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COORDINATORE Prof. Bernardi Francesco

EDENTULISM PROBLEM:

USE OF STEM CELLS FOR BONE REGENERATION AND BONE INFLAMMATION

Settore Scientifico Disciplinare MED/04

Dottorando

Dott. Bressan Eriberto

Tutore

Prof. Pinton Paolo

Anni 2010/2014

Indice

Summary

In this study we have started from a real odontoiatric clinic problem, which affects a high percentage of patients: edentulism.

Implant therapy, started by Prof. Branemark in the seventies thanks to the discovery of osseointegration, has developed in the last 15 years.

This therapy represents a valid solution for edentulous patients, although it cannot always be used.

Severe osseous resorption or anatomical limitations hinder the use of implant and force the employ of biomaterials and/or autologous bone, which, according to the latest scientific research, represents the gold standard.

However, intraoral bone graft is not always well tolerated by patients and, in cases of severe atrophy, the quantity of bone that can be taken from the oral cavity is not enough for complete regeneration.

Although implant therapy represents the best solution for edentulous patients, in the latest years a biological complication has become more and more frequent: peri-implantitis.

It represents a serious problem since it begins with an inflammation of peri-implant hard tissues that clinically leads to a loss of peri-implant alveolar bone and, eventually, to the loss of the implant as a whole.

According to the most recent literature, this serious problem affects 10% of the patients and 4% of the implant sites after 10 years of follow-up.

To make this pathology even more serious is the little knowledge that clinicians and researchers have about early diagnosis, etiopathogenesis and therapy.

The purpose of this study is to address both edentulism and consequent osseous regeneration, as well as the problem of peri-implantitis from a biological point of view and with the help of tissue engineering.

In particular, we wanted to test the ability of stem cells taken from adult tissue to favor - in shorter time - osseous regeneration and implant osseointegration both *in vitro* and on small and large animals.

Furthermore, we have tested the anti-inflammatory ability of stem cells in bone tissue. Finally, with regard to the problem of peri-implantitis, we have searched for predictive genetic factors in order to possibly identify patients at risk.

Preliminary results of studies performed both *in vitro* and on animal enable us to

state that:

- It is possible to isolate stem cells from different adult tissues (adipose tissue, dental pulp) and test their genetic stability.
- For the cells isolated from dental pulp, we could identify an important connection between patient's age and ability to differentiate and proliferate.
- Stem cells combined with different scaffolds are able to foster osseous regeneration faster.
- It has been recognized an actual anti-inflammatory ability of stem cells in bone tissue.
- Preliminary results on the use of CGH as genetic predictive technique for peri-implantitis are encouraging since they foreground a correlation between the genetic alteration of some chromosomal tracts and clinical onset of the disease.

Introduction

Edentulous patients

The absence of good oral health in adults can manifest into social, physical, and emotional health issues. Poor oral health, therefore, negatively impacts on quality of life. Oral health is instrumental to older people's health, life satisfaction, quality of life and their perception of self. [Mitchell et al. 2013; Mariño et al. 2013]
In addition, clinical data demonstrates that poor oral health increases the risks to health in the same way as any disease of the body system [health 2001]. The interconnections between poor oral health, in particular periodontal disease, and other acute and chronic medical conditions (e.g. pneumonia, cerebrovascular and cardiovascular disease as acute myocardial infarction stroke and coronary heart disease [Emingil et al. 2000; Elter et al. 2003; Hung et al. 2007] diabetes, nutritional deficiencies are now established [Genco and Van Dyke 2010; LOESCHE et al. 1998].

The ability to chew and swallow food comfortably, to speak and to interact socially, can be compromised by common oral diseases and partial or full edentulism conditions. (Figure 1, Figure 2)
Partial edentulsim is the absence of at least one natural tooth, and full edentulism is the complete absence of all natural teeth.

Thorstensson H. and Johansson B. [Thorstensson and Johansson 2010] suggests that the main cause of tooth loss is due to caries in about 55% of swedish individuals of the study, up to the oldest age substantial increase in frequency to 75%. Periodontitis, as a reason for tooth loss, is of minor importance compared with caries but increase steadily over the lifespan from 18 to 33%. Other reasons are toothache, endodontics and tooth/root fractures.

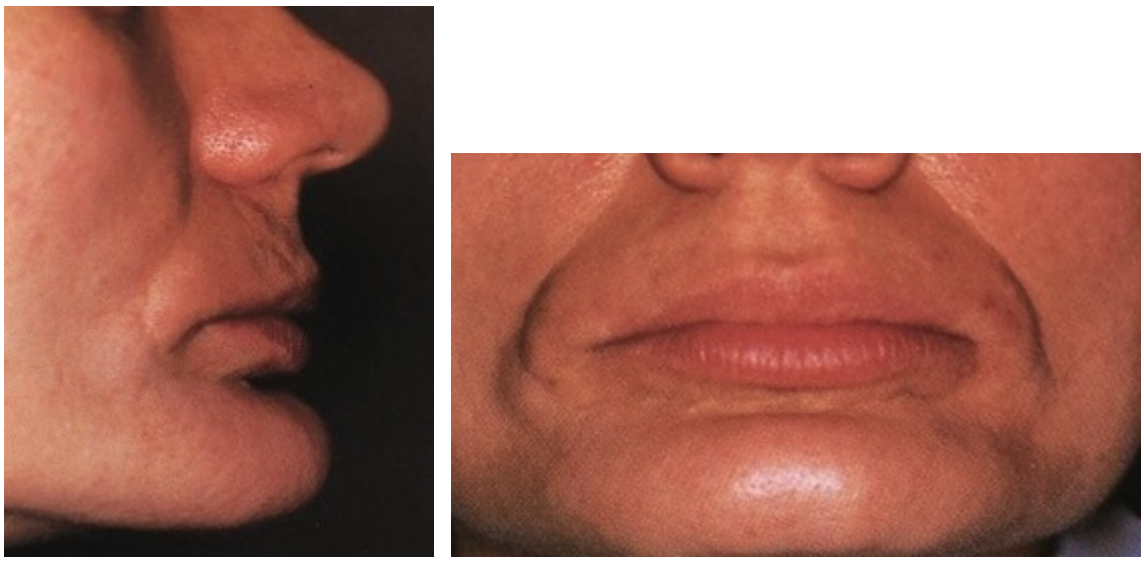


Figure 1: edentulous patient with severe atrophy: lateral view, frontal view

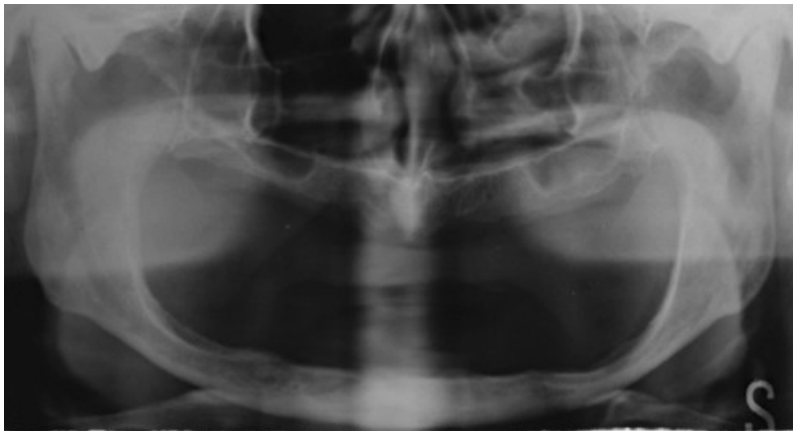


Figure 2: orthopantomography

Oral health status and quality of life are strictly related.

The ability to chew food may affect dietary choices and nutritional intake and have consequences for general health [Joshipura et al. 1996a; KRALL et al. 1998]. Edentates have been shown to have a significantly lower fruit and vegetable intake than the fully dentate. Tsakos G et al.[Tsakos et al. 2010] suggests that edentate individuals consumed 50.7 g (27.0, 74.3) fewer fruits/vegetables per day than the dentate. Joshipura et al. [Joshipura et al. 1996b] investigated the association between tooth loss and diet. The edentulous had a higher intakes of total fat and saturated fat and a lower intake of non-starch polysaccharide (NSP) (dietary fiber), β-carotene, and fruits and vegetables than subjects with 25 or more teeth. Consumption of fruit and vegetables is also positively related with the number of natural teeth. Patients with loss of functional dentition and denture-wearing result in selective food intake, hard foods and foods cointaining seeds and pips are

avoided (such as tomatoes, grapes and whole grain bread) with the effects of material deprivation on nutrient intake [Moynihan and Bradbury 2001].

Oral health is not only related to masticatory function and chew ability. Esthetic and psycological problems are often related with loss of teeth. Missing teeth can have negative consequences on self-image, social interaction and psychological health. Interviews done on edentulous patients by Fiske J et al. [Fiske et al. 1998] suggests the main themes identified in reaction to tooth loss were: lowered self-confidence, altered self-image, dislike of appearance, an inability to discuss this taboo subject, a concern about prosthodontic privacy, behaving in a way that keeps the tooth loss secret, altered behaviour in socialising and forming close relationships, premature ageing, and lack of preparation. Active ageing requires maintenance of oral health status: desire for physical attractiveness and interest in appearance does not decline with age [Xiaoxian Meng et al. 2007]; consequently in edentates patients complete prosthetic treatment contributes to maintaining aesthetic appearance, fluent speech and suitable occlusal arrangements for masticatory efficiency [Quran et al. 2001].

Papadaki E. and Anastassiadou V. [Papadaki and Anastassiadou 2012] correlate emotional reactions to tooth loss with denture satisfaction attributes in elderly complete denture wearers. Questionnaire for Emotional reactions to tooth loss showed that 60% of patients had not only difficulties in accepting their tooth loss, but 65% of the younger participants and 47% of the older ones required more than 6 months to come to terms with it. The same pattern was revealed regarding time of acceptance. Four key feelings associated with losing the last tooth/teeth identify sensation of relieved in 1/4 of the subjects, sadness in 1/4 and resignation and oldness in 1/5. Older subjects more often felt relieved with tooth clearance, in contrast to the younger subjects who were more likely to develop negative feelings of bereavement. The study suggests that a substantial proportion of patients were satisfied with their complete dentures rehabilitation but some patients experienced increased social and psychological problems related to their edentulousness and the wearing of complete dentures. The aesthetic and functional aspects of complete dentures affected both patients' social behaviour and self-confidence as going out and laughing in public, speaking difficulties correlated with avoiding social interaction.

The edentoulus patient has a resorption of alvelar bones. This condition in association with the absence of teeth create physiognomy changes as leak of intraoral volume and deflation of perioral tissues (lips and cheeks). Esthetics changes could create psychological problems and loss of self-esteem.

Carossa S. et al [Carossa et al. 2000] investigated the correlation between edentulism, sleep disorders and arterial hypertension. Respiratory disturbances during sleep are considered risk factors for arterial hypertension and cardiovascular diseases. Edentulism, by decreasing retro-pharyngeal space, may favor upper airway occlusion during sleep. In edentulous subjects, removing dentures during sleep may favor respiratory disorders, and increase the risk for hypertension and cardiovascular disease.

Different studies demonstrate that oral health conditions and edentulism is strictly related to age, education, socio-economic status, ethnicity and smoking [Thorstensson and Johansson 2010; Wu et al. 2012; Kim et al. 2012; Brennan et al. 2008; Elani et al. 2012].
In economically developed countries, the trend of edentulism has declined consistently. In England and Wales, the prevalence of edentulism for the adult population declined from 37% in 1968 to 12% in 1998 [Kelly et al. 1998]. In Australia, the prevalence of edentulism for the adult population declined from 20.5% in 1979 to 8% in 2002. Among older adults aged 65 and above, the reduction for males was from 59.7% to 26.5% and for females was from 71.5% to 40.3% [Sanders et al. 2004]. Similarly, in the United States, the few studies available on middle-aged and older adults have shown that edentulism in these age groups has been dropping for the past several decades. One study revealed that within the period of 1971 and 2001, for those in a low socioeconomic position (SEP), the prevalence of edentulism declined from 50% to 32% in adults age 55–64 and 58–43% in age 65–74; the comparable declines for these age groups for individuals in a high SEP were 22–6% and 30–9%,respectively [Cunha-Cruz et al. 2007]. A report conducted by the National Centers for Health Statistics using the National Health and Nutrition Surveys of 1988–1994 (NHANES III) and NHANES 1999–2004 [Kim et al. 2012] found that the prevalence of edentulism declined in the United States over these two-time periods from 34% to 27% among adults aged 65 and older over [BA et al. 2007]. Thanks to improvements in oral health,

the proportion of the population who are edentulous has declined over the past 20 years [Sanders et al. 2004]. However, the number of people requiring complete dentures has been predicted to increase over the next 20 years in the United States. Moreover, although implant treatment is reportedly increasing, the need for complete denture treatment is likely to remain substantial in the future rise [Starr and Hall 2009].

Musacchio et al. [Musacchio et al. 2007] reported the prevalence of edentulism in north-est of Italy was 43.8%; this was more pronounced in women and increased with age. It was 31.8% in the 65-69 years age group and more than twice (63.9%) in the 90 years group. The prevalence of edentulous subjects was much higher in heavy smokers than in non-smokers (55.6% versus 26%) and in subjects with 0-3 years of education (52.4%) than 4-8 years (44.3%) and >8 years (3.3%).

Douglass et al. [Douglass et al. 2002] indicated that edentulism has declined by 10% every decade and that only 90% of edentulous adults obtain and wear complete dentures. However, when the number of adults in each specific age group is multiplied by the percentage who need a complete maxillary or mandibular denture, the results suggest that the adult population in need of 1 or 2 complete dentures will increase from 33.6 million adults in 1991 to 37.9 million adults in 2020. The 10% decline in edentulism experienced each decade for the past 30 years will be more than offset by the 79% increase in the adult population older than 55 years. the number of people in the United States who need complete dentures will increase over the next 20 years despite an anticipated decline in the age-specific rates of edentulism.
The assumption that the most of edentulous persons wear and utilize dentures was proven Redford et al and Marcus et al. [Redford et al. 1996; Marcus et al. 1996] in their analysis of denture use in the United States and New England. These 2 research groups found that 89.6% [Redford et al. 1996] and 89.9% [Marcus et al. 1996], respectively, of the edentulous population used dentures.

For past decades, conventional dentures were the only available treatment for edentulism. This treatment relies on the retention and support provided by remaining bone ridge, but many denture-wearing patients have a poor diet and cannot speak clearly due to lack of denture retention and stability [Sánchez-Ayala

et al. 2010]. Inevitable ridge resorption, at the onset of edentulism or over time, may further decrease oral function if dentures do not remain retentive and stable. Ill-fitting dentures can prevent enjoyment of food and affect overall nutrition [Marcus et al. 1996]. The success of conventional complete dentures treatment is variable and depends on a patient's adaptive capacity to overcome the limitations of complete dentures by an habituation process. The inadequacy of conventional treatment makes implant therapy an alternative to provide significant improvement in stability, retention and quality of life in denture-wearing patients. [Sánchez-Ayala et al. 2010]. Moreover implant-supported fixed dental prosthesis could be considered a restitutio ad integrum of oral health problems.

The introduction of osseointegrated implants has opened new possibilities for improving chewing capacity of edentulous subjects wearing removable dentures. In addition to fixed dental prosthesis, implants can also be used to improve the function of removable prosthesis by the use of various retention systems.
The limit of implant therapy is inadequate quantity of bone: resorption and remodelling of the alveolar ridge is a process that occurs especially after tooth extraction and results in a decrease ridge dimension [Schropp et al. 2003; Araujo and Lindhe 2005]. The volume and rate of bone loss depends by different factors such as gender, hormones, general disease, denture rehabilitation and metabolism [Güler et al. 2005]. The greater amount of resorption occurs during the first 3 months of healing with a significative loss of height and width of the alveolar bone [Schropp et al. 2003; Kerr et al. 2008]. Horizontal dimensional changes consist of 50% alveolar ridge reduction after 1 year from tooth extraction [Schropp et al. 2003]. The resorption occurs primarily from the buccal aspect, with significantly less resorption from the lingual aspect [Araujo and Lindhe 2005]. Alveolar ridge resorption is usually more rapid in the premolar and molar region than the anterior region af the mandible [AA 2002].
Furthermore after teeth extraction in the anterior mandibular region there is higher basal bone disponibility and there are not anatomical landmarks to be compromised for implants insertion such as in the maxillary anterior region. Oikarinen et al suggest that implants of 8mm or longer could be inserted in the anterior maxilla in more than 50% of patients. The mandible showed that implantation was possible in almost every jaw with fixture of 8mm or longer in the canine regions [Oikarinen et al. 1995]. Instead in the maxillary posterior region the

proportion of sites with bone height ≥8mm and bone width ≥6mm was 28.3%, 18.4%, 8.0% and 18.2% at first premolar, second premolar, first molar and second molar sites, respectively [Pramstraller et al. 2011] The use of removable dentures significantly increases the severity of alveolar bone resorption in edentulous areas. Xie et al. reported that the edentulous maxilla has a much greater reduction in radiologic heights compared to the edentulous mandible for both sexes. The finding is considered to be in agreement with the study of Tallgren [Xie et al. 1997; Tallgren 1972].

