;137:1115–9.
54. Badros A, Weikel D, Salama A, Goloubeva O, Schneider A, Rapoport A, et al. Osteonecrosis of the jaw in multiple myeloma patients: clinical features and risk factors. J Clin Oncol. 2006;20:945–52.
55. Wozney J. The bone morphogenetic protein family and osteogenesis. Mol Reprod Dev. 1992;32:160–7.
56. Boyne PJ. Application of bone morphogenetic proteins in the treatment of clinical oral and maxillofacial osseous defects. J Bone Joint Surg. 2001;83A:146–50.
57. Cicciù M, Herford AS, Juodžbalys G, Stoffella E. Recombinant human bone morphogenetic protein type 2 application for a possible treatment of bisphosphonates-related osteonecrosis of the jaw. J Craniofac Surg. 2012;23:784–8.
58. Cetiner S, Sucak GT, Kahraman SA, Aki SZ, Kocakahyaoglu B, Gultekin SE, et al. Osteonecrosis of the jaw in patients with multiple myeloma treated with zoledronic acid. J Bone Miner Metab. 2009;27:435–43.
59. Curi MM, Cossolin GS, Koga DH, Araújo SR, Feher O, dos Santos MO, et al. Treatment of avascular osteonecrosis of the jaw in cancer patients with a history of bisphosphonate therapy by combining bone resection and autologous platelet-rich plasma: report of 3 cases. J Oral Maxillofac Surg. 2007;65:349–55.
60. Lee CY, David T, Nishime M. Use of platelet-rich plasma in the management of oral bisphosphonate-associated osteonecrosis of the jaw: a report of 2 cases. J Oral Implantol. 2007;33:371–82.
61. Bocanegra-Perez S, Vicente-Barrero M, Knezevic M, Castellano-Navarro JM, Rodrıguez-Bocanegra E, Rodrıguez-Millares J, et al. Use of platelet-rich plasma in the treatment of bisphosphonate-related osteonecrosis of the jaw. Int J Oral Maxillofac Surg. 2012;41:1410–5.
62. Adornato MC, Morcos I, Rozanski J. The treatment of bisphosphonate associated osteonecrosis of the jaws with bone resection and autologous platelet-derived growth factors. J Am Dent Assoc. 2007;138: 971–7.
63. Mozzati M, Gallesio G, Arata V, Pol R, Scoletta M. Platelet-rich therapies in the treatment of intravenous bisphosphonate-related osteonecrosis of the jaw: a report of 32 cases. Oral Oncol. 2012; 48:469–74.
64. Coviello V, Peluso F, Dehkhargani SZ, Verdugo F, Raffaelli L, Manicone PF, et al. Platelet-rich plasma improves wound healing in multiple myeloma bisphosphonate-associated osteonecrosis of the jaw patients. J Biol Regul Homeost Agents. 2012; 26:151–5.
65. Dohan Ehrenfest DM, Rasmusson L, Albrektsson T. Classification of platelet concentrates: from pure platelet-rich plasma (PPRP) to leukocyte- and platelet-rich fibrin (L-PRF). Trends Biotechnol. 2009;27:158–67.
66. Marx RE. Platelet-rich plasma: evidence to support its use. J Oral Maxillofac Surg. 2004;62:489–96.
67. Oliver R. Bisphosphonates and oral surgery. Oral Surg. 2009;2:56–63.
68. Tischler M. Platelet rich plasma. The use of autologous growth factors to enhance bone and soft tissue grafts. N Y State Dent J. 2002;68:22–4.
69. Carlson NE, Roach RB. Platelet-rich plasma: clinical applications in dentistry. J Am Dent Assoc. 2002;133:1383–6.
70. Nikolidakis D, Jansen JA. The biology of platelet-rich plasma and its application in oral surgery: literature review. Tissue Eng Part B Rev. 2008;14:249–58.
71. Scoletta M, Arata V, Arduino PG, Lerda E, Chiecchio A, Gallesio G, Scully C, Mozzati M. Tooth extractions in intravenous bisphosphonate-treated patients: a refined protocol. J Oral Maxillofac Surg. 2013;71(6):994–9. doi:10.1016/j.joms.2013.01.006. Epub 2013 Feb 21.
72. Curi MM, Cossolin GS, Koga DH, Zardetto C, Christianini S, Feher O, et al. Bisphosphonate-related osteonecrosis of the jaws–an initial case series report of treatment combining partial bone resection and autologous platelet-rich plasma. J Oral Maxillofac Surg. 2011;69:2465–72.
73. Ziebart T, Koch F, Klein MO, Guth J, Adler J, Pabst A, Al-Nawas B, Walter C. Geranylgeraniol – a new potential therapeutic approach to bisphosphonate associated osteonecrosis of the jaw. Oral Oncol. 2011;47(3):195–201.