In addition to the problem of edentulism, Injuries caused by trauma, tumor or cyst resection, infectious diseases, and also congenital and developmental conditions (i.e., cleft palate de- fects) may result into serious functional, aesthetical and psychological sequelae [Cohen 1995; Hunt and Hobar 2003]. In such situations, absence of hard and soft tissues can be disfiguring and often compromise basic functions such as mastication, speech, swallowing, and also lead to limited thermal and physical protection of important anatomical structures (i.e. brain, nerves, arteries, veins) [Davis and Telischi 1994; Kadota et al. 2008; Curtis et al. 1997; Urken et al. 1991]. The progression of certain oral conditions may also result in craniofacial defects of difficult resolution. For istance periodontitis is a chronic inflammatory disease of bacterial etiology, characterized by the loss of support around teeth, including alveolar bone resorption and soft tissue alterations [Genco 1992; Kinane and Bartold 2007; Feng and Weinberg 2006].
Achieving predictable regeneration in the treatment of craniofacial defects is remarkably challenging in most clinical scenarios

Another current cause of bone resorption is peri-implantitis. Peri-implantitis is defined as inflammation of peri-implant tissues accompanied with changes in the level of crestal bone and with the presence of bleeding on probing and/or suppuration, with or without concomitant deepening of peri-implant pockets [Lang et al. 2011].
Peri-implantitis is a serious current problem because neither the causes neither therapies are currently unclear.
However, this disease will be discussed in the next chapter

Regenerative procedures, applying the concept of guided bone regeneration, use of bone grafts, and membranes, are implemented to rebuild peri-implant supporting bone, rebuild bone after implant-extraction for peri-implant inflammation and for alveolar ridge regeneration.

Tooth anatomy

Each tooth consists of three main parts: the crown, the neck and the root, that we can define with anatomic o clinical criteria. Here follow a brief review of the tooth anatomy (Figure 3) involved on stem cells-tissue engineering field.

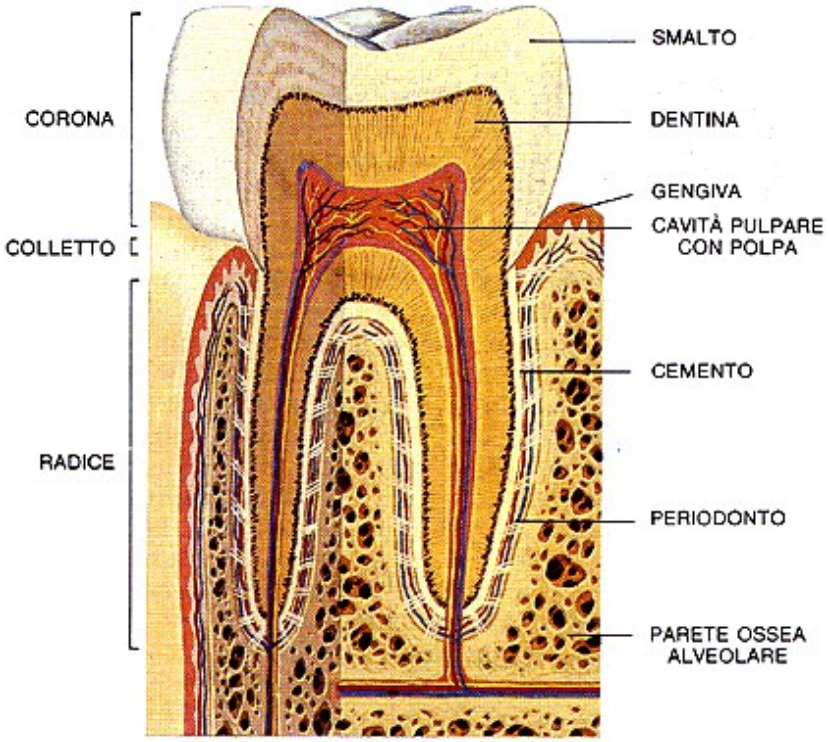


Figure 3: tooth anatomy

Dentin

Dentin is a mesenchymal derived tissue lying between enamel or cementum and dental pulp (pulp chamber and root canal). It is a mineralized connective tissue with an organic matrix. It is made up of 70% inorganic materials (especially hydroxyapatite crystals), 20% organic materials and 10% water by weight. The bulk of organic matrix (85-90%) consists of type I collagen, there is also a minor amount of type V and VI collagen. Noncollagenous molecules of dentin are dentin phosphorines, Gla proteins, acidic glycoproteins, growthrelated factors, serum derived proteins, lipids and proteoglycans. Dentin has microscopic channels (0,5-3

μm), called dentinal tubules, radiating outward from pulp cavity to dentinoenamel or dentino-cementum junction. These tubules contain projections of cells secreting dental matrix, known as odontoblasts. The most peripheral aspect of the pulp is lined by the body of these odontoblasts [Yoshiba et al. 2002]

Pulp

Pulp consists of a loose connective tissue enclosed by rigid predentin and dentin. Along the border between the dentin and the pulp are odontoblasts. The thickness of dentinal layer increases with age due to the deposition of secondary and tertiary dentin, reducing the volume of the pulp chamber and the root canals. The most peripheral aspect of the pulp contains four layers of cells: the odontoblastic layer (the most external one), the cell-free zone, the cell-rich zone and the parietal plexus of nerves. Deep inside is the pulp proper, composed of a great amount of fibroblasts and ECM. Blood vessels and nerves enter the tooth mostly through the apical foramen. Other cells in the pulp include undifferentiated mesenchymal cells, deriving from dental papilla, fibrocytes, macrophages and lymphocytes.

Alveolar bone

The bone that supports the teeth is called alveolar bone. It is composed of compact bone and trabecular or spongy bone. The outside wall of the bone is compact bone, such as the thin layer that lines the socket known as lamina dura.
The spongy bone is inside and contains bone marrow. The number and the size of the trabeculae in this bone are determined by the function activity of the organ.
Alveolar bone proper is the part just around the tooth and it gives attachment to the PDL fibres (bundle bone). The alveolar bone proper is also called cribiform plate, due to the presence of perforation for the entry of vessels and nerves.
Bone is made of 65% inorganic material (mainly hydroxyapatite) by weight, 15% water, 20% organic material. The organic matrix is composed of collagen type I (90-95%), Gla proteins, glycoproteins, phosphorines, proteoglycans, growth factors and bone morphogenetic proteins (e.g. osteogenin) [Lindhe et al. 2009a].

Odontogenesis

During the sixth week of embryogenesis, after the migration of neural crest cells into head and neck mesenchyme, the ectoderm of the first brachial arch begins to proliferate giving rise to the vestibular lamina and the dental lamina.

Dental lamina is a band of ectodermic cells growing from the epithelium of the stomodeum into the underlying mesenchyme and giving rise to the enamel organs of teeth, along the horse shoe shaped dental arches.

Several transcription factors are implicated in odontogenesis, including Pax9, Pitx2, Runx2, Msx1, Msx2, Bmp2, Bmp4, Fgf8 and Fgf9 [Bei and Maas 1998]. The development is commonly divided into the following stages: the bud stage, the cap stage,the bell stage.

The early bell stage of odontogenesis is characterized by epithelial expansion and differentiation into the inner and outer enamel epithelium, stratum intermedium and stellate reticulum.

During the late bell stage, two tooth specific cell types are formed: ameloblasts, which derive from the inner enamel epithelium and produce enamel, and odontoblasts, which differentiate from dental papilla and synthesize dentin.

Dentinogenesis starts before enamel formation with the secretion of an organic matrix in the area directly adjacent to the inner enamel epithelium. Dentin is formed by the production of organic matrix (predentin) and the simultaneous mineralization of this matrix [Hao et al. 2009].

After crown formation, root development begins. The cells of the inner and of the outer enamel epithelium become in contact and give rise to the cervical loop at the base of enamel organ.

The cells of the cervical loop continue to grow away from the crown and become Hertwig's epithelial root sheath. It induce the adjacent cells of dental papilla to differentiate into odontoblasts and produce dentin. Once this structure fragments, the dentin of the root comes in contact with the dental follicle and stimulates the cementoblasts to begin cementum secretion.

The dental follicle also gives rise to the other supporting structure of the tooth: the periodontal ligament and the alveolar bone proper [Luan et al. 2006].

Bone tissue

Bone tissue is a connective tissue specialized in providing support. Affinity of bone tissue with connective tissues is confirmed by both its origin from mesenchyme, the embryonic tissue where connective tissues is derived from, and its composition since the extracellular matrix is made up of collagen fibers and amorphous fundamental substance.

Bone tissue is characterized by mineralization. The presence of minerals and the peculiar distribution of organic components in the extracellular matrix lend to this tissue remarkable mechanic properties: hardness, resistance to pressure, traction and torsion. Thanks to these properties, bone tissue represents an ideal material for the formation of skeletal bones, which as a whole can be seen as a supportive scaffolding of the organism. Furthermore, given the relevant amount of calcium salts, bone tissue represents the principal store of calcium ions for the metabolic needs of the entire organism.

Calcium deposition in the bone and its mobilization, finely controlled by endocrine mechanisms, provide a crucial contribution to the regulation of plasma levels of this ion.

Morphology of bone tissue

From a macroscopic point of view, two varieties of bone can be distinguished: compact bone and spongy bone (Figure 4)

Compact bone

Compact bone is found in the outer layer of short, flat and long bones and constitutes the diaphysis of the latter as well.

The bone matrix of the compact bone is organized in lamellae which form cavities named bone lacunae containing osteocytes. Compact bone lamellae usually arrange themselves in three different ways:

- Concentrically around vascular channels to form cylindrical structures, named *Haversian systems* or *osteons*, oriented along the major axis of the bone;
- In the spaces around osteons, taking on various dimensions and irregular shapes, to form *interstitial systems* ;

- Under the periosteum (a layer of connective tissue on the outer surface of the bone) and over the endostium (connective tissue on the inner surface of the bone) to form *inner and outer circumferential lamellae*.

In the compact bone two categories of vascular channels can be identified:

- *Harvesian canals*: longitudinal canals located at the center of osteons containing one or two vessels;
- *Volkmann's canals*: transverse canals interconnecting Harvesian canals

Spongy bone

Spongy bone can be found primarily inside short bone, flat bones and epiphysis of long bones.

It is made up by a tridimensional network of branched bone spicules named *trabeculae,* that limit a labyrinth of interconnected spaces occupied by hemopoietic bone marrow.

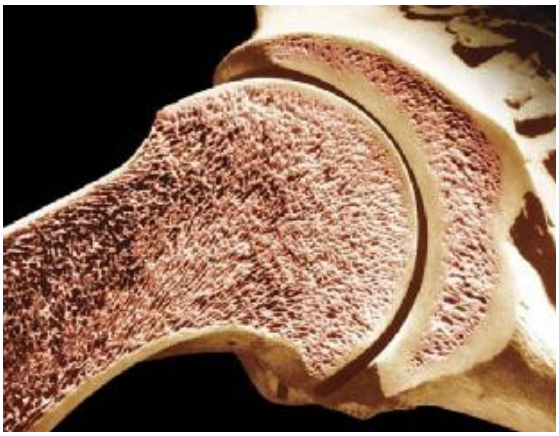


Figure 4: Distribution of compact and spongy bones in the bone.

Bone extracellular matrix

Being a connective tissue, bone tissue contains a relevant amount of organic extracellular matrix, composed of connective fibers and amorphous substance enriched by the mineral component.

Organic matrix

It favors resistance to traction and pressure. It consists of:

- *connective fibers*: almost totally made up of Type I collagen fibers, are characterized by a great number of crossed bundles that maintain united the sigle molecules of tropocollagen. The abundant presence of collagen is the

main cause of the marked acidophilia that characterizes the intracellular substance of the bone.

Collagen fibers aggregate to form remarkably thick collagen fibers (5-10 µm) only in the *fibrous bone tissue*, whereas in the *lamellar bone tissue* collagen microfibrils group to form a homogeneous tridimensional network. Connective fibers are particularly abundant in the periosteum therefrom thick bundles of collagen fibers start to penetrate into the cortical bone tissue and get lost in the intracellular substance of the bone. These bundles constitute *Sharpey's perforating fibers*, which fasten the periosteum to the surface of the bone.

- *elastic fibers*: virtually absent from the bone tissue, reticular fibers are located at the level of the basal membrane that surrounds intraosseous blood vessels;
- *amorphous substance*: characterized by a peculiar and relevantly different composition in respect to other connective tissues, it is composed of various macromolecules:
    - Proteoglycans: made up of sulfated glycosaminoglycan acids (cheratan sulfate, chondroitin sulfate) which are kept together by short protein chains.
        - Glycoproteins: include several molecules, some of which are believed to play a fundamental role in the mineralization processes. Among these:
            a) Osteonectin: the most abundant protein. It possesses high affinity for calcium, both as a free ion and as an associated ion in crystal-like complexes. It is believed that it works as nucleation agent for mineral crystals since it is considered capable of concentrating calcium located nearby, thus creating the conditions to start the precipitation of calcium phosphatase.
            b) Fibronectin: is an adhesion molecule located primarily in the pericelluar matrix and characterized by a portion capable of binding with collagen. It is believed that fibronectin is involved in the following processes: migration, adhesion to the matrix and organization of the bone cells.
            c) Alkaline phosphatase: is an enzyme capable of hydrolyzing phosphate groups of organic substrates in a basic environ-

ment. It is involved in the processes of mineralization, providing phosphate ions to form mineral crystals.

- Sialoproteins (BSP): specific glycoproteins containing traces of sialic acid which possess the RGD (Arg-Gly-Asp) aminoacid sequences responsible for the adhesion of the cells to the bone matrix. They include osteopontin.
- Proteins containing gamma linolenic acid (GLA): thanks to GLA they are able to chelate bivalent cations such as calcium ion. Two proteins containing GLA can be identified in the bone:
  a) Osteumcalcin: a small protein containing 3 or 5 GLA residues. It is involved in the inhibition of the matrix mineralization since it binds calcium ions and makes them available for the combination with phosphate ions thus inhibiting the dimensional growth of crystal minerals. It is abundant in the mature bone tissue whereas is scarce in the developing bone tissue (osteoid tissue).
  b) GLA protein of the matrix: it has a higher molecular weigh than osteumcalcin and can be found both in the mature bone and in the osteoid tissue as well as in the cartilage which is about to be replaced by osseous tissue.

Inorganic matrix

The mineral component represents 65% of the dry weight of the bone.

Its function is to provide hardness and rigidity to the bone tissue.

It is made up of calcium crystals - mainly calcium phosphate - and of calcium carbonate, calcium fluoride and magnesium phosphate in smaller quantities. Calcium phosphate can be found in the form of apatite crystals ($Ca_{10}(PO_4)_6^{2+}$) whose positive charges are normally neutralized by the binding with two hydroxide ions thus forming hydroxyapatite.

Hydroxyapatite crystals appear like long and thin needles about 2 nm thick and 20-40 nm long. They tend to arrange themselves parallel to each other and to collagen microfibrils, covering their surface and permeating their porosities.

During the mineralization process of the bone, calcium phosphate precipitates at first in the form of tiny amorphous aggregates.

These initial nuclei of mineral concretion are rapidly replaced by very thin crystals positioned parallel to the filamentous molecules of the fundamental substance

called axial filaments.These crystals assume the typical shape of apatite crystals, progressively occupying most space inbetween collagen microfibrils and permeating the microfibrils themselves. Once the apatite crystals have formed, the deposition of new mineral can occur both through the formation of new crystals and through apposition on pre-existing crystals. This phenomenon is finely regulated by bone cells thanks to the production of specific molecules of the bone matrix.

Bone cells

Four types of cells can be identified in the bone tissue:
Osteoprogenitors (also called pre-osteoblasts), o*steoblasts, osteocytes* and osteoclasts. Among these, osteoprogenitors, osteoblasts and osteocytes are in fact subsequent functional phases of the same cell type and are derived from the pluripotent mesenchymal cell of connective tissues. Osteoclasts, instead, are derived from precursors migrated from the blood to the bone tissue, the so called pre-osteoclasts, which are derived from the stem cells of the hematopoietic bone marrow.

Osteoprogentitor cells

Pre-osteoblasts have a spindle or oval shape, a dispersed chromatin nucleus (euchromatic) with a large nucleolus and scarce and basophilic cytoplasm because of the presence of a number of free polyribosomes, whereas other granules are scantily represented.
Osteoprogenitor cells place themselves on the free surfaces of the bones: the can be recognized at level of the inner layer of the periosteum, the so called Ollier's osteogenic layer, rich in vessels and at the level of the endostium close to capillaries.
Osteoprogenitor cells are able to proliferate, a feature which is shown especially when their body is growing but can be observed also during adult life. They can produce and secrete growth and differentiaton factors, the so called *bone morphogenetic proteins* (BMP). When they start the differentiation process, osteoprogenitor cells change into osteoblasts.

Osteoblasts

Osteoblasts are primarily responsible for synthesizing the extracellular bone matrix and for its mineralization. They have a spherical or polyhedric shape and tend to align to form epithelious laminae by the developing bone surfaces. In the active phase of the deposition of the bone matrix, osteoblasts have relevant dimensions (about 20 μm), a euchromatic spherical nucleus with a large nucleolus, a basophilic abundant cytoplasm with PAS-positive granulations, a well-developed Golgi's apparatus and a number of long-shaped mitochondria. Osteoblasts are characterized by the positivity for alkaline phosphatase and by the presence of small cytoplasmatic granules pink in colour containing the precursors of the bone matrix glycoproteins. On the side facing the mineralizing bone matrix, they show several vescicles rich in proteoglycan which, once expelled, will form the mineralization nuclei.

Osteoclasts are interconnected with each other and with nearby osteocytes by means of gap junctions to exchange signal molecules which coordinate metabolic activity and bone matrix deposition.

Osteocytes

Osteocytes are cells which are typically present in the mature bone and are responsible for its maintenance and turnover. They are terminal cells with a finite lifespan.

An osteocyte is a star-shaped cell, with a cellular body resembling a biconvex lens and with several cytoplasmatic extensions.

It presents a heterochromatic nucleus (with condensed chromatin) a small nucleolus and a perinuclear cytoplasm rather scarce and basophilic. Cytoplasmatic organules, RER and Golgi's apparatus tend to decrease their dimension as the cell grows older until it dies due to apoptosis.

In the bone tissue, the osteocyte is enclosed in a niche carved in the bone matrix, called *bone lacuna*, whose shape replicates that of the cell, whereas its extensions reach thin canals called *bone canaliculi*.

Each osteocyte is in contact with surrounding osteocytes through gap junctions at the extremities of their extensions. Water and metabolites can reach all osteocytes, even the farthest away from blood vessels, through non-mineralized osteoid tissue which covers the inner communicating surface of lacunae and canaliculi. Moreover, metabolites and signal molecules dissolved in the cytoplasm can be exchanged between osteocytes through the gap junctions.

Osteoclasts

Osteoclasts are cells specialized in bone resorption. They derive from pre-osteoclasts originating in the hematopoietic bone marrow and are carried by the blood stream up to the sites of bone resorption, where they melt together generating active osteoclasts, i.e. syncytial elements capable of dissolve and digest organic components of the bone tissue.

Mature osteoclasts are giant plurinucleated cells (100-200 μm) with acidophilic cytoplasm; in a single osteoclast can be detected up to 50 nuclei with lax chromatin and clearly visible nucleolus. When a osteoclasts is activated it adheres to the mineralized matrix and due to its erosive action a cavity called *Howship's lacuna* is formed. The side of the cell which is clinged to the bone is characterized by the so called ruffled border, a thickening of the cell surface made up of a large number of cytoplasmatic lamellae, having different sizes and lengths, which considerably widen the extension of the plasmalemma.

Through the ruffled border osteoclasts adhere tightly to the surface of the bone to be resorbed, delimiting the extracellular environment where ostoelytic substances are released, an area called *sealing zone*.The resorbtion of the bone matrix begins with the dissolution of the mineral component due to the acidification of the microenvironment in the *sealing zone*, followed by exocytosis of lysosomal enzymes which digest the organic components of the bone matrix. Furthermore, osteoclasts stimulate osteobalsts to release collagenase enzyme which contributes, through its lytic activity, to the digestion of the organic matrix of the bone. Once the first lacuna has been formed, the osteoclast separates from the bone matrix and migrates with amoeboid movement to a portion of an adjacent bone, adhering again and forming a new lacuna. Osteoclastic function is finely regulated by hormonal and local factors.

Bone histogenesis

Bone always develops replacing a pre-existing tissue, be it mesenchyme or a differentiated connective tissue. The processes which lead to the genesis of the bone tissue within another tissue are called *ossification* or *osteogenesis*.

These processes are maximized during prenatal life and continue to maintain a high pace throughout the period of the somatic development.

Two types of ossification can be identified:

- Direct or intramembranous ossification;
- Indirect or chondral ossification.

Direct ossification

Direct or intramembranous ossification is typical of flat bones. It starts from ossification centers which develop in the mesenchyme at early stages of foetal life or in membranes of fibrous, dense connective tissue derived from mesenchyme at later stages of intrauterine and postnatal life.

Direct ossification begins when, beside a rich vascular network, mesenchymal cells differentiate into osteoprogenitor cells which, in turn, change into osteoblasts. Through gap junctions osteoblasts align themselves in epithelial-like rows and start depositing the organic matrix of the bone, i.e. osteoid tissue. When the osteoid tissue undergoes mineralization, it changes into spongy bone and osteoblasts remain enclosed in bone lacunae transforming into osteocytes. Afterwards preosteoclasts differentiate into osteoclasts which dissociate fibrous bone, later replaced by lamellar bone.

Indirect ossification

Indirect or chondral ossification is the most widespread variant. Typically, the bone is preceded by a cartilage scaffold with about the same shape of the bone segment to be and which is later reabsorbed and replaced by bone tissue. Bone can develop both inside the cartilage scaffold (endochondral ossification) and on the outer surface of the cartilage in contact with the perichondrium (perichondral ossification).

Perichondrial ossification starts in the perichondrium where osteoprogenitor cells differentiate into osteoblasts which deposit osteoid tissue. This tissue is committed to become fiber bone through mineralization and is later rearranged by osteoclasts with subsequent deposition of lamellar bone. The bone thus generated binds to the surface of the cartilage, under the perichondrium which then develops into periosteum.

Endochondrial ossification starts when the chondrocytes of the cartilage scaffold undergo hypertrophy. In their cytoplasm drops of glycogen accumulation and PAS-positive granulations resembling the calcifying globules of the osteoclasts can be detected. They release vesicles inducing the calcification of the cartilage matrix and, finally, are destined to undergo apoptosis.

Calcified cartilage matrix is partially subject to erosion thanks to the intervention of cells of osteoclastic nature, coming from the nearby already-formed bone and generating wide cartilage lacunae which merge into each other and where blood vessels, departing from the perichondrium and accompanied by mesenchymal cells, penetrate. Mesenchymal cells differentiate into osteoprogenitor cells and afterwards in osteoblasts which deposit fibrous bone by the remnants of the calcified cartilage matrix. Finally, osteoclasts intervene to resorb both the fiber bone and the mineralized bone matrix, whereas new osteoblasts deposit lamellar bone. Some of the mesenchymal cells, penetrated with blood vessels, originate new vessels and hematopoietic bone marrow. [Zallone 2007; Capitani et al. 1996]

Implant therapy

Missing teeth and supporting oral tissues have traditionally been replaced with removable dentures or fixed bridges permitting restoration of masticatory, phonetic function, and aesthetics.

Replacement of lost dentition has been traced to ancient Egyptian and South American civilizations. In ancient Egyptian writings implanted animal and carved ivory teeth were the oldest examples of primitive implantology. In eighteenth and nineteenth century England and colonial America, poor individuals sold their teeth for extraction and transplantation to wealthy recipients. The clinical outcomes of these transplanted dentitions were either ankylosis or root resorption. Continued research prolonged allotransplant survival but did not appreciably improve predictability.

In 1809 Maggiolo placed an immediate single-stage gold implant in a fresh extraction site with the coronal aspect of the fixture protruding just above the gingiva. Postoperative complications included severe pain and gingival inflammation. Since then various implant materials were used ranging from roughened lead roots holding a platinum post to tubes of gold and iridium. Adams in 1937 patented a submergible threaded cylindrical implant with a ball head screwed to the root for retention for an overdenture in a fashion similar to that done today.

Up to this point implant success was marginal with a maximum longevity of only a few years. Strock placed the first long-term endosseous implant at Harvard in

1938. This implant was a threaded cobalt-chrome-molybdenum screw with a cone-shaped head for the cementation of a jacket crown. The implant remained stable and asymptomatic until 1955, at which time the patient died in a car accident. Strock wrote, “The histological sections of implants in the dog study showed remarkable complete tolerance of the dental implant and the pathologist report so indicated to our gratification.” Strock demonstrated for the first time that metallic endosteal dental implants were tolerated in humans, with a survival rate of up to 17 years.

Due to inadequate alveolar bone height in certain sites of the jaws, subperiosteal implants were developed. In 1943 Dahl placed a metal structure on the maxillary alveolar crest with four projecting posts. Multiple variations to this initial design were fabricated but these devices often resulted in wound dehiscence. Blade implants were introduced by Linkow and by Roberts and Roberts. There were numerous configurations with broad applications, and the implants became the most widely used device in implantology in the United States and abroad.

A two-staged threaded titanium root-form implant was first presented in North America by Brånemark in 1978. He showed that titanium oculars, placed in the femurs of rabbits, osseointegrated in the femurs of rabbits after a period of healing.

Two-staged titanium implants were first placed in patients in 1965 and studies showed prolonged survival, free-standing function, bone maintenance, and significant improvement in benefit-to-risk ratio over all previous implants. This breakthrough has revolutionalized maxillofacial reconstruction. Subsequently, various implant designs have been manufactured and research in implantology has grown exponentially. The frontiers of implantology are rapidly being advanced and esthetics continue to be an integral part of this progress [Brånemark et al. 1985].

In 1977, Brånemark presented his research work carried out over 10 years showing that bone can grow intimately onto the surface of titanium implants (Brånemark 1977). The now well-accepted concept, termed osseointegration, has undoubtedly been one of the most significant scientific breakthroughs in dentistry over the past 30 years. A multitude of implant designs have been marketed since, and the clinical situations in which osseointegrated implantretained prostheses are used have expanded enormously.

One of the key factors for the long-term success of dental implants is the

maintenance of healthy tissues around them. A cause-effect relationship between bacterial plaque accumulation and the development of inflammatory changes in the soft tissues surrounding dental implants has been shown [Pontoriero et al. 1994]. If this reversible condition, called ’peri-implant mucositis’, is left untreated, it may lead to the progressive destruction of the tissues supporting an implant (peri-implantitis) and ultimately to its failure. [Mombelli 1999] [Esposito et al. 2011]

The 20-year cumulative survival rates of short and standard implants were 92.3 and 95.9%, respectively. The cumulative success rates were 78.3 and 81.4%. The survival rates of short implants in posterior and anterior regions were comparable: 95 and 96.4%, respectively.[Lops et al. 2012]

Implant therapy nowadays is a major branch of dentistry that is constantly evolving also thanks to the scientific interest of many companies. The patients themselves requiring implant therapy to get a fixed prosthesis that mimics the most of the natural tooth (Figure 5, Figure 6). Implant therapy can also be used to stabilize dentures and thus ensuring greater comfort to the patient both masticatory that phonetic and aesthetic (Figure 7).

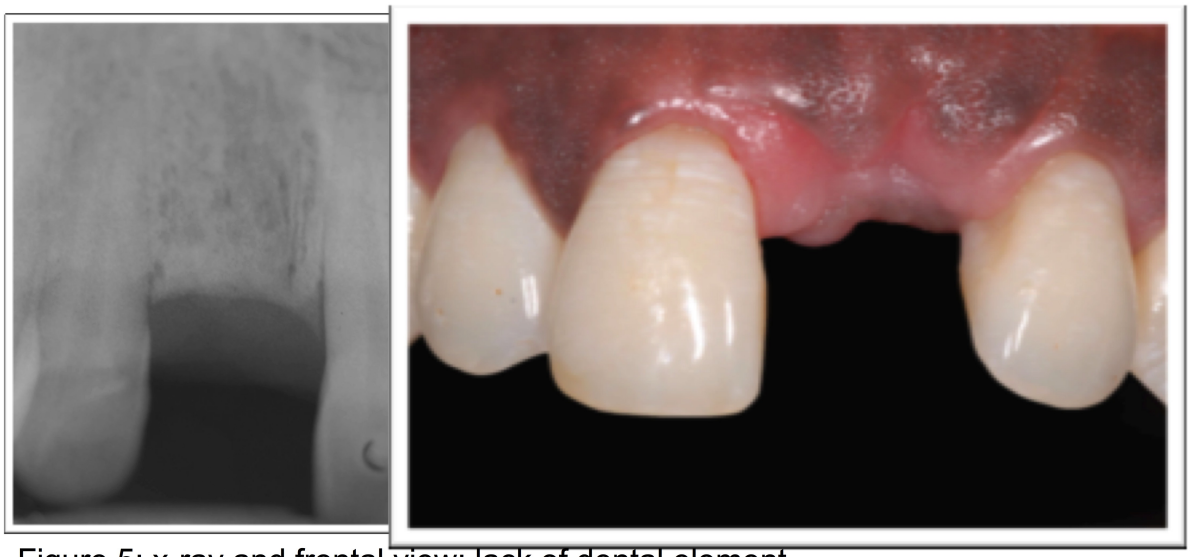


Figure 5: x-ray and frontal view: lack of dental element

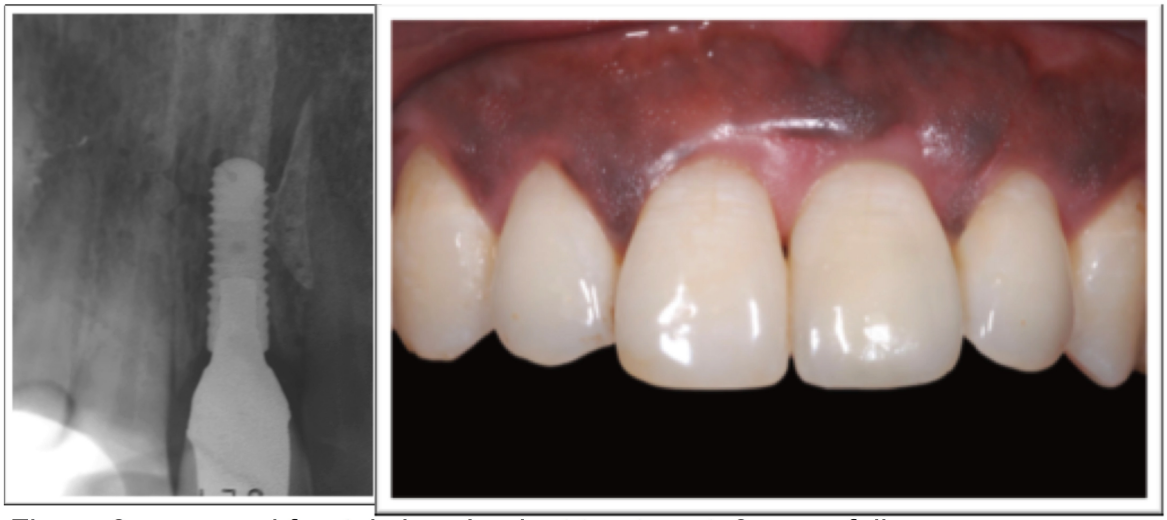


Figure 6: x-ray and frontal view. Implant treatment: 3 years follow up

Figure 7: patient before and after treatment

Peri-implantitis

Implant therapy is a well established method of replacing missing teeth. Excellent long- term results can be achieved, but biologic complications may occur.