6 Pathogenesis of antiresorptive drug-related osteonecrosis of the jaw

Riham Fliefel, Sven Otto

1 Introductory questions

In this chapter, three questions are raised and discussed:

- Which theories exist for the pathogenesis of antiresorptive drug-related osteonecrosis of the jaw (ARONJ)?
- Why are the jaw bones predominantly affected?
- Why can nitrogen-containing bisphosphonates and denosumab cause ARONJ?

2 Background

Bones are constantly remodeled through osteoblastic (bone formation) and osteoclastic (bone resorption) activity to maintain skeletal strength and integrity. However, imbalance between these phenomena affects bone mineral density leading to such bone disorders as osteoporosis, Paget's disease, myeloma, bone metastases secondary to cancer, as well as osteogenesis imperfecta and inflammatory bone loss. One of the recent treatments of bone disorders is the use of antiresorptive drugs including hormone replacement therapy, selective estrogen receptor modulators, bisphosphonates, and denosumab, which reduce the occurrence of bone pain, pathological fracture, and spinal cord compression [1–4].

Among the antiresorptive drugs, bisphosphonates (BPs) are stable analogues of natural inorganic pyrophosphates [5–7]. They can be classified into nonnitrogen BPs, which metabolically interfere with adenosine triphosphate-dependent (ATP) intracellular pathways, and nitrogen BPs, which inhibit farnesyl pyrophosphate synthase [8, 9]. Denosumab is a new antiresorptive drug with a novel mechanism of action [10]. Both denosumab and bisphosphonates target osteoclasts, however, their effects on osteoblasts are largely indirect [11].

The mechanisms of action of BPs in bone metabolism are complex and multifactorial, altering the osteoclast cytoskeleton, stimulating apoptosis, and reducing proton-pump expression [12–14]. They interfere with chemotaxis and the attachment of osteoclast to bone together with suppressing mature osteoclast function by defective intracellular vesicle transport, which in turn prevents osteoclasts from forming a tight scaling zone or ruffled border required for bone resorption [15–17]. In addition, they inhibit recruitment, activation, and differentiation of osteoclast precursors [18]. The clinical efficacy of BPs rises from their ability to bind strongly to bone mineral [7]. The initial clearance of BPs occurs through renal excretion or adsorption to bone mineral extending over a period of weeks to years [19]. During bone resorption, the acidic pH in the resorption lacuna increases the dissociation of BP from bone [20]. This is followed by the uptake of the BP most likely by fluid-phase endocytosis [21].

Bone resorption is regulated through what is known as RANK/RANKL/OPG pathway [11, 22]. The receptor activator of nuclear factor kappa-B ligand (RANKL) is a transmembrane and soluble protein highly expressed by osteoblasts [23, 24]; its receptor, receptor activator of nuclear factor kappa-B (RANK), is located on the cell membrane of osteoclasts and preosteoclasts [24, 25]. Increased bone resorption results from RANK/RANKL binding, which stimulates the formation, activity, and survival of osteoclasts [26]. Osteoprotegerin (OPG) is a naturally occurring soluble, nonsignaling "decoy receptor" for RANKL. Osteoprotegerin inhibits osteoclast activity by binding to RANKL, preventing its interaction with RANK [26–28]. Both RANKL and OPG are produced by osteoblasts [29].

Denosumab is a fully human monoclonal antibody that was developed specifically to interact with the RANK/RANKL/OPG pathway [7]. By binding to RANKL, it prevents the maturation and differentiation of preosteoclasts in the extracellular environment and promotes apoptosis of osteoclasts [30]. It has several advantages over BPs including better tolerability, ease of subcutaneous injection, shorter half-life, and reduced incidence of nephrotoxicity, rendering it the drug of choice for patients with renal diseases or

prostate cancer [31]. In contrast to the BPs, denosumab does not become embedded within bone tissue [10, 11]. Denosumab is cleared from the bloodstream through the reticuloendothelial system, with a half-life of approximately 26 days without inducing the formation of neutralizing antibodies [32].

Antiresorptive drugs have serveral side effects including upper gastrointestinal, where nausea, vomiting, epigastric pain, and dyspepsia can occur after oral administration of drugs for the treatment of osteoporosis. Subsequently, several cases of renal failure were reported following the use of intravenous BPs. A possible mechanism of renal toxicity was the strong affinity of the BP for metal ions and their tendency to form complexes and aggregates with metal ions. Nonspecific conjunctivitis is the most common ocular side effect of BPs, which usually improves without therapy and despite continuing treatment with BPs. Transient hypocalcaemia with secondary hyperparathyroidism is also a side effect of BP administration. There is a possibility of severe and sometimes incapacitating bone, joint, and/or muscle (musculoskeletal) pain in patients taking BPs [33, 34].

3 Theories for the pathogenesis of ARONJ

No potential adverse effect of antiresorptive drugs has caused more scientific attention than ARONJ, which ranges in severity from painless small areas of exposed bone, to significant bone exposure associated with severe pain, sequestration, infection, fistula, or jaw fracture [35–38]. The pathogenesis of the disease is certainly associated with many questions regarding the potential mechanisms underlying the pathophysiology [22, 39, 40]. Five main mechanisms have also been proposed: 1) impaired remodeling; 2) inhibition of angiogenesis; 3) local toxicity; 4) immunomodulation; and 5) infections. It is most likely that a combination of these facilitate the development of ARONJ [41]. However, the most cited theory to explain the mechanism suggests that it is caused by cessation of bone remodeling and bone turnover by the inhibition of osteoclasts [42].

Antiresorptive drug-related osteonecrosis of the jaw most commonly occurs in the oral cavity as the jaws are covered and protected only by a thin layer of periosteum and epithelium against the multitude of bacteria in the oral cavity making it prone for infections. The alveolar bone of the jaws is daily remodeled with a high rate of bone turnover, and the presence of teeth and gum provides an easy entrance for bacterial infection [40, 43]. The oral structures are subjected to a wide variety of stresses, which may be physiologic, iatrogenic, or inflammatory. The constant stress leads to trauma to the mucosa with exposure of bone [40]. Prolonged use of BPs can suppress bone turnover with accumulation of microcracks resulting in decreased biomechanical competence [35, 44]. Bisphosphonates cause excessive reduction of bone turnover resulting in an increased risk of bone necrosis in osseous repair [45, 46]. However, this theory failed to explain why exposed necrotic lesions are rarely seen in bones other than the jaw. Antiresorptive drug-related osteonecrosis of the jaw does not appear to occur in other conditions associated with reduced bone turnover, such as hypoparathyroidism, and in patients with reported ARONJ the bone turnover markers were not overly suppressed [47, 48]. In patients with breast cancer and bone metastases treated with zoledronate or denosumab, bone scintigraphy images suggest that the bone turnover of the mandible and maxilla is not overly changed when compared to other bones [49].

Blood supply may play a role in ARONJ as its reduction might lead to delayed wound healing due to the antiangiogenic effect [50]. Antiresorptive medications may inhibit angiogenesis by inhibiting the formation of blood vessels, endothelial cells, fibroblast growth factor, and endothelial growth factor impairing endothelial cell (EC) functions leading to altered adhesion and migration. Furthermore, there is reduced proliferation, increased apoptosis, and decreased capillary-like tube formation in ECs that might cause bone necrosis [51–53]. In a study by Wehrhan et al [54], mucoperiosteal tissue samples from ARONJ patients under BPs and controls were assessed for vascularization with CD31 staining and neoangiogenesis by CD105. Although there was no difference in the vascularization between sample groups, there were significantly fewer CD105-positive vessels in ARONJ samples suggesting that neoangiogenesis was suppressed in ARONJ patients. Histological evaluation of ARONJ tissue revealed decreased p63 gene expression, indicating a reduction in basal cell progenitors, and might lead to impaired healing of the oral mucosa [55]. Although BPs, bevacizumab, and sunitinib all have antiangiogenic effects, the effects of denosumab on angiogenesis is largely unknown [56–58]. As such, impaired vascularization may play only a minor role in the development of ARONJ [59].

Soft-tissue cytotoxicity might also play a role explaining why bone is directly exposed to the oral environment through teeth and periodontal ligaments [60]. Local infection and

tooth extraction could result in the release of BPs into the local tissues. Provided that the local concentration of drugs is high enough, the proliferation of adjacent epithelial cells could be inhibited and thus slow down the healing of the breached mucosal barrier [61]. However, soft-tissue toxicity has not been reported with denosumab. Use of BPs was explored on a variety of cells, including gastrointestinal cells, cervical epithelial cells, renal cells, prostate epithelial cells, and oral mucosal cells [40]. Antiresorptive drugs also act on immunity, resulting in impairment of myeloid cell function [62, 63], and dendritic cell [64] and T-cell upregulation [65]. They increase the antigenicity of cancer cells as targets and increase adaptive immunity. This impairment of local immunity with an infectious tendency may be a key element in ARONJ [41].