The most common biological complications were hygiene-related: 30.2% of patients displayed peri-implant mucositis and 10.4% peri-implantitis [Francetti et al. 2013].

Perimplantitis is defined as: plaque-induced progressive marginal bone loss observed on radiographs with clinical signs of infection of the peri-implant soft tissues (Figure 8).

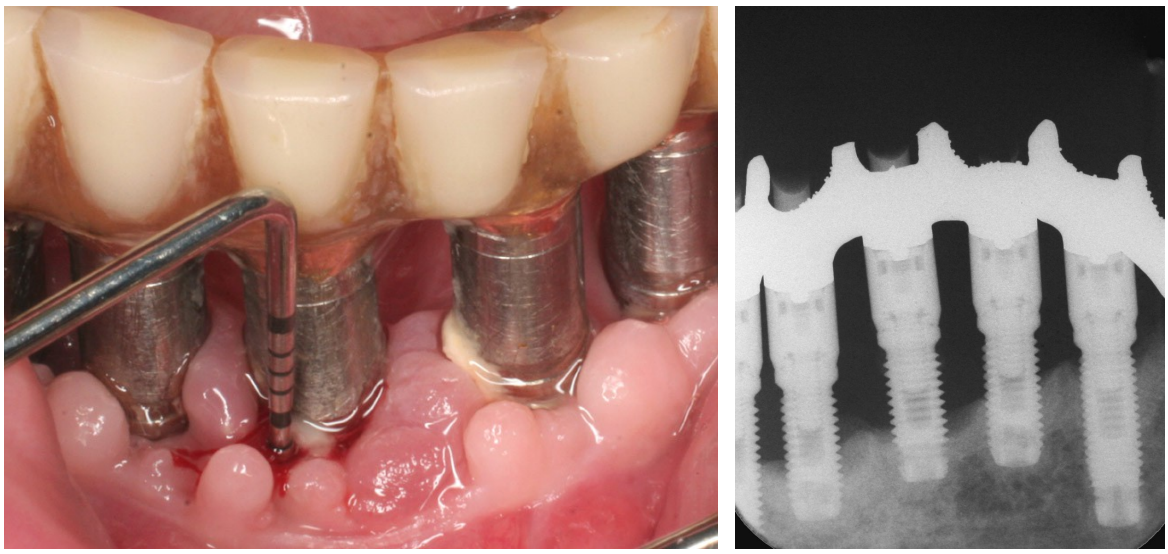


Figure 8: patient with severe peri-implantitis: presence of suppuration and bone loss

The occurrence of peri-implantitis is not rare. In a single-cohort study [Roos-Jansåker et al. 2006] peri-implantitis, defined as a marginal bone loss of 3 mm or more in combination with bleeding on probing or pus or both, was diagnosed in 16% of patients treated with turned (machined) Brånemark implants 9 to 14 years after loading. The occurrence of peri-implantitis around implants with roughened surfaces is likely to be even higher, since it was observed in another Cochrane systematic review [Esposito et al. 2007] that statistically significantly more peri-implantitis occurred at 3 years of loading around implants with roughened surfaces when compared to turned (machined) Brånemark implants [Esposito et al. 2011].

In a recent consensus conference Klinge et Meyle reported the prevalence of peri-implantitis over a 5-10 year period following implant placement has been in the order of 10% of implants and 20% of patients [Klinge et al. 2012].

Cecchinato showed that during the 10-year follow-up period, 12% of patients and 5% of implants displayed signs of peri-implantitis (bone loss >0.5 mm, BoP+, PPD ≥6 mm) [Cecchinato et al. 2013].

Keratinized gingiva has been shown to promote soft tissue health around teeth. However, around dental implants, the presence of keratinized gingiva may or may not be important for preservation of crestal bone. Krekeler and colleagues suggested that there is a strong correlation of keratinized gingiva with implant failure and the absence of an adequate band of keratinized mucosa surrounding the abutment. This suggested relationship was based on the ability of the keratinized mucosa to withstand bacterial insult and ingression, which can lead to

peri-implantitis [Krekeler et al. 1985].

The keratinized gingiva allows a better marginal seal, mantaining a mucosal collar that prevents the access of bacteria into the underlying tissues. These bacteria can maintain inflammatory conditions harmful to the peri implant soft and hard tissues. The Brito's systematic review concludes that the presence of an adequate zone of keratinized tissue may be necessary because it was shown to be related to better peri-implant tissue health [Brito et al. 2013].

From studies of Lang, Lindhe and Schou conducted on animal models in which they were created peri-implantitis and experimental periodontitis, is evident that to support peri-implant pathology plaque is a key factor. Plaque is a biofilm rich in bacteria. The bacteria around teeth and implants have very similar characteristics: sites showing periodontal and peri-implant inflammation with diffuse biofilms, contain a significant amount of gram-negative bacteria.

The main treatment of peri-implantitis is based on the resolution of the inflammatory lesion. In experiments on animals conducted by Ericsson, Persson and Lindhe, peri-implant experimentally induced lesions were subsequently subjected to therapy. The animals were administered systemic antibiotics, while the local treatment was performed only on some of the implant sites affected. After several months of healing, the implant sites were also receiving local therapy, ie the subgingival mechanical cleaning, inflammatory lesions had resolved, While in non-exposed to local treatment sites had been maintained the inflammatory infiltrate in the mucosa and bone.

Peri-implantitis antibiotics treatment must be combined with a meticulous removal of biofilm from contaminated implant surface.

To conclude it can be stated that lesions caused by peri-implantitis are little encapsulated, extend inside the marginal bone tissue and can, if left to progress, lead to loss of the implant. Symptoms of peri-implantitis are related to infectious/inflammatory lesion. Therefore you are having radiographic signs of bone loss, which looks lijke a crater. Swelling and redness of the mucosa occur with frequency, but also bleeding after slight probing and suppuration. However the implant may still remain stable for extended periods of time [Lindhe et al. 2009b].

Bone regeneration

Autogenous tissues have been widely used and are still considered as the gold standard to which all other biomaterials are compared [Dimitriou et al. 2011a]. Nevertheless, even the most advanced reconstructive techniques using autologous materials are often insufficient to restore extensive or complex maxillofacial defects [Susarla et al. 2011]. Autografts contain all of the basic elements necessary to induce effective tissue regeneration, provided cells, extracellular matrix and cytokines [Pape et al. 2010; Khan et al. 2005]. However, the use of autogenous tissue involves the need of harvesting it from a donor site, with the consequent drawbacks in terms of costs, procedure time, patient discomfort and possible complications.

Additionally, oftentimes the volume of harvested tissues is not sufficient to fill or cover a defect, given the limited availability of autogenous tissues [Dimitriou et al. 2011b; Zouhary 2010]. To overcome these limitations, a variety of exogenous substitute materials, including allografts, xenografts and alloplasts, have been introduced in clinical practice over the last three decades [Bauer and Muschler 2000; De Long et al. 2007]. These materials primarily act as scaffolds, supporting the migration of cells from the periphery of the grafted area. Substitutes are indicated in the treatment of cases where the application of autografts alone may not be possible [Finkemeier 2002]. Unfortunately, when comparing these biomaterials to autografts other limitations emerge.

The presence of cellular populations, orchestrate the release of growth factors, maintenance of a stable scaffold, and stimulate angiogenesis and are key for successful tissue regeneration as they play a fundamental role on the healing process [Taba et al. 2005]. Controlling the dynamics of these elements allows for a more predictable treatment of challenging alveolar bone loss or extremily atrophy.

Novel tissue engineering therapies aimed at enabling clinicians to achieve predictable regeneration have been recently developed.

Tissue engineering has a great potential in the clinical area for the regeneration of both hard and soft tissues and could represent a new important instrument to enhance wound healing in different scenarios.

Tissue engineering

The great progress in the knowledge in the field of cellular biology and of biotechnologies has led to the development of technologies aimed at the *in vitro* growing and reconstruction of tissues or organs, thus defining a new branch of biomedical sciences known as *tissue engineering*. The combination of these technologies make possible the *ex vivo* expansion of autologous cells and their employment in the repair of lesions and in the regeneration of tissues through the use of biocompatible three-dimensional matrices. By suitably modulating the chemical, mechanical and physical characteristics of such matrices, it is possible to use them as supports for the inoculation, growth and differentiation of autologous cells for the *in vitro* regeneration of specialized tissues.

Tissue engineering allows to combine the potentials of living cell transplantation with the technology of artificial organs for the realization of functional structures. For this reason, tissue engineering, with the introduction of bioartificial structures, represents an evolution with respect to the substitutes of first generation: traditional artificial organs like heart valve prostheses, pacemakers and orthopaedic prostheses, whose clinical alternative was represented by the transplantation of organs from donors.

The *in vitro* reconstruction of a tissue or of a whole organ, however, requires not only an in-depth study of the composition and structure of the three-dimensional matrices, but also of the physical forces acting on it. Furthermore, it requires knowledge about the chemical and molecular factors that regulate the growth and differentiation of cells and tissues.

Therefore, it can be reasonably stated that tissue engineering is based on two main components: **three-dimensional support** and **biological component**. The three-dimensional support is represented by biomaterials, whereas the biological component comprises both the cells and the molecular factors.

Biomaterials

A biomaterial is the three-dimensional support necessary for cells to be distributed in the three dimensions and to lay the extracellular matrix. Therefore, a biomaterial is an element or a combination of several elements used in the treatment, improvement or replacement of a tissue or of a whole organ.

Biomaterials, in order to be suitable for tissue engineering, must possess the following characteristics:

- Biocompatibility with the receiving tissue or organ;
- Biodegradability: the ideal degradation speed corresponds to the speed of formation of the new tissue;
- Non-toxicity;
- Non-immunogenicity;
- Optimal mechanical properties to be inserted in the surrounding tissue;
- Suitable porosity and morphology for the transport of cells, gases, metabolites, nutrients and molecules both within the biomaterial and between the biomaterial and the surrounding environment.

In general, biomaterials are designed and built drawing inspiration from the extracellular matrix, as this guarantees communication between the cells and the stability of the tissues by means of the adhesion molecules. In general, the extracellular matrix is made up of the ground substance, a very hydrous gel that provides the matrix with compressive strength, of fibres that provide tensile strength, and of water that favours the rapid diffusion of substances. These elements, common to all extracellular matrices, are combined with different ratios in each tissue. Natural, synthetic or semi-synthetic biomaterials were used in order to obtain three-dimensional structures compatible with the extracellular matrix of the tissue to be regenerated. Natural biomaterials are, for example, collagen, gelatin, fibrin, hyaluronic acid, cellulose, chitin, alginates, hydroxyapatite and materials from cadavers or from animals. They provide several advantages, such as selective adhesion (collagen), biodegradability (gelatin and chitin) and mechanical properties similar to those of natural tissues (heart valves and blood vessels from animals). Natural biomaterials have some disadvantages as well: the possibility to transmit viral infections, antigenicity and instability. On the other hand, synthetic biomaterials are: polyglycolic acid (PGA), polylactic acid (PLA), polytetrafluoroethylene (PTFE), ceramic and alloys. They feature a satisfactory three-dimensional architecture but questionable biocompatibility; furthermore, most of the synthetic biomaterials have a poor content of information and signals for the cells. Finally, semi-synthetic biomaterials derive from alterations of the natural biomaterials for the purpose of improving their performance. Some examples are modified hyaluronic acid and hydroxyapatite derivatives.

On the basis of their application, biomaterials can be divided into supports for the

reconstruction of soft tissue or for the reconstruction of hard tissue [Bressan et al. 2011] [Gardin et al. 2011].

Soft Tissue

At present, the strategies for the reconstruction of soft tissue are based on grafts of autologous tissue or on bioengineered implants made up of cells inoculated in biocompatible supports. To this purpose, different natural biomaterials are used, also in association with synthetic or organic materials.

Some examples of biomaterials used in soft tissue regeneration are:

- ***Collagen***: it is the main component of the extracellular matrix of the connective tissue. Thanks to its biocompatibility, strength and flexibility, it is widely used to generate dermal substitutes. Several scaffolds based on collagen are currently marketed as dermal substitutes, in particular in the form of hydrogel. These substitutes are usually obtained through the suspension of dermal fibroblasts in a collagen hydrogel. However, their use as a permanent graft is limited due to their low resistance to degradation, especially if the hydrogel is a low-concentration hydrogel [Helary et al. 2011]. Dermal substitutes made up of a collagen layer associated with dermal glycosaminoglycans superimposed to a silicone layer are available on the market. These substitutes were successfully used in the treatment of chronic cutaneous wounds [Kahn et al. 2011].
- ***Gelatin***: it is produced from the hydrolysis of collagen, it has high haemostatic power and does not cause antigenicity. Thanks to its properties, gelatin was widely used as a tissue adhesive for the closure of wounds [Dhandayuthapani et al. 2010].
- ***Fibrin***: it derives from the polymerization of fibrinogen in the presence of thrombin. It is not part of the extracellular matrix but is temporarily present during the healing of wounds as it is involved in blood clotting. In the field of regenerative medicine, fibrin glue was widely used as a tissue adhesive. Fibrin glue is marketed in the form of two separate solutions: one of fibrinogen and the other of thrombin, applicable by means of a double syringe or a spray. When they are mixed together, they mime the last phases of the clotting cascade and form a fibrin clot [Thompson et al. 1988]. Fibrin glue was used, as an alternative to clips, in burn patients subjected to the removal of the wound and to skin graft. It has proven to be safe and efficient, giving better results than clipping. Furthermore, the use of fibrin glue has obtained

compliance from patients, since the removal of clips is often painful and requires more time [Foster et al. 2008].

- ***Hyaluronic acid***: it is a polysaccharide of the extracellular matrix diffused in a ubiquitous way. In an aqueous environment, it generates viscous matrices guaranteeing tissue hydration, it regulates the organization of matrix proteoglycans, and it is also involved in cellular adhesion, proliferation, migration and differentiation. *In vitro* and *in vivo* studies have widely demonstrated its potentials in the construction of three-dimensional supports useful for tissue engineering [Solchaga et al. 1999] . Hyaluronic acid derivatives are available on the market, such as HYAFF® 11 (Fidia, Italy), a linear derivative of hyaluronic acid modified by complete esterification of the carboxyl groups of glucuronic acid with benzyl groups. This modification determines lower hydrophilicity and degradation by the hyaluronidases. The esterified derivatives maintain the same biological characteristics as hyaluronic acid, but they have a longer permanence time when they are implanted and they can be processed by means of various weaving techniques producing various articles like gauzes, sponges, microspheres, granules, membranes and non-woven felts, with characteristics that can be adapted to many clinical applications [Rastrelli et al. 1990].
- ***Laminin***: it is a glycoprotein of the extracellular matrix generally present in the basement membrane. It promotes cellular adhesion, migration, growth and differentiation. In the field of tissue engineering laminin is used to improve the functionalities of three-dimensional supports and to prevent or minimize transplant rejection. It has been demonstrated that associating laminin with a chitin-based support (a structural element of the exoskeleton of crustaceans) promotes the healing of wounds by fastening re-epithelialization through the reduction of inflammatory infiltrates and the higher proliferation of fibroblasts [Min et al. 2010].

Hard Tissue

In general, by speaking of *hard tissue* reconstruction one means the regeneration of the hard tissue par excellence: bone tissue. At present, progresses in the field of nanotechnologies have allowed to produce bone substitutes alternative to bone tissue grafts deriving from the same patient (autotransplantation) or from donors (allotransplantation). Today *autotransplantation* is still the “gold standard” in bone reconstruction as it features some important qualities:

- Osteoconductivity: ensuring cellular adhesion and proliferation in a structure that allows for cellular migration and for the formation of new vessels;
- Osteogenicity: ensuring the presence of osteoblasts in the graft adhesion seat to favour the generation of new bone tissue;
- Osteoinductivity: capability to stimulate the surrounding stem or osteoprogenitor cells to differentiate in osteoblasts.