4 Special properties of jaw bones

Infection and periodontal disease are critical factors associated with ARONJ. However, controversy exists as to whether: 1) BP inhibition of bone remodeling results in necrosis with subsequent infection or 2) the direct toxic effects of BPs on the oral mucosa allow for invasion of oral pathogens causing infection with subsequent necrosis [66, 67]. Among all the bones, the jaw seems to be the most liable to bacterial infection since mucosa covering the alveolar bone is very thin and vulnerable and teeth easily become a pathway for bacteria from the outside into the bone. After administration, BPs accumulate in the bone and during physiological remodeling, osteocytes are exposed to BPs in bone [68]. Bisphosphonates bind to bone at neutral pH and are released from bone in an acidic milieu; thus, pH and infections might play an important role in the pathogenesis of ARONJ. This physiologic mechanism takes place in the resorption lacunas during bone resorption, where acid pH increases the dissociation between BP and hydroxyapatite. To date, this well-known feature has usually not been brought into connection to the pathogenesis of ARONJ, but may prove to be the missing part in the multifactorial puzzle [69, 70].

Aghaloo et al [71] found that necrosis of the alveolar bones developed after the placement of a wire ligature around the crown of a maxillary molar in a rat periodontal disease model. The results showed that periodontitis, which is presumably infection-related, can trigger osteonecrosis. When periodontitis occurs, inflammatory cells are recruited to the sites to eliminate the causative pathogens. However, the blockade of bone resorption with BPs may render it difficult for these cells to access the pathogens, allowing the infection to persist. The resulting accumulation of bacterial toxins and inflammation-generated superoxides will promote bone necrosis [68]. The mechanism of ARONJ is highly related to immunity and infection rather than being aseptic or avascular in origin [56]. It mostly follows invasive dental procedures, suggesting that ARONJ likely involves a drug-related compromise in the bone response to invasive trauma. Antiresorptive drug-related osteonecrosis of the jaw often manifests after dental extractions but it has to be taken into consideration that the majority of those extractions are performed due to dental infections, especially apical and periodontal infections. For a direct in vivo mechanism to be identified, it is yet unclear whether invasive trauma by itself is sufficient to precipitate ARONJ in individuals treated with antiresorptive drugs [36, 48]. Polymicrobial infection and periodontal disease are very likely to contribute to the development of ARONJ as a biofilm-associated infection. Filleul et al [72] found out that actinomyces were present in 70% of all cases. Thumbigere-Math et al [73] found actinomyces-like microorganisms in all bone specimens of patients during microbiological examination. In animal models treated with BPs, bacterial infection was sufficient enough to cause ARONJ [36]. Sterile inflammation alone in the soft tissues surrounding the jaw seems insufficient to induce ARONJ [74]. Treatment with antibiotics in animal models [75] and mucoperiosteal coverage on the day of tooth extraction in a rat model prevented the development of ARONJ [76].

The presence of the infectious component in ARONJ seems to be the most dangerous aspect. Oral pathogens should be prevented from reaching the bone surface, and optimum oral hygiene is essential. The current regimens, which consist of oral antiseptics and antibiotics, are not always successful. Ideally, treatment aims to eradicate the underlying infection, prevent secondary infection, stop the disease process, and control symptoms [77]. Traumatic intervention should be avoided, but where it must be undertaken, strict adherence is necessary. The proposed sequence of events in the development of ARONJ with infection could justify temporary discontinuation of the drug to allow recovery of macrophage production and function [78]. A potential scheme for the pathogenesis of ARONJ taking together the above mentioned aspects but stressing the role of local infections is illustrated in **Fig 6-1**. Infection might also be the initiating event for ARONJ in patients receiving denosumab as there is also a strong remodeling suppression and therefore only limited capacity to deal with odontogenic infections.

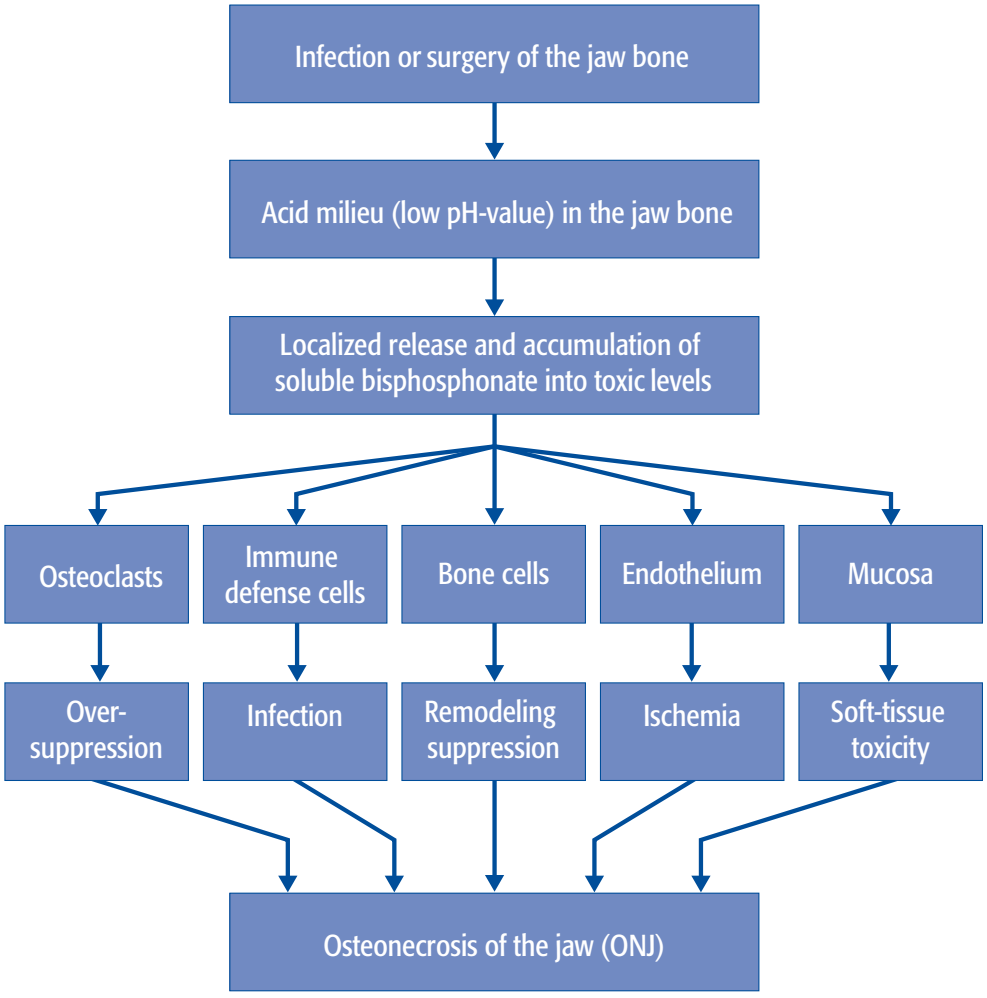

Fig 6-1 Potential scheme for the pathogenesis of ARONJ.

5 Conclusion

While various theories for the etiology of ARONJ are discussed, there is more and more data supporting the important role of local infections. Consequently, the jaw bones, especially in areas with dentoalveolar infections and surgeries, are mainly affected. The similarities and potential differences between ARONJ lesions caused by BPs and denosumab still have to be elucidated.