Despite these characteristics, autotransplantation is associated with several collateral effects, among which: post-surgery pain, formation of hematomas, haemorrhages, nervous lesions, infections and aesthetic defects. *Allotransplantation*, on the other hand, implies a high risk of disease transmission and immune responses; for this reason, bone grafts, before being implanted, are frozen or freeze-dried. An alternative to allotransplantation is the *demineralized bone matrix*, produced from decalcified cortical bone treated with radiation and chemical agents. The result, a bone matrix made up of denatured proteins only, is not a stable structure but an osteoconductive substrate to be used in case of structurally stable bone lesions. Although there exist different forms of bone transplantation, they all share reduced osteoinductivity and the absence of a cellular component, as the donor grafts are devitalized through radiation or freeze-drying [Finkemeier 2002].

A way of getting round the drawbacks related to bone tissue transplantation is to treat the bone defects with bioengineered substitutes. In addition to the already mentioned characteristics of osteoconductivity, osteogenicity and osteoinductivity, the ideal bone substitute must possess other precise characteristics [Gardin et al. 2012b]:

- Osseointegration: the capability to establish connections with the original bone tissue;
- Biodegradability;
- Morphology similar to the human bone;
- Easy clinical use;
- Economical.

A great number of bone substitutes, of natural and synthetic origin, which promote the proliferation, differentiation and migration of cells, are available on the market. Among these there are biomaterials made up of:

- *Metals*: they were widely used, especially titanium, as bone substitutes thanks to their mechanical strength. However, after 10-15 years metal im-

plants must be replaced due to their poor or incomplete osseointegration with the surrounding bone. In order to improve osseointegration, metal implants were developed with nanostructured surfaces that increase their surface wrinkledness and wettability favouring the absorption of proteins, cellular adhesion and proliferation and calcium deposition [Tran and Webster 2009].

- *Ceramics*: based on calcium phosphate, such as hydroxyapatite (HA) and β-tricalcium phosphate (TCP), they are widely used in bone tissue regeneration thanks to their high biocompatibility with cells and bone tissue. Ceramics are also bioactive, that is to say, they are able to support cellular adhesion, proliferation and differentiation. Although HA and TCP resemble the inorganic components of the bone matrix, their use is limited by their fragility and low resistance to pressure. Moreover, like metals, ceramics too feature a poor osseointegration, which can be made up for through the development of nanoscale surfaces. With respect to conventional ceramics, nanoscale ceramics feature a greater cellular adhesion and proliferation and calcium deposition thanks to the greater wettability and adsorption of vitronectin, one of the proteins involved in cellular adhesion [Webster et al. 2001].
- *Polymers*: of natural or synthetic origin. Natural polymers include polysaccharides (alginate, chitin/chitosan, hyaluronic acid and derivatives) and proteins (collagen, fibrin and silk). The advantage of natural polymers lies in their high biocompatibility, but synthetic polymers have no immunogenicity and can be processed to obtain particular characteristics [Balasundaram and Webster 2007]. The most used synthetic polymers are polylactic acid (PLA), polyglycolic acid (PGA), and their co-polymer, polylactic-co-glycolic acid (PLGA). The osseointegration of polymers, too, can be increased if modified on a nanoscale level, for example, through the bond with proteins or peptides that can increase cellular adhesion. Since the biological activity of the adhesion proteins is expressed through peculiar RGD sequences (arginin-glycin-aspartat), the functionalization of synthetic polymers with such sequences increases their interaction with integrins, the membrane receptors of the RGD sequences, favouring cellular adhesion [Paletta et al. 2009].

Since the bone substitutes made up of one component only do not have all the characteristics of an ideal substitute, research in recent years has been focussing

on the combination of the above-listed materials.

Cell therapy applications for craniofacial regeneration

Interest about novel stem cell based therapies has exponentially been increasing over the past years, not only in the scientific community but also within the society. Indeed, stem cells seem to give the best chance for human tissue engineering, and particularly, mesenchymal stem cells (MSCs) represent a great tool in regenerative medicine because of their ability to differentiate into a variety of specialized cells in addition to their immune privileged characteristics

Hovewer both somatic and stem cells can be used in cell based therapy (Table 1). Somatic cells can be harvested, cultured and implanted with the aim of engineering new tissues. Limitations in their use are related to the lack of self-renewal capability and limited potency; characteristics that are exclusive of stem cells [Garcia-Godoy and Murray 2006]. Somatic cell delivery and stem cell therapy cells have been evaluated in different areas of regenerative medicine.

Somatic cells in the craniofacial region, fibroblast-like cells derived from the periodontal ligament have been used to promote periodontal regeneration [Dogan et al. 2002; Dogan et al. 2003]. As demonstrated through in vivo investigations using a labeling technique, oral-derived periodontal cells are able to stimulate alveolar bone formation [Lekic et al. 2001]. Cloned tooth-lining cementoblasts, periodontal ligament fibroblasts, and dental follicle cells seeded onto three-dimensional polylactic-co-glycolic acid scaffolds, exhibit mineral formation in vitro [Jin et al. 2003b]. Immortalized cementoblasts delivered to large periodontal defects via biodegradable PLGA polymer sponges contributed to complete bone bridging and PDL formation, while dental follicle cells inhibited bone formation [Zhao et al. 2004].

Another study showed that skin fibroblasts transduced by the BMP- 7 gene promoted the regeneration of periodontal defects including new bone, functional PDL and tooth root cementum [Jin et al. 2003a]. In the management of soft tissue defects cultivated fibroblasts have also been used for the treatment of interdental papillary insufficiency [McGuire and Scheyer 2007]. A human oral mucosa equivalent, made of autogenous keratinocytes on a cadaveric dermal carrier (Alloderm®) was able to favor wound healing when compared to the dermal carrier alone [Izumi et al. 2003]. An ex vivo synthesized oral mucosa equivalent

(EVPOME) produced without using animal-derived serum or feeder layer cells [Hotta et al. 2007; Song et al. 2004] has demonstrated its ability to promote early initiation of epithelialization, short healing period and minimal scar contraction.

This can be partially explained by the ability of this living construct to secrete growth factors as VEGF, promoting initial vascularization, which is critical to subsequent graft survival [Nakanishi et al. 2007; Xu et al. 2009]. EVPOME has been successfully used to treat patients affected by squamous cell carcinoma of the tongue, leukoplakia of the tongue, gingiva, and buccal mucosa or hypoplasia of the alveolar ridge [Hotta et al. 2007]. In other soft tissue applications, allogenic foreskin fibroblasts have been utilized to promote keratinized tissue formation at mucogingival defects [McGuire and Nunn 2005]. A tissue-engineered living cellular construct comprised of viable neonatal keratinocytes and fibroblasts rendered similar clinical outcomes when compared to conventional gingival autografts [McGuire et al. 2011]. This construct has a strong potential to promote tissue neogenesis through the stimulation of angiogenic signals [Morelli et al. 2011]. Another interesting product consists of the application of neonatal keratinocytes and fibroblasts for increasing keratinized gingiva around teeth [McGuire et al. 2008]

This cell construct can stimulate the expression of angiogenic-related biomarkers as compared with autogenous free gingival grafts during early wound-healing stages [Morelli et al. 2011] and, therefore, constitutes a promising material for gingival grafting without the need of a donor site.

The benefits of using somatic cells for the regeneration of soft and hard tissues in the craniofacial district have been illustrated by several preclinical and clinical studies [Mao et al. 2006]. Although, the lack of selfrenewal capability and their commitment toward a single cellular. Phenotype limit their use in the treatment of more challenging craniofacial defects, in which a more orchestrated cellular response may be critical to gain success. Given their higher characteristics, stem cells might have a greater potential in this area.

Table 1: Cell therapy applications for periodontal/craniofacial tissue engineering.

	Regenerative cell construct	Study model	References
Autologous Stem Cells	Bone block allografts impregnated with autogenous bone marrow	Patients with severely atrophic maxillary and mandibular ridges	[Soltan et al. 2007]
	Autologous MSCs isolated from a bone marrow aspirate and expanded in vitro	Periodontal regeneration in class III furcations in a dog model	[Kawaguchi et al. 2004]
	Engineered porous scaffold seeded with BMSCs	Postextraction socket in rabbits	[Marei et al. 2005]
	PRP+MNCs from bone marrow aspirate	Alveolar ridge augmentation in humans	[Filho Cerruti et al. 2007]
	PRP + in vitro-expanded bone marrow derived MSCs	Trephined defects in dog mandibles	[Yamada et al. 2004c; Yamada et al. 2004b; Yamada et al. 2004a]
	PRP+in vitro-expanded bone marrow derived MSCs	Periodontal defects in humans	[Yamada et al. 2006]
	Adipose-derived stem cells	Periodontal defects in Wistar rats	[Tobita et al. 2008]
	BMSCs incorporated with a PLCL scaffold	Osteochondral defect on the medial femoral condyles at a high load- bearing site on a rabbit's knee joint	[Xie et al. 2010]
	NELL-1 modified autogenous BMSCs in PLGA scaffold	Surgically-created osteochondral defects in goats' mandibular condyles	[Zhu et al. 2011]
	Autologous periodontal ligament cells from extracted teeth in a hyaluronic acid carrier	Dehiscence defects in beagle dogs	[Akizuki et al. 2005]
	PDL stem cells from extracted teeth	Surgically-created periodontal defects in miniature pigs	[Liu et al. 2008]
	mp2-supplemented dental pulp stem cells	On amputated pulp to stimulate reparative dentin formation	[Iohara et al. 2006]
	BMSCs cryopreserved for 1 month and freshly isolated BMSCs (control)	Periodontal fenestration on beagle dogs	[Li et al. 2009]
Allogenic Somatic Cells	Fibroblast-like cells from expanded regenerated periodontal ligament cells	Artificial class II furcal defect in a dog model	[Dogan et al. 2002; Dogan et al. 2003]
	Periodontal ligament cells	Periodontal defects created in Sprague–Dawley male rats	[Lekic et al. 2001]
	Cultured cementoblasts, periodontal ligament fibroblasts, and dental follicle cells	Ectopic tissue regeneration in mice using 3-D poly lactic-co-glycolic acid (PLGA) scaffolds	[Jin et al. 2003b]
	Cultured primary follicle cells and immortalized cementoblasts	Buccal periodontal defects in mandibular molar of athymic rats	[Zhao et al. 2004]
	Syngeneic skin fibroblasts transduced by the BMP-7 gene	Periodontal ligament regeneration at sites with periodontal bone defects in rats	[Jin et al. 2003b]
	Living human fibroblast-derived dermal substitute (Allogenic foreskin fibroblasts and keratinocytes)	Patients with insufficient attached gingiv	[McGuire and Nunn 2005]
	Living human fibroblast-derived dermal substitute (Allogenic foreskin fibroblasts and keratinocytes)	Multi center study treating patients with insufficient attached gingiva but no need for root coverage	[McGuire and Scheyer 2007]
Autologous Somatic Cells	Periodontal ligament cell sheets with reinforced hyaluronic acid carrier	Surgically create dehiscence defects	[Akizuki et al. 2005]
	Cultured and expanded autologous fibroblasts	Injections for papilla priming procedure to augment open interproximal spaces	[McGuire and Scheyer 2007]
	Ex vivo produced oral mucosa equivalent (EVPOME, Autogenous keratinocytes seeded on Alloderm®)	Patients with either a premalignant or cancerous mucosal oral lesion	[Izumi et al. 2003]
	Ex vivo produced oral mucosa equivalent (EVPOME, Autogenous keratinocytes seeded on Alloderm®	Patients affected by squamous cell carcinoma of the tongue, leukoplakia of the tongue, gingiva, and buccal mucosa or hypoplasia in the alveolar ridge	[Hotta et al. 2007]
	Autogenous chondrocytes expanded in presence of FGF-2 and TGFß1	Cartilage defects in the knee	[Brittberg et al. 1994; Jakob et al. 2001; Dozin et al. 2005; Dozin et al. 2002]
	Engineered cartilage generated in vitro from chondrocytes cultured on a biodegradable scaffold	Osteochondral defect in a rabbit knee joint	[Schaefer et al. 2002]
	PDL-derived cells cultured and placed on the surface of Ti pins	Implantation on nude mice, beagle dogs and human patients	[Gault et al. 2010]

Stem cells

Stem cells are cells which can continuously divide generating undifferentiated (*self-renewing*) or specialized (*multilineage differentiation*) daughter cells. They can divide symmetrically, to increase the number of stem cells, or asymmetrically to keep the number of stem cells unvaried and generate parent cells, which in turn will differentiate into mature cells. [Bluteau et al. 2008].

Commonly stem cells are grouped in Embryonic Stem Cells (ESC) and Adult Stem Cells (ASC).

ESC are derived from inner cell mass of a blastocyst of an early-stage embryo. They are named pluripotent stem cells since they are able to differentaiate into all types of cells of an adult individuum.

On the contrary ASC are simply multipotent as their differentiation potential is limited to specific cell lines.

ASC reside in different organs and tissues in microenvironments named “stem cell niches” consisting of heterogeneous cells, extracellular matrix and soluble factors which support the maintenance and self-renewal of stem cells. [Yen and Sharpe 2007]

For this reason ASC are also named postnatal or somatic cells. ASC cannot be identified with certainty but only on the basis of indirect properties such as the expression of a specific range of surface markers, slow cell cycle, clonogenicity and undifferentiated state.

The best way to determine staminality is the *self-renewal* assessment based on the isolation of likely-to-be stem cells and their subsequent transplant in order to evaluate their ability to reconstruct host tissue [Bluteau et al. 2008].

Mesenchymal stem cells (MSC) are non-hematopoietic cells of mesodermal and neuroectodermal origin. They can be found in many soft tissues (Table 2) such as bone marrow, adipose tissue, umbilical cord and dental pulp. They differentiate to form mesoderm derived cells e.g. adipocytes, chondrocites and osteocytes but also cells belonging to the others germ layers (Table 3). As a matter of fact, it is known that MSC have a higher plasticity degree in respect to other ASC populations and are able to differentiate *in vitro* to form non-mesodermal cell lines such as neurons and astrocytes.

It appears that their function in the human body is to grant a supply of repairing cells without distinctive characteristics but receptive to signals which can guide

them to generate different specialized cell lines. The destiny of MSC can be influenced by signals of different nature: by tissue damages such as trauma, fracture, inflammation, necrosis and tumor, by chemotactic signals and by signals coming from the surrounding micro environment. [Pountos and Giannoudis 2005]. Besides multipotency, MSC possess further important qualities: they can be easily amplified in a laboratory using standard cultivating conditions and the source they are derived from is not encountering ethical problems.

Table 2 List of tissues where MSC have been found and related references.

Source	*Reference*
Bone marrow stroma	Jones E. A., 2002
Adipose tissue	Zuk P. A., 2001
Trabecular bone	Tuli R., 2003
Joint cartilage	Alsalameh S., 2004
Synovial membrane	De Bari C., 2003
Muscle	Young H. E., 2001
Tendon	Salingcarnboriboon R.,2003
Umbilical cord	Romanov Y. A., 2003
Blood vessels	Abedin M., 2004
Blood	Kuznetsov S. A., 2001
Skin	Toma J. G., 2005
Dental pulp	Gronthos S., 2000
Foetal tissues	Muench M. O., 2002

Table 3: List of cell lines generated by MSC and related references.

Differentiation	*Reference*
Osteoblasts	Jones E. A., 2002
Chondrocytes	Jones E. A., 2002
Adipocytes	Jones E. A., 2002
Cardiomyocytes	Orlic D., 2001
Fibroblasts	Ogawa M., 2006
Myofibroblasts	Ogawa M., 2006
Skeletal muscle	De Bari C., 2003
Tenocytesi	Pittenger M., 2002
Neurons	Long X., 2005
Astrocytes	Mimura T., 2004
Hepatocytes	Lee K. D., 2004
Pancreatic cells	Chen L. B., 2004

Their high ability to proliferate together with their multipotency and their elevated differentiation potential make MSC interesting candidates for tissue regeneration.
MSC have been discovered and first characterized in the bone marrow stroma and named bone marrow-derived mesenchymal stem cells (BMMSC) [Friedenstein et al. 1970]. They can be easily obtained, considerably amplified *in vitro* and utilized in cell-mediated therapy and tissue engineering.
However, clinical use of BMMSC is limited by a range of problems among which painful sample taking, often combined with a low number of cells per sample. For this reason, a number of researchers have begun to look for more accessible and abundant alternative sources from where MSC can be obtained with less invasive sample-collection procedures.