6 References

1. **Feurer E, Chapurlat R.** Emerging drugs for osteoporosis. *Expert Opin Emerg Drugs.* 2014 Sep; 19(3):385–395.
2. **Boyle WJ, Simonet WS, Lacey DL.** Osteoclast differentiation and activation. *Nature.* 2003 May; 423(6937):337–342.
3. **Russell RG.** Pharmacological diversity among drugs that inhibit bone resorption. *Curr Opin Pharmacol.* 2015 Jun; 22:115–130.
4. **Van den Wyngaert T, Huizing MT, Fossion E, et al.** Bisphosphonates in oncology: rising stars or fallen heroes. *Oncologist.* 2009 Feb; 14(2):181–191.
5. **Fleisch H.** Development of bisphosphonates. *Breast Cancer Res.* 2002; 4(1):30–34.
6. **Russell RG, Watts NB, Ebetino FH, et al.** Mechanisms of action of bisphosphonates: similarities and differences and their potential influence on clinical efficacy. *Osteoporosis International.* 2008 Jun; 19(6):733–759.
7. **Hanley DA, Adachi JD, Bell A, et al.** Denosumab: mechanism of action and clinical outcomes. *Int J Clin Pract.* 2012 Dec; 66(12)1139–1146.
8. **Russell RG.** Bisphosphonates: the first 40 years. *Bone.* 2011 Jul; 49(1):2–19.
9. **Reszka AA, Rodan GA.** Mechanism of action of bisphosphonates. *Curr Osteoporos Rep.* 2003 Sep; 1(2):45–52.
10. **Moen MD, Keam SJ.** Denosumab: a review of its use in the treatment of postmenopausal osteoporosis. *Drugs Aging.* 2011 Jan; 28(1):63–82.
11. **Baron R, Ferrari S, Russell RG.** Denosumab and bisphosphonates: different mechanisms of action and effects. *Bone.* 2011 Apr; 48(4):677–692.
12. **Sato M, Grasser W, Endo N, et al.** Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. *J Clin Invest.* 1991 Dec; 88(6):2095–2105.
13. **Hughes DE, Wright KR, Uy HL, et al.** Bisphosphonates promote apoptosis in murine osteoclasts in vitro and in vivo. *J Bone Miner Res.* 1995 Oct; 10(10):1478–1487.
14. **Miller SC, Jee WS.** The effect of dichloromethylene diphosphonate, a pyrophosphate analog, on bone and bone cell structure in the growing rat. *Anat Rec.* 1979 Mar; 193(3):439–462.
15. **Green J.** Cytosolic pH regulation in osteoblasts. *Miner Electrolyte Metab.* 1994; 20(1-2):16–30.
16. **Flanagan AM, TJ Chambers.** Inhibition of bone resorption by bisphosphonates: interactions between bisphosphonates, osteoclasts, and bone. *Calcif Tissue Int.* 1991 Dec; 49(6):407–415.
17. **Coxon FP, Helfrich MH, Van't Hof R, et al.** Protein geranylgeranylation is required for osteoclast formation, function, and survival: inhibition by bisphosphonates and GGTI-298. *J Bone Miner Res.* 2000 Aug; 15(8):1467–1476.
18. **Hughes DE, MacDonald BR, Russell RG, et al.** Inhibition of osteoclast-like cell formation by bisphosphonates in long-term cultures of human bone marrow. *J Clin Invest.* 1989 Jun; 83(6):1930–1935.
19. **Russell RG, Rogers MJ.** Bisphosphonates: from the laboratory to the clinic and back again. *Bone.* 1999 Jul; 25(1):97–106.
20. **Ebetino FH, Francis MD.** Mechanisms of action of etidronate and other bisphosphonates. *Rev Cont Pharmaco.* 1998; 9:233–243.
21. **Thompson K, Rogers MJ, Coxon FP, et al.** Cytosolic entry of bisphosphonate drugs requires acidification of vesicles after fluid-phase endocytosis. *Mol Pharmacol.* 2006 May; 69(5):1624–1632.
22. **Yamashita J, McCauley LK.** Antiresorptives and osteonecrosis of the jaw. *J Evid Based Dent Pract.* 2012 Sep; 12(3 Suppl):S233–247.
23. **Collin-Osdoby P.** Regulation of vascular calcification by osteoclast regulatory factors RANKL and osteoprotegerin. *Circ Res.* 2004 Nov; 95(11):1046–1057.
24. **Lewiecki EM.** Treatment of osteoporosis with denosumab. *Maturitas.* 2010 Jun; 66(2):182–186.
25. **Hsu H, Lacey DL, Dunstan CR, et al.** Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. *Proc Natl Acad Sci USA.* 1999 Mar; 96(7):3540–3545.
26. **Lacey DL, Timms E, Tan HL, et al.** Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell.* 1998 Apr; 93(2):165–176.
27. **Burgess TL, Qian Y, Kaufman S, et al.** The ligand for osteoprotegerin (OPGL) directly activates mature osteoclasts. *J Cell Biol.* 1999 May; 145(3):527–538.
28. **Simonet WS, Lacey DL, Dunstan CR, et al.** Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell.* 1997 Apr; 89(2):309–319.
29. **Schwarz EM, Ritchlin CT.** Clinical development of anti-RANKL therapy. *Arthritis Res Ther.* 2007; 9(1 Suppl):S7.
30. **Bekker PJ, Holloway D, Nakanishi A, et al.** The effect of a single dose of osteoprotegerin in postmenopausal women. *J Bone Miner Res.* 2001 Feb; 16(2):348–360.
31. **Uyanne J, Calhoun CC, Le AD.** Antiresorptive Drug–Related Osteonecrosis of the Jaw. *Dent Clin North Am.* 2014 Apr; 58(2):369–384.
32. **Amgen Canada Inc.** Prolia product monograph. Canada; Oct 2011.
33. **Kennel KA, Drake MT.** Adverse effects of bisphosphonates: implications for osteoporosis management. *Mayo Clin Proc.* 2009 Jul; 84(7):632–7;quiz 638.

34. **Papapetrou PD.** Bisphosphonate-associated adverse events. *Hormones (Athens).* 2009; 8(2):96–110.
35. **Khosla S, Burr D, Cauley J, et al.** Bisphosphonate-associated osteonecrosis of the jaw: report of a task force of the American Society for Bone and Mineral Research. *J Bone Miner Res.* 2007 Oct; 22(10):1479–1491.
36. **Ruggiero SL, Dodson TB, Fantasia J, et al.** American Association of Oral and Maxillofacial Surgeons position paper on medication-related osteonecrosis of the jaw—2014 update. *J Oral Maxillofac Surg.* 2014 Oct; 72(10):1938–1956.
37. **Lipton A, Fizazi K, Stopeck AT, et al.** Superiority of denosumab to zoledronic acid for prevention of skeletal-related events: a combined analysis of 3 pivotal, randomised, phase 3 trials. *Eur J Cancer.* 2012 Nov; 48(16):3082–3092.
38. **Ruggiero SL, Dodson TB, Assael LA, et al.** American Association of Oral and Maxillofacial Surgeons position paper on bisphosphonate-related osteonecrosis of the jaw—2009 update. *J Oral Maxillofac Surg.* 2009 May; 67(5 Suppl):S2–12.
39. **Allen MR, Burr DB.** The pathogenesis of bisphosphonate-related osteonecrosis of the jaw: so many hypotheses, so few data. *J Oral Maxillofac Surg.* 2009 May; 67(5 Suppl):S61–70.
40. **Landesberg R, Woo V, Cermers S, et al.** Potential pathophysiological mechanisms in osteonecrosis of the jaw. *Ann NY Acad Sci.* 2011 Feb; 1218:62–79.
41. **Wimalawansa SJ.** Insight into bisphosphonate-associated osteomyelitis of the jaw: pathophysiology, mechanisms and clinical management. *Expert Opin Drug Saf.* 2008 Jul; 7(4):491–512.
42. **Marx RE, Sawstari Y, Fortin M, et al.** Bisphosphonate-induced exposed bone (osteonecrosis/osteopetrosis) of the jaws: risk factors, recognition, prevention, and treatment. *J Oral Maxillofac Surg.* 2005 Nov; 63(11):1567–1575.
43. **Yoneda T.** Bisphosphonate-related osteonecrosis of the jaw: position paper from the Allied Task Force Committee of Japanese Society for Bone and Mineral Research, Japan Osteoporosis Society, Japanese Society of Periodontology, Japanese Society for Oral and Maxillofacial Radiology, and Japanese Society of Oral and Maxillofacial Surgeons. *J Bone Miner Metab.* 2010; 28(4):365–383.
44. **Woo SB, Hellstein JW, Kalmar JR.** Narrative [corrected] review: bisphosphonates and osteonecrosis of the jaws. *Ann Intern Med.* 2006; 144(10):753–761.
45. **Chapurlat RD, Arlot M, Burt-Pichat B, et al.** Microcrack frequency and bone remodeling in postmenopausal osteoporotic women on long-term bisphosphonates: a bone biopsy study. *J Bone Miner Res.* 2007 Oct; 22(10):1502–1509.
46. **Stepan JJ, Burr DB, Pavo I, et al.** Low bone mineral density is associated with bone microdamage accumulation in postmenopausal women with osteoporosis. *Bone.* 2007 Sep; 41(3):378–385.
47. **Pazianas M.** Osteonecrosis of the jaw and the role of macrophages. *J Natl Cancer Inst.* 2011 Feb; 103(3):232–240.
48. **Reid IR, Cornish J.** Epidemiology and pathogenesis of osteonecrosis of the jaw. *Nat Rev Rheumatol.* 2012 Nov; 8(2):90–96.
49. **Ristow O, Gerngross C, Schwaiger M, et al.** Effect of antiresorptive drugs on bony turnover in the jaw: denosumab compared with bisphosphonates. *Br J Oral Maxillofac Surg.* 2014 Apr; 52(4):308–313.
50. **Ruggiero SL, Mehrotra B, Rosenberg TJ, et al.** Osteonecrosis of the jaws associated with the use of bisphosphonates: A review of 63 cases. *J Oral Maxillofac Surg.* 2004 May; 62(5):527–534.
51. **Pickett FA.** Bisphosphonate-associated osteonecrosis of the jaw: a literature review and clinical practice guidelines. *J Dent Hyg.* 2006; 80(3):10.
52. **Wood J, Bonjean K, Ruetz S, et al.** Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid. *J Pharmacol Exp Ther.* 2002 Sep; 302(3):1055–1061.
53. **Fournier P, Boissier S, Filleur S, et al.** Bisphosphonates inhibit angiogenesis in vitro and testosterone-stimulated vascular regrowth in the ventral prostate in castrated rats. *Cancer Res.* 2002 Nov 15; 62(22):6538–6544.
54. **Wehrhan F, Stockmann P, Nkenke E, et al.** Differential impairment of vascularization and angiogenesis in bisphosphonate-associated osteonecrosis of the jaw-related mucoperiosteal tissue. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* 2011 Aug; 112(2):216–221
55. **Scheller EL, Baldwin CM, Kuo S, et al.** Bisphosphonates inhibit expression of p63 by oral keratinocytes. *J Dent Res.* 2011 Jul; 90(7):894–899.
56. **Roelofs AJ, Thompson K, Gordon S, et al.** Molecular mechanisms of action of bisphosphonates: current status. *Clin Cancer Res.* 2006 Oct 15; 12(20 Pt 2):6222s–6230s.
57. **Koch FP, Walter C, Hansen T, et al.** Osteonecrosis of the jaw related to sunitinib. *Oral Maxillofac Surg.* 2011 Mar; 15(1):63–66.
58. **Misso G, Porru M, Stoppacciaro M, et al.** Evaluation of the in vitro and in vivo antiangiogenic effects of denosumab and zoledronic acid. *Cancer Biol Ther.* 2012 Dec; 13(14):1491–1500.
59. **Compston J.** Pathophysiology of atypical femoral fractures and osteonecrosis of the jaw. *Osteoporos Int.* 2011 Dec; 22(12):2951–2961.
60. **Badel T, Pavicin IS, Carek AJ, et al.** Pathophysiology of osteonecrosis of the jaw in patients treated with bisphosphonate. *Coll Antropol.* 2013 Jun; 37(2):645–651.
61. **Cornish J, Bava U, Callon KE, et al.** Bone-bound bisphosphonate inhibits growth of adjacent non-bone cells. *Bone.* 2011 Oct; 49(4):710–716
62. **Melani C, Sangaletti S, Barazzetta FM, et al.** Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. *Cancer Res.* 2007 Dec; 67(23):11438–11446.
63. **Dieli F, Vermijlen D, Fulfaro F, et al.** Targeting human {gamma delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res.* 2007 Aug; 67(15):7450–7457.
64. **Fiore F, Castella B, Nuschak B, et al.** Enhanced ability of dendritic cells to stimulate innate and adaptive immunity on short-term incubation with zoledronic acid. *Blood.* 2007 Aug; 110(3):921–927.