Adipose tissue-derived stem cells

Adipose tissue, just like bone marrow, derives from mesenchyme and is characterized by an easy-to-isolate supportive stroma. Adipose tissue could easily represent an alternative source to bone marrow when isolating stem cells. Through liposuction operations it is possible to collect a large amount of adipose derived stem cells (ADSC) which can be effortlessly amplified *in vitro*. In fact, in standard

cultivating conditions and without special caution, ADSC grow rapidly showing a stable proliferation kinetics [Zuk et al. 2001].

It has been demonstrated that ADSC, just like BMMSC, can differentiate *in vitro* into osteocytes, adipocytes, myocytes and chondrocytes, if treated with specific differentiation factors. Such a plasticity further confirms that adipose tissue is composed of a population of multipotent stem cells whose ability to differentiate equals BMMSC.

ADSC staminality has been confirmed by the characterization of CD (cluster of differentiation) antigen markers. Analogously to BMMSC, ADSC express markers on the cell surface: CD29, CD44, CD71, CD90, CD105/SH2 e SH3, which together with SH2, is considered a marker for MSC [Haynesworth et al. 1992].

Besides these markers, ADSC and BMMSC alike express STRO-1, a marker utilized to isolate bone marrow mother cells [Dennis et al. 2002]. On the contrary ADSC do not express the markers of the hemopoietic lineage CD31, CD34 e CD45. In addition they are positive for CD13 but negative for CD14, CD16, CD56, CD61, CD62E, CD104 e CD106. ADSC differ from BMMSC just for the expression of two antigen markers: CD49d and CD106. In particular, ADSC express CD49d but not CD106, whereas the converse has been observed in BMMSC [Zuk et al. 2002]. The expression of CD106 is functionally associated to hematopoiesis [Lévesque et al. 2001], consequently the lack of expression of this antigen marker in ADSC is coherent with their localization in a non-haematopoietic tissue.

Since adipose tissue - just like bone marrow stroma - derives from mesoderm, it is non unlikely to observe ADSC differentiation into cells of the mesodermal lineage. However, their differentiation into cells of the neuronal phenotype - generating cells with similar morphology and expression of protein markers similar to nervous cells - has likewise been documented [Franco Lambert et al. 2009].

It is concluded that ADSC possess all the characteristcs typical of MSC: fibroblastoid morphology, ability to form single colonies named CFU-F (fibroblastoid colony-forming units), expression of the typical set of cell surface markers, multipotency as well as ability to differentiate into cells of the mesodermal lineage [Kern et al. 2006].

Dental pulp stem cells

Dental pulp is a soft connective tissue of mesenchymal origin trapped inside the pulp cavity of the tooth. It is possible to identify four layers in the pulp: the outermost layer composed of odontoblasts which generate dentine; a second layer called *"cell free zone",* poor in cells but rich in collagen fibers; a third layer called *"cell rich zone"* containing progenitor cells and undifferentiated cells, some of which are stem cells; an innermost layer, located in the center of the pulp chamber, rich in both vessels and nerve networks. Stem cells and undifferentiated cells contained in the "*cell rich zone*" can migrate to variuous district where - driven by different stimuli – can originate new differentiated cells and specialized tissues [d'Aquino et al. 2007]. Dental pulp of the third molar, although often discarded, represents an easily accessible source of MSC. It was only in the year 2000 that dental pulp stem cells (DPSC) were first identified and isolated according to their clonogenic abilities and their high proliferation speed. [Gronthos et al. 2000]. Later, more than four types of MSC cells derived from dental pulp have been identified: stem cells from human exfoliated deciduous teeth (SHED) [Miura et al. 2003], stem cells derived from periodontal ligaments (PDLSC) [Seo et al. 2004] stem cells from the apical papilla (SCAP) [Sonoyama et al. 2006] and dental follicle progenitor cells (DFPC) [Morsczeck et al. 2005]. They all show multipotency and ability to regenerate different dental and periodontal tissues *in vitro* and *in vivo* [Huang 2009].

In addition, they are positive for markers STRO-1, CD13, CD24, CD29, CD44, CD73, CD90, CD105, CD106, CD146, Oct4, Nanog e β2 integrin, but negative for CD14, CD34, CD45 e HLA-DR. The persistence of negativity for CD45 and positivity for CD34 demonstrates that DPSC are not derived from a hematopoietic source, but are of mesenchymal origin [d'Aquino et al. 2007]. Just like all MSC, DPSC are a heterogeneous population and the different markers listed above can be expressed in subpopulations of the same cells [Huang 2009].

They can survive in culture for long periods and be maintained for several passages: clear signs of senescence have not been detected up to 80 passages. Furthermore, they can be cryopreserved for long periods without losing their multipotency [Laino et al. 2005].

It has been demonstrated their differentiation into odontoblasts, the cells producing the mineralized matrix of dentine [Gronthos et al. 2000]. However, DPSC can also

differentiate into adipocytes and neurons showing both morphology and respective markers. In addition, their chondrogenic and myogenic differentiation *in vitro* has been observed. DPSC plasticity and multipotency can be related to the origins of dental pulp, consisiting both of ectodermic and mesodermic components and of neural crest derived cells. [d'Aquino et al. 2009].

Scaffolds for cell therapy delivery to oral and craniofacial defects

As underline previously, Scaffolds play a pivotal role in providing a three-dimensional template for tissue neogenesis [Ma 2008]. Scaffolds can not only be used as carriers for cell delivery but also serve as synthetic extracellularmatrix environments to define a 3D geometry for tissue regeneration and provide an adequate microenvironment in term of chemical composition, physical structure and biologically functional moieties [Rice et al. 2005; Liu and Ma 2004].

Thus far, the most widely adopted scaffolds for craniofacial bone regeneration are xenogenic and allogenic bone substitutes, hydroxyapatite, calcium phosphates, and gelatin or collagenous sponges [Kawaguchi et al. 2004; Lekic et al. 2001; Nakahara et al. 2004; Kaigler et al. 2010a; Kaigler et al. 2010b]. Limitations in their use are related to the lack of degradability of certain materials or too fast degradability of others, poor processability into porous structures, brittleness, inability to generate structures to be tailored to the specific needs of the patient or inability to maintain the desired volume under mechanical stimuli. In order to overcome these limitations synthetic scaffolds specifically designed to mimic the wound healing extracellular matrix are being evaluated.

This biomimetic concept applied to materials synthesis intends to generate biodegradable scaffolds with a highly porous structure and adequate mechanical properties for bone engineering [Ma 2008]. Ideally, a scaffold material should be degradable at a rate similar to that of the new tissue formation, large interconnected pores are required to allow for cell incorporation, migration, and proliferation [Zhang and Ma 2000]. Bone formation occurs over a structured collagen matrix with fiber bundle diameter varying from 50 to 500 nm [Hay 1991; Elsdale and Bard 1972], therefore nanofibrous scaffolds appear to provide better cellular attachment [Woo et al. 2003], increased differentiation of osteoblastic cells [Chen et al. 2006], and enhanced mineral deposition compared to solid-walled

scaffolds [Woo et al. 2007].

Electrospinning, self-assembly, and phase separation are three different methods employed in the fabrication of nano-fibrous polymeric scaffolds for tissue engineering. Electrospinning is a simple method, which utilizes an electric field to draw a polymer solution from an orifice to a collector, producing polymer fibers [Dosunmu et al. 2006; Jia et al. 2002]. It can be used to produce thin two-dimensional sheets, while three-dimensional nanofibrous scaffolds have been fabricated by layering these 2D sheets [Matthews et al. 2002] or by combining electrospinning with 3D printing [Moroni et al. 2008]. Molecular self-assembly uses non-covalent bonds such as hydrogen bonds, van der Waals interactions, electrostatic interactions, and hydrophobic interactions for fabricating supramolecular architectures [Whitesides et al. 1991]. Limitations in the use of self-assembly methods are related to difficulties in forming macropores and limited mechanical properties [Smith et al. 2009]. Finally, thermally induced phase separation (TIPS) technique can be used to fabricate nano-fibers through polymer dissolution, phase separation and gelation, solvent extraction, freezing, and freeze-drying under vacuum [Ma and Zhang 1999]. This technique can also be combined with processing techniques such as particulate leaching or 3D printing to design complex 3D structures with well-defined pore morphologies [Chen et al. 2006; He et al. 2010; Wei and Ma 2006].

Another interesting aspect of polymer scaffolds is that CAD/CAM technologies can be applied to create patient-specific, anatomically shaped scaffolds. As craniofacial defects and anatomical structures may greatly vary among different individuals a scaffold unique to each patient can be helpful in regenerating defects with complex geometry [Ma 2008].

Polymers have great design flexibility and their composition and structure can be designed to match the specific needs of the tissue to be engineered. Moreover, benefits can be reached by adding nano-crystalline hydroxyapatite to the scaffolds as it has a strong potential for attracting osteoblasts (osteoconductivity), it improves its mechanical properties [Wei and Ma 2004], and may reduce adverse effects associated with the degradation of some synthetic polymers [Smith et al. 2009]. Hydroxyapatite crystals can be incorporated during processing of polymer scaffolds or they could be biomimetically grown onto a prefabricated polymer scaffold. Since all interactions with biological components occur at the pore surface, the non-exposed ceramic is in effect wasted [Smith et al. 2009] and could

affect biodegradability and mechanical properties of the scaffold. It is therefore recommended to allow apatite to form as a coating of the polymer scaffold in order to enhance its surface characteristics.

An interesting technology has been described in which prefabricated polymer scaffold are soaked in simulated body fluid in order to allow apatite crystals to grow onto its pore surfaces [Boskey 2003; Boskey 1998].

Growth factors can easily be incorporated in polymeric scaffolds [Elisseeff et al. 2001; Wei et al. 2007; Jin et al. 2008], which would allow for a more sustained release of the molecules and better properly orchestrated tissue formation. As such, 3D porous, nanofibrous scaffolds have supported various stem cells and differentiated cells to regenerate many hard and soft tissues.

It should be pointed out that significant technical challenges remain for the synergistic integration of structural cues with biological cues for cell-based therapies to achieve functional dental and craniofacial tissue regeneration [Gupte and Ma 2012]. However, it is likely that the continuous expansion of biomimetic approaches in the scaffolding materials design will substantially advance the field of tissue engineering and regenerative medicine. Recently, a biomimetic fiber-guiding scaffold using solid free-form fabrication methods that custom fit complex anatomical defects to guide functionally-oriented ligamentous fibers in periodontal regeneration has been successfully tested in vivo [Park et al. 2012] and work is being done to incorporate biomimetic scaffolds in cellular delivery for craniofacial bone regeneration in many other clinical scenarios

Aim

The percentages related to total edentulism and to the connected social consequences represent an important problem.
Dental implant tooth replacements, one of the most popular therapies for total or partial edentulism, however not always is a possible solution.
Extremely atrofy or bone loss need to reconstruction before inserting implant.
Autogenous tissues have been widely used and are still considered as the gold standard to which all other biomaterials are compared.
Nevertheless, even the most advanced reconstructive techniques using autologous materials are often insufficient to restore extensive or complex maxillofacial defects.
However, the use of autogenous tissue involves the need of harvesting it from a donor site, with the consequent drawbacks in terms of costs, procedure time, patient discomfort and possible complications.
Otherwise implant may be affected by a similar condition of periodontics known as periimplantitis.
The latter can cause severe destruction of the peri-implant hard and soft tissues.
The Aims of this thesis are:

1. To evaluate whether the tissue engineering, through the use of stem cells, can be an effective therapy for the alveolar bone regeneration.

   For this first purpose, we wanted to test :

   a) the properties and safety of stem cells taken from different postnatal tissues;
   b) the ability of stem cells to promote bone regeneration both in vitro and on small and large size animal.

2. Use the tissue engineering in the area of bone inflammation both in the therapeutic environment and in prevention, and more specifically to:

   a) test stem cells anti inflammatory capability;
   b) try to identify a genetic predisposition in the peri-implantitis development.

Materials and methods

In Vitro

To test the properties and safety of stem cells taken from different postnatal tissues we have used those materials and methods with experimental *in vitro*.

Isolation and culture of adult stem cells

Adult stem cells from adipose tissue (ADSCs)

Adipose-derived stem cells (ADSCs) were isolated from samples of subcutaneous adipose tissue of patients subjected to abdominoplasty, upon informed consent. [Gardin et al. 2012a]

Abdominal fat, washed in PBS and broken into small pieces, was digested in agitation for 3 hours at room temperature. Digestion was carried out with a 0.075% solution of *Collagenase from Clostridium histolyticum type II* (Sigma) in *Hank's Balanced Salts Solution* (HBSS, Lonza). The enzymatic activity of collagenase was blocked with an equal volume of complete *Dulbecco's Modified Eagle Medium* (DMEM, Lonza), made up of DMEM, 10% Fetal Bovine Serum (FBS, Gibco) and 1% Penicillin/Streptomycin (P/S, Lonza). The cells, collected by centrifugation at 1200 revolutions per minute (rpm) for 4 minutes, were washed in PBS and filtered through a 70 ∓m strainer (BD Biosciences) to eliminate the residues of digestion. Finally, the collected cells were inoculated in F75 flasks (BD Falcon) with complete DMEM *low glucose* (Lonza) and placed in a humidified incubator with 5% CO_2 and at 37°C.

Two days after digestion, the non-adherent cells (mature adipocytes and red blood cells) and the culture medium were eliminated and replaced with fresh complete DMEM *low glucose*. Afterwards, the culture medium was changed every 2 days.

Adult stem cells from dental pulp (DPSCs)

The dental pulp was extracted from healthy third molars of adults, upon informed consent. Donors of both genders, non-smokers, not subjected to pharmacological therapy and with no oral and systemic infections were chosen. The extraction of the third molars was preceded by a dental hygiene session.[Bressan et al. 2012]

The dental pulp was isolated by mechanical disruption of the crown within 24

hours from the extraction of each molar (Figure 9)

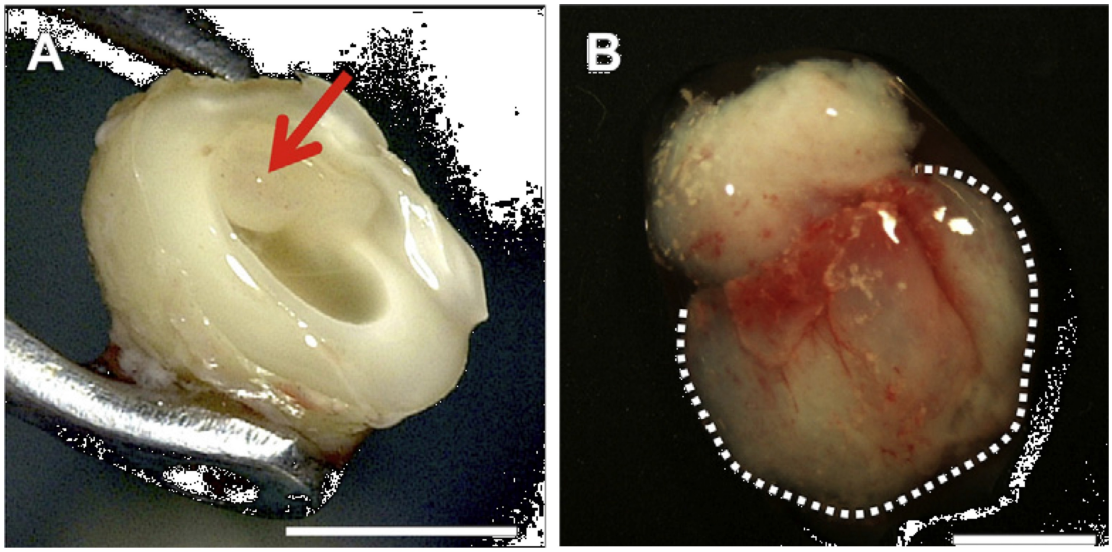


Figure 9: Sources of adult stem cells in dental tissues. (A) After a tooth was cut horizontally, the pulp tissue (arrow) in the pulp chamber was exposed; this pulp provides dental pulp stem cells (DPSCs). (B) Extracted impacted third molar (10-year-old female) containing the dental follicle (dotted line) that provides dental follicle stem cells (DFSCs). Bar: 5 mm.