65. **Sato K, Kimura S, Segawa H, et al.** Cytotoxic effects of gammadelta T cells expanded ex vivo by a third generation bisphosphonate for cancer immunotherapy. *Int J Cancer.* 2005 Aug 10; 116(1):94–99.
66. **Roodman GD.** Mechanisms of bone metastasis, pathophysiology of osteonecrosis of the jaw, and integrins, platelets and bone metastasis: meeting report from skeletal complications of malignancy V. *IBMS BoneKEy.* 2008; 5(8):294–296.
67. **Anavi-Lev K, Anavi Y, Chaushu G, et al.** Bisphosphonate related osteonecrosis of the jaws: clinico-pathological investigation and histomorphometric analysis. *Oral Surg Oral Med Oral Pathol Oral Radiol.* 2013 May; 115(5):660–6
68. **Ikebe T.** Pathophysiology of BRONJ: Drug-related osteoclastic disease of the jaw. *Oral Science Internatl.* 2013; 10(1):1–8.
69. **Otto S, Pautke C, Opelz C, et al**. Osteonecrosis of the Jaw: Effect of Bisphosphonate Type, Local Concentration, and Acidic Milieu on the Pathomechanism. *J Oral Maxillofac Surg.* 2010 Nov; 68(11):2837–2845
70. **Otto S, Hafner S, Mast G, et al.** Bisphosphonate-related osteonecrosis of the jaw: is pH the missing part in the pathogenesis puzzle? *J Oral Maxillofac Surg.* 2010 May; 68*(5):1158–1161.*
71. **Aghaloo TL, Kang B, Sung EC, et al.** Periodontal disease and bisphosphonates induce osteonecrosis of the jaws in the rat. *J Bone Miner Res.* 2011 Aug; 26(8):1871–1882.
72. **Filleul O, Crompot E, Saussez S.** Bisphosphonate-induced osteonecrosis of the jaw: a review of 2,400 patient cases. *J Cancer Res Clin Oncol.* 2010 Aug; 136(8):1117–1124.
73. **Thumbigere-Math V, Sabino MC, Gopalakrishnan R, et al.** Bisphosphonate-Related Osteonecrosis of the Jaw: Clinical Features, Risk Factors, Management, and Treatment Outcomes of 26 Patients. *J Oral Maxillofac Surg.* 2009 Sep; 67(9):1904–1913.
74. **Bonnet N, Lesclous P, Saffar JL, et al.** Zoledronate effects on systemic and jaw osteopenias in ovariectomized periostin-deficient mice. *PLoS One.* 2013; 8(3):e58726.
75. **López-Jornet P, Camacho-Alonso F, Martínez-Canovas A, et al.** Perioperative antibiotic regimen in rats treated with pamidronate plus dexamethasone and subjected to dental extraction: a study of the changes in the jaws. *J Oral Maxillofac Surg.* 2011 Oct; 69(10):2488–2493.
76. **Abtahi J, Agholme F, Aspenberg P.** Prevention of osteonecrosis of the jaw by mucoperiosteal coverage in a rat model. *Int J Oral Maxillofac Surg.* 2013 May; 42(5):632–636.
77. **McLeod NM, Patel V, Kusanale A, et al.** Bisphosphonate osteonecrosis of the jaw: a literature review of UK policies versus international policies on the management of bisphosphonate osteonecrosis of the jaw. *Br J Oral Maxillofac Surg.* 2011 Jul; 49(5):335–342.
78. **Katsarelis H, Shah NP, Dhariwal DK, et al.** Infection and medication-related osteonecrosis of the jaw. *J Dent Res.* 2015 Apr; 94(4):534–539.