The dental pulp, broken into small pieces and washed in PBS, was digested with a HBSS solution made up of *Worthington Collagenase type I* (3 mg/ml) and of *Dispase II (Roche)* (2 mg/ml) for 1 hour in a humidified incubator with 5% CO_2 and at 37°C.

Digestion was blocked by adding an equal volume of complete DMEM. The cells, collected by centrifugation at 1200 rpm for 4 minutes, were inoculated in 24-well plates (BD Falcon) with complete DMEM and placed into a humidified incubator with 5% CO_2 and at 37°C.

After 3 days from digestion, the residues of digestion and the non-adherent cells were eliminated and replaced with fresh complete DMEM. Afterwards, the culture medium was changed every 2 days.

Biomaterials:

The following biomaterials were used:

- Hyaluronic acid;
- Fibrin;

- Hydroxyapatite.

A hyaluronic acid derivative was used for the *in vitro* reconstruction of dental-like tissue: HYAFF 11® (Fidia, Abano Terme, PD, Italy). Since hyaluronic acid is a highly hydrophilic linear polymer, in an aqueous environment it generates a viscous gel that drastically reduces its workability. In order to reduce its hydrophilicity, keeping its biological properties unchanged, the hyaluronic acid was submitted to an esterification process of the carboxyl groups with benzyl alcohol.

HYAFF 11® is the 100% benzyl ester of hyaluronic acid. It is insoluble in aqueous solution but biodegradable, as the hydrolysis of the esteric bond determines the release of benzyl acid (eliminated with the urine) and of hyaluronic acid, which follows the same metabolic pathway as endogenous hyaluronic acid.

HYAFF 1‿® was used in the form of *non-woven* tissue with a specific weight of 100 g/m^2, made up of 20-µm-thick fibres and having a size of 1 cm^2.

Two different hydroxyapatite-based biomaterials were used for vascularized bone tissue reconstruction: Orthoss® (Geistlich Pharma AG, Switzerland) for *in vitro* experiments and Bio-Oss® (Geistlich Pharma AG, Switzerland) in *in vivo* experiments.

Orthoss is made up of carbonate-natural nanocrystalline hydroxyapatite deriving from the purification and sterilization of bovine bones. Therefore, it is an inorganic bone matrix with a microporous and macroporous structure similar to human spongy bone. In the *in vitro* experiments it was used in the form of blocks having a size of 1 cm × 1 cm × 2 cm. Bio-Oss, too, derives from the mineral component of bovine bone matrix, but it is in the form of granules having a size of 0.25-1 mm.

Differentiation

A culture medium containing both osteogenic differentiation factors and endothelial differentiation factors was used for *in vitro* vascularized bone tissue reconstruction.

Osteo-Endothelial Differentiation Medium:

- Endothelial Basal Medium;
- 2% FBS;
- 1% P/S;
- 10 ng/ml FGF-b;
- 10 ng/ml EGF;
- 10 mg/ml Heparin;

- 1 mg/ml Hydrocortisone;
- 10 mM ↕-Glycerophosphate;
- 10 nM Dexamethasone.

Finally, *in vitro* dental-like tissue reconstruction was carried out by imbibing the biomaterial with a mixture of neuronal factors in the presence of endothelial differentiation medium.

Mixture of Neuronal Factors: - 50 ng/ml NGF-↕≃
- 50 ng/ml BDNF;
- 10 ng/ml NT-3;
- 4 mM Forskolin;
- 10 ng/ml Heregulin ↕‿≃
- 40 ng/ml FGF-b;
- 20 ng/ml EGF.

Endothelial Differentiation Medium:
- Endothelial Basal Medium;
- 2% FBS;
- 1% P/S:
- 3 ng/ml FGF-b;
- 10 ng/ml EGF;
- 10 mg/ml Heparin;
- 1 mg/ml Hydrocortisone.

Proliferation tests

MTT Assay

The cell proliferation rate was assessed by MTT assay, according to the method by Denizot et al. [Denizot F., 1986]. It is a colorimetric assay that measures in a quantitative manner the activity of the mitochondrial enzyme succinate dehydrogenase of the cells being cultured. This enzyme, active in the mitochondria of viable cells only, is normally used as a marker of the cells' metabolic activity, viability and growth.

The assay is based on the reduction of the chemical compound MTT, that is to say, *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*, a yellow tetrazolium dye, in blue formazan salts, by mitochondrial succinate dehydrogenase. The

amount of formazan produced is proportional to the activity of mitochondrial succinate dehydrogenase and, therefore, directly proportional to the number of viable cells. By means of spectrophotometric reading, at a wavelength of 570 nm, it is possible to quantify the formazan salts and, then, correlate the optical density (O.D.) value to the number of cells.

The assay was carried out by incubating for 3 hours the cell samples with 1 ml MTT solution - 0.5 mg/ml - in PBS in a humidified incubator with 5% CO_2 and at 37°C. Afterwards, the MTT solution was eliminated gently and the formazan salts were dissolved in 500 ∓l extraction solution (90% isopropanol and 10% dimethyl sulfoxide) for 15 minutes at room temperature. Finally, the formazan blue solutions were read at the spectrophometer at a wavelength of 570 nm.

Population Doubling Time

The assessment of the proliferative capacity of the DPSC cells included the study of the Population Doubling Time (PDT).

The analysis was carried out at passages p2, p5 and p8 inoculating 5 x 10^4 cells in 12-well plates (BD Falcon). At intervals of 24 hours from inoculation, the number of adherent cells was determined for each passage by detaching them with an EDTA 0.02% and tripsin 0.25% solution (Lonza) and counting them with the Bürker chamber (Marienfeld Superior).

The PDT was calculated by applying the following formula:

$$PDT = (T - T_0) \times \log 2 / (\log N_t - \log N_0)$$

wherein $(T - T_0)$ indicates the culture time expressed in days, N_t is the average number of cells collected at time T, and N_0 is the number of cells inoculated in each well/plate at time 0.

Morphological study

Immunofluorescence

Monolayer immunofluorescence analyses were carried out by culturing the cells on slides (having a diameter of 19 mm) pre-treated with a 2% aqueous solution of Gelatin B extracted from bovine skin (Sigma Aldrich) in the presence of complete DMEM or of differentiation medium. The analysis required the fixation of the cells on the slides with a 3.7% formalin solution for 15 minutes at room temperature.

The three-dimensional constructs, on the other hand, were fixed in formalin for two hours and then dehydrated through ascending alcohols: the samples were soaked in ethyl alcohol solutions with growing concentration (from 50% to 100%) with double passages of 15 minutes each, to gradually eliminate the water content, before passing through xylene until clearing. After the inclusion in paraffin the samples were cut into slices having a thickness of 6 ∓m and submitted to the immunofluorescence reaction.

The samples, after two washes in PBS, were treated for one hour with a 2% Bovine Serum Albumin (BSA, Sigma) solution in PBS to reduce the non-specific bonds of primary antibodies. Afterwards, the following primary antibodies diluted in BSA at 2% were incubated for one night at 4°C:

- Chicken anti Human CNPase (Millipore);
- Chicken anti Human III ↕-tubulin (Millipore);
- Mouse anti Human CD31 (Abcam);
- Mouse anti Human Nestin (Covance);
- Mouse anti Human Vimentin (Sigma);
- Rabbit anti Human CD105 (Santa Cruz);
- Rabbit anti Human CD73 (Abcam);
- Rabbit anti Human CD90 (Abcam);
- Rabbit anti Human GFAP (Millipore);
- Rabbit anti Human S100 (Sigma);
- Rabbit anti Human von Willebrand Factor (Dako).

After a wash in PBS, the samples were incubated for one hour at room temperature with the following secondary antibodies:

- Goat anti Chicken IgG (H + L) TRITC labeled (KPL);
- Goat anti Mouse IgG (H + L) FITC labeled (KPL);
- Goat anti Rabbit IgG (H + L) TRITC labeled (KPL).

Following a wash in PBS, the nuclei were marked with Hoeches 33342 (Sigma) for 15 minutes at room temperature. After a short wash in PBS, the mounting on slide holders was carried out with ProLong® Gold antifade reagent (Invitrogen).

The immunofluorescence images were acquired by means of the Zeiss Axioplan microscope (Carl Zeiss, Germany), coupled with the Leica DC500 camera and with the Leica IM1000 Image Manager v1.20 software (Leica, Germany). The images were acquired at 40x enlargements.

Scanning Electron Microscopy (SEM)

The constructs, previously washed in PBS, were washed with a 2% glutaraldehyde solution in a 0.1 M sodium phosphate buffer with 7.2 pH and preserved at 4° C until the moment of dehydration. The latter, preceded by three washes in 0.1 M sodium phosphate buffer, was carried out through ascending alcohols: the samples were soaked in solutions of ethyl alcohol with growing concentration (from 20% to 96%) with double passages of 5 minutes each, to gradually eliminate the water content from the samples. The samples were preserved one night in 96% alcohol and after a passage in absolute alcohol were submitted to Critical Point Drying and metallised with gold. The preparations obtained were observed with a scanning electron microscope (model: JMS Jeol 6490) in use at the CUGAS Interdepartmental Service Centre of the University of Padua.

Gene expression by Real-Time PCR

RNA extraction and quantification

The RNA extraction of the samples was carried out by using a monophasic solution based on phenol and guanidine isothiocyanate, TRIzol® Reagent (Invitrogen).

The homogenization of the samples with such reagent causes the lysis of the cell membranes guaranteeing the integrity of RNA inhibiting the activity of RNase enzymes.

The constructs were transferred to microtubes with 1 ml TRIzol® Reagent and incubated for 5 minutes at 30°C to allow for their complete dissociation. Afterwards, 200 ∓l chloroform were added to the samples and these were stirred for 30 seconds before incubation at room temperature for 2 further minutes. From the following centrifugation at 14000 rpm for 15 minutes at 4°C three phases were obtained: an upper, aqueous, limpid phase containing the RNA, a central, white interphase containing the DNA and a lower, pink phase containing proteins, phenol and chloroform.

The aqueous phase was transferred into a new microtube and 500 ∓l isopropanol were added to precipitate the RNA. After incubating the samples for 10 minutes at room temperature, these were centrifuged at 14000 rpm for 10 minutes at 4° C.

The pellet deriving from centrifugation, made up of the RNA, was washed with 1 ml cold 75% ethanol, stirred and centrifuged at 14000 rpm for 5 minutes at 4° C.

Afterwards, the RNA was dried at room temperature for ten minutes and re-suspended in DNase RNase-free water.

RNA quantification was carried out with the NanoDrop spectrophotometer (NanoDrop™ 1000, Thermo Scientific), which, besides estimating the concentration of the samples, determines their purity from contaminants. The instrument provides the absorbance ratios 260/280 and 260/230, whose values must be respectively included in the ranges of 1.8-2 and 2-2.2 in order to consider the RNA samples pure from contaminants. Low values of such ratios indicate the presence of proteins, phenol and other organic contaminants that absorb around 280 and 230 nm.

Synthesis of complementary DNA

Complementary DNA (cDNA) was synthesized following the Invitrogen protocol: First-Strand cDNA synthesis using M-MLV RT. Such protocol provides the use of the M-MLV RT (Moloney Murine Leukemia Virus Reverse Transcriptase) enzyme that, using the RNA strand as a template, synthesizes a complementary DNA strand.

For each sample, 500 ng RNA were retrotranscribed in a reaction volume equal to 20 ∓l. In nuclease-free microtubes were mixed: 1 µl Oligo $(dT)_{12-18}$ [500 µg/ml] (Invitrogen); 500 ng total RNA; 1 µl dNTP Mix [10 mM] (Invitrogen) and nuclease-free distilled water up to the volume of 12 µl. The mixtures were heated to 65°C for 5 minutes and then placed into ice for some minutes.

After a short centrifugation step to collect the content on the bottom of the microtube, we added: 4 µl First-Strand Buffer 5X (Invitrogen); 2 µl DTT [0.1 M] (Invitrogen) and 1 µl RNaseOUT Recombinant Ribonuclease Inhibitor [40 U/µl] (Invitrogen). The reaction mixtures, after being stirred gently, were incubated at 37° C for 2 minutes. Afterwards, by pipetting gently, 1 µl M-MLV RT enzyme [200 U/µl] (Invitrogen) was added to each sample. After incubating the samples at 37°C for 50 minutes, the reaction was inactivated by heating the samples to 70°C for 15 minutes.

The so obtained cDNA was preserved at -20°C until its use as a template for PCR amplification.

Real-Time PCR

Real-Time PCR is an advanced version of polymerase chain reaction (PCR) that allows, through the use of dyes or fluorescent probes, to quantify in real time DNA

amplification by correlating fluorescence intensity with the concentration of the PCR products. Each Real-Time PCR reaction is characterized by an accumulation of fluorescent signal that is defined by the Ct (cycle threshold), that is to say, by the number of cycles required to obtain a signal intensity higher than the ground noise of the system. To high amounts of initial DNA corresponds a fast increase in the fluorescent signal and, therefore, a low Ct value.

The fluorescent signal was generated by adding to the reaction mixture the SYBR Green I fluorescent dye. It is an asymmetric cyanine intercalating the double-strand DNA. When the SYBR Green I molecules are free in solution they show no relevant fluorescence; on the contrary, when they bind to the double-helix DNA (in correspondence of the minor groove), they undergo a conformational change and considerably increase the amount of fluorescence emitted.

For each sample, 2.5 ŧl cDNA were amplified in a reaction volume equal to 25 ŧl. In nuclease-free 200-ŧl microtubes were mixed: 12.5 µl FastStart SYBR Green Master [2X] (Roche); 0.75 µl Forward primer [10 ŧM]; 0.75 µl Reverse primer [10 ŧM]; 8.5 ŧl Dnase-Rnase free water (GIBCO). After gently mixing and shortly centrifuging each mixture, 2.5µl cDNA were added.

The Real-Time PCR reactions were carried out in a Rotor-Gene RG-3000A thermal cycler (Corbett Research, Australia), following the amplification programme shown in Table 4.

Table 4: Amplification programme of the Real-Time PCR.

Cycles	*Passage*	*Temperature*	*Time*
1	Initial denaturation of cDNA	95 °C	10 minutes
40	Denaturation	95 °C	10 seconds
	Primer pairing	60 °C	30 seconds
	Extension	72 °C	30 seconds
1	Final extension	72 °C	10 minutes

The cDNA samples were analysed in triple copy using the Primer pairs shown in Table 5.

The genic expression analysis was determined by normalizing the amount of

transcript of the gene of interest with that of the gene of reference (GAPDH) in the same sample. Normalization was carried out by applying the following formula:

$$2^{\lambda Ct}$$

wherein 2 represents amplification efficiency equal to 100%,

$$\lambda C_t = C_{t\ \text{gene of reference}} - C_{t\ \text{gene of interest}}$$

Finally, the genic profiles were presented as the ratio (R) between the $2^{\lambda Ct}$ of the condition of reference (cell culture in the presence of differentiation factors) and the normalized $2^{\lambda Ct}$ of the control condition (cell cultures in non-differentiation medium).

Table 5: Forward and Reverse Primers used in the Real-Time PCR analyses.

Gene	*Forward Primer (5'→3')*	*Reverse Primer (5'→3')*
CD31	TCCAGCCAACTTCACCATCC	TGGGAGAGCATTTCACATACGA
I Collagen ↦1	TGAGCCAGCAGATCGAGA	ACCAGTCTCCATGTTGCAGA
CNPase	AGATGCGGTGGCTAAAGGTC	TCTTAGGCAGCTCTTTGGGA
GAPDH	TCAACAGCGACACCCAC	GGGTCTCTCTCTTCCTCTTGTG
GFAP	AGATCCGCACGCAGTATGAG	AGGTCGCAGGTCAAGGA
Nestin	TCAGAGGGAAGGAGATAGAGAGTC	AGCCAGAAACCATATGTCAAGAGA
Osteocalcin	GCAGCGAGGTAGTGAAGAGAC	AGCAGAGCGACACCCTA
Osteonectin	TGCATGTGTCTTAGTCTTAGTCACC	GCTAACTTAGTGCTTACAGGAACCA
Osteopontin	TGGAAAGCGAGGAGTTGAATGG	GCTCATTGCTCTCATCATTGGC
PPAR•	CAGGAGATCACAGAGTATGCCAA	TCCCTTGTCATGAAGCCTTGG
RUNX2	AGCCTTACCAAACAACACAACAG	CCATATGTCCTCTCAGCTCAGC
S100	GACAAGTACAAGCTGAGCAAGAAG	CCACAAGCACCACATACTCCTG
VEGFA	GGACAGAAAGACAGATCACAGGTAC	GCAGGTGAGAGTAAGCGAAGG
Vimentin	CAGATGCGTGAAATGGAAGAGAAC	GGTGGCAATCTCAATGTCAAGG
vWF	ACGTATGGTCTGTGTGGGATC	GACAAGACACTGCTCCTCCA
III ↕≥ Tubulin	CTCAGGGGCCTTTGGACATC	CAGGCAGTCGCAGTTTTCAC

Cytogenetic analyses

Karyotype

The karyotype is a cytogenetic analysis that allows to visualize the chromosomes that make up the whole genome of an eukaryotic cell. Therefore, it allows to distinguish the chromosomes on the basis of their number, shape and size and to appreciate any anomaly both in the number (trisomies and monosomies) and in

the structure (translocations, deletions and inversions).

In the cells the DNA appears like a non-analyzable disorganized mass, except for a particular phase of cell division, the metaphase, when it condenses in tidy structures: the chromosomes. The metaphase chromosomes can be marked with particular substances that bind to specific chromosome regions and that give them their typical band aspect. The marked chromosomes appear at the microscope like well-defined and easily identifiable structures; this allows to develop a cariogram, a tidy representation of the chromosomes paired on the basis of size, of the position of the centromere and of the banding. Furthermore, in accordance with international nomenclature, each pair is assigned a number from 1 to 22, and the sexual chromosomes are placed next to them.

The cells, previously inoculated in chamber slides (Lab Tek), were treated for 3 hours with the alkaloid Colchicine 10 ∓g/ml (Sigma Aldrich) to stop cell division at the metaphase stage. Afterwards, they were treated for 15 minutes with a hypotonic solution (1% solution of sodium citrate in water) to break the cell membranes and disperse the chromosomes in a larger area. Afterwards the action of the hypotonic solution was blocked for 5 minutes with a fixative made up of ethanol and acetic acid in a 3:1 ratio. Then the fixative was applied twice more for 15 minutes and the slides were left to dry in a humid environment.

The chromosomes were marked using the dye Quinacrine and were observed with the fluorescence microscope.

CGH array

The Comparative Genomic Hybridization (CGH) array is a technique that allows to identify any changes in the number of copies of the genes distributed all along the genome. It allows to quantify deletions or amplifications in the genome with an average resolution of 100 Kb, precisely defining the sizes and the start and end points of the regions involved in the unbalance.

It is possible to carry out a CGH array on any tissue from which it is possible to extract DNA, such as: peripheral blood, skin biopsy, solid tumours, marrow, chorial villi, amniotic fluid, fetal blood and cell cultures. For this reason, the CGH array finds application in several fields, such as: neuropsychiatry (in the diagnosis of mental retardation, autism, epilepsy and malformation of the cerebral cortex), paediatrics (to confirm congenital anomalies, developmental alterations), obstetrics (in a suspected case of fetal chromosomal anomaly) and oncology (in the diagnosis of tumours of the hematopoietic lineage and solid tissues).

The CGH array was carried out by following the protocol *Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis* (Agilent Technologies), which is divided into the following phases:

- *DNA Extraction*: the target DNA was extracted from the samples using a genomic DNA extraction kit (Sigma).
- *DNA Digestion*: 500 ng target DNA and 500 ng control DNA were digested for 2 hours at 37°C with the enzymes RsaI and Alu I (Promega), which were subsequently inactivated for 20 minutes at 65°C.
- *DNA marking and purification*: The samples were marked for two hours using the marking Kit Quick Amp, two-colour (Agilent) by means of the "random priming" technique with the cyanines Cy5-dUTP for the target DNA and Cy3-dUTP for the control DNA. Afterwards the marked products were purified with Amicon Ultra-0.5 30 K (Millipore).
- *Hybridization on slides*: The purified products were denatured and co-hybridized on a 44K slide (Human Genome CGH Microarray, 4x44K, Agilent) loaded with probes (60bp oligomers) with an average resolution of about 100 Kb. Hybridization was carried out in rotation at the controlled temperature of 65°C for 40 hours.
- *Scanner reading and data analysis*: At the end of incubation the slides were washed with particular washing buffers (Agilent) and read with the scanner G2505B Agilent. Data were processed by means of specific software provided by Agilent (Feature Extraction and CGH-Analytics).

Use of CGH array for perimplantitis

To assess whether there is a relation to genetic predisposition and risk of peri-implantitis, we have used this materials and methods.

We identified 20 patients with peri-implantitis and requested a blood test.

From a clinical point of view the diagnosis was performed by probing and radiographic examination.

All patients had infection with suppuration and marginal bone loss.

As a test, we have identified 5 patients with implants but without peri-implantitis.

All patients had received treatment with implants at least 3 years with at least 2 implants.

For all patients we required informed consent

Array CGH has been conducted using the Agilent Human Genome CGH Microarray Kit 44K and 180K (Agilent Technologies, Palo Alto, CA, USA) with a resolution of w75 kb.

Labeling and hybridization has been performed following the supplier's protocols.

We have used 4 mg of purified DNA obtained from 20 patient's blood (with perimplantitis) and DNA obtained from 5 patients without perimplantitis as control . For all patients we required informed consent

It has been double-digested with RsaI and AluI for two hours at 37°C.

After column purification, 1 mg of each digested sample has been labeled by random priming (Invitrogen) for 2 hours using Cy5-dUTP for the patient's DNA and Cy3- dUTP for the control DNA.

Labeled products has been column purified and prepared according to the Agilent protocol. After probe denaturation and pre-annealing with 50 mg of Cot-1 DNA, hybridization has been performed at 65°C under rotation for 40 h.

After two washing steps the array has been analyzed with the Agilent scanner and the Feature Extraction software (v8.0).

Graphical overview has been obtained using the CGH analytics software (v3.1) (Agilent Technologies).

In Vivo

To test the ability of stem cells to promote bone regeneration and to test stem cells anti inflammatory capability we have have used those materials and methods with in vivo experiments. We have used both on small and large size animal.

In vivo rat model

The *in vivo* experiments were carried out on four immunocompromised female Wistar rats (Charles River) of 8 weeks. At the level of the cranial bones some lesions were made, which were then covered with the sample to be tested (a hydroxyapatite-based support previously cultured for one week with ADSCs in the presence of osteogenic and endothelial factors) or with the control (the hydroxyapatite-based support only).

All the operations were carried out upon general anaesthesia by means of the

intraperitoneal injection of ketamine hydrochloride (Ketaras, Yuhan Corporation, Korea, 40mg/kg) mixed with xylazine (Rompuns, Bayer Korea, Korea, 10 mg/kg). After disinfecting the cutis of the head with Betadine 10% (Potadine, Sam-Il Pharmacology, Korea) and injecting lidocaine (2% lidocaine containing. 1:100000 epinephrine, Lidocaine HCL Injs.Yuhan Corp., Korea) into the skull bone, an incision was made along the sagittal suture, the periost was lifted and a lesion of 5 mm of diameter was made using a trephine without perforating the dura.

Then the lesions were filled with the hydroxyapatite-based scaffold (control) only or with the scaffold inoculated with ADSCs. After the operation the animals were placed separately in cages thermostated at 22°C with a day/night cycle of 12 hours and with food *ad libitum*. Three weeks after the operation the rats were sacrificed by cervical dislocation.

All the animals were treated following the “Recommendations for the management of laboratory animals in biomedical research” issued by the Ethical Committee for Animal Experimentation of the University of Padua.

Goldner trichrome staining

The samples recovered from the *in vivo* experiments were fixed for one night in 3.7% formalin at 4°C, decalcified for 20 days with a solution based on formalin and formic acid, and included in paraffin. The inclusion in paraffin was carried out with the following passages:

- two 30-minute passages in 50% ethanol;
- three 30-minute passages in 70% ethanol;
- two 30-minute passages in 95% ethanol;
- two 30-minute passages in 100% ethanol;
- xylene until clearing;
- two one-hour passages in liquid paraffin in a heater at 60°C;
- inclusion.

The included samples were cut into slices having a thickness of 7 ∓m, which were rehydrated through ascending alcohols and submitted to Goldner trichrome staining, in order to distinguish the mineralized bone from the (unmineralized) osteoid.

Goldner trichrome staining (also known as Masson-Goldner) uses several dyes to stain the different structures. In particular, we used: ferric hematoxylin to make the nuclei brown-black, Light Green to make the collagen fibres green, acid fuchsin

and xylidine ponceau to make the cytoplasm red, and Orange G to make the erythrocytes orange.

Staining was carried out as follows:

- ferric hematoxylin for 10 minutes;
- washed in distilled water;
- differentiated in spring water;
- acid fuchsin and xylidine ponceau for 5 minutes;
- washed in 1% acetic acid;
- differentiated in 1% phosphomolybdic acid for 5 minutes;
- washed in 1% acetic acid;
- Light Green for 2 minutes;
- washed in 1% acetic acid.

In vivo sheep model

Twenty adult female sheep were used for the experiment. During surgical procedures, the animals were premedicated with IM methadone (0.2-0.3 mg / kg) and xylazine (0.1 mg / kg), and then anesthesia was induced with propofol (3-5 mg / kg IV). After the oro-tracheal intubation, general anesthesia was maintained with inhaled Isoflurane (1-1.6%, from 1.19 to 1.56 MAC) in oxygen/medical air.

The surgical field was prepared by shaving the skin to visualize main landmarks, namely, the angular vein of the eye and the transverse artery of the face. The sheep were prepped and draped in a customary manner for a sterile surgical procedure. The surgical site and the incision line were located and prepared with iodine. Approximately 3.6 mL of local anesthesia (Polocaine 2%, 1:20 000 levonordefrin; Astra Pharmaceuticals, Westborough, Mass) was administered in the surgical site.

An oblique caudodorsal, rostroventral, extraoral incision approximately 5 cm in length was made over the most ventral aspect of the maxillary sinus. Subcutaneous tissue and the masseter muscle were divided to expose the maxillary periosteum, which was incised and elevated dorsally.

The lateral wall of the sinus was approached with a surgical rotating tungsten bur to perform a rectangular surface antrostomy (Figure 10) under abundant irrigation with saline solution.

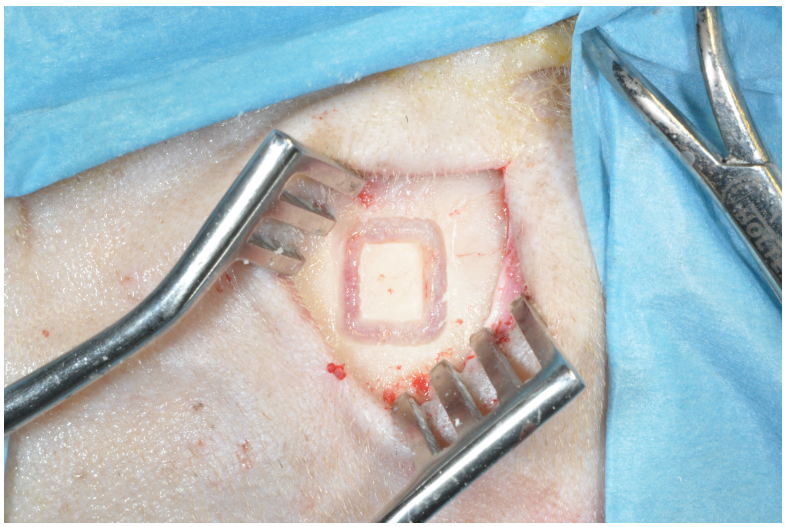


Figure 10: rectangular antrostomy performed with surgical bur to access the maxillary sinus

The antrum window was removed by fracturing along the osteotomy with a chisel instrument (Figure 11).

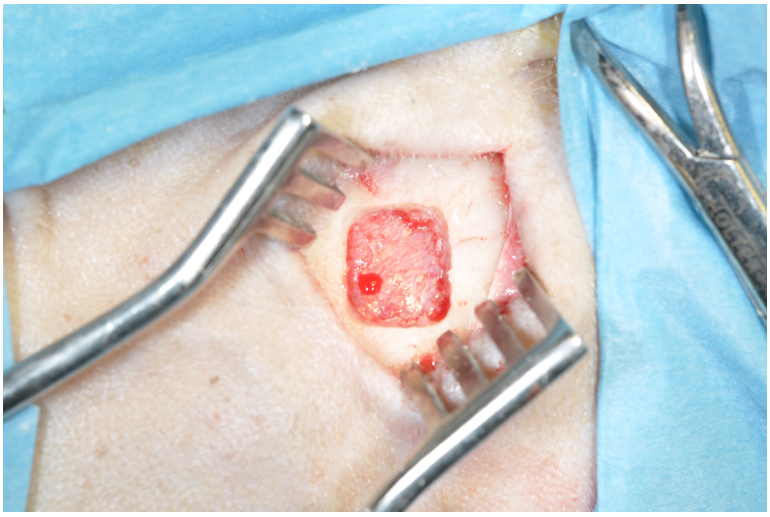


Figure 11: antrum window was removed. Schneiderian membrane was exposed

The sinus lining and floor were meticulously evaluated to remove any remaining soft tissue.

Maxillary sinus elevation was performed bilaterally in each sheep.

Sheep were divided into two groups of healing before sacrifice: 15 and 30 days.

Each group consisted of 10 sheep of which six have received as a graft material in

one maxillary sinus hydroxyapatite based granular scaffolds biomaterial (Bio-oss® Bio-Oss; Geistlich Pharma AG, Wohlhusen, Switzerland) alone whereas the contralateral side received granular scaffolds biomaterial (Bio-oss® Bio-Oss; Geistlich Pharma AG, Wohlhusen, Switzerland) in presence of ADPSc. (Figure 12)

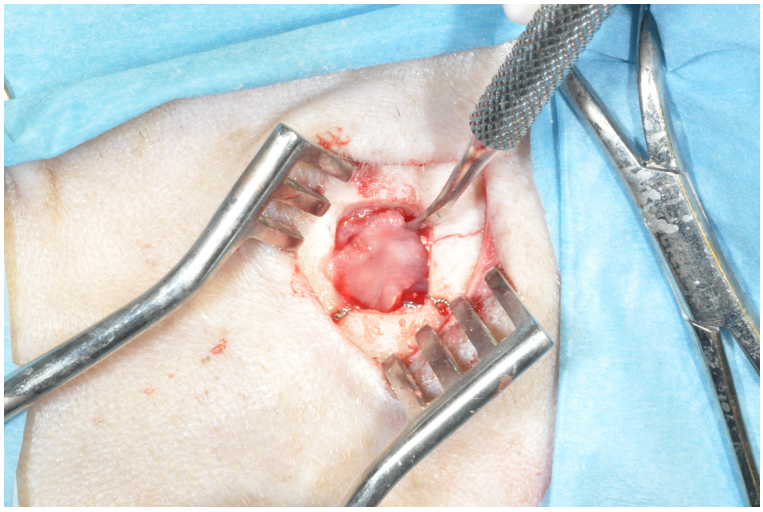


Figure 12: Schneiderian membrane was elevated. Sinus was grafted with biomaterial and non woven scaffold with ADPSc.

The others four sheep received as a graft material in one maxillary sinus hydroxyapatite based granular scaffolds biomaterial (Bio-oss® Bio-Oss; Geistlich Pharma AG, Wohlhusen, Switzerland) alone plus TNFα (10 mM) to induce an inflammatory state whereas the contralateral side received hydroxyapatite based granular scaffolds biomaterial (Bio-oss® Bio-Oss; Geistlich Pharma AG, Wohlhusen, Switzerland) plus ADSc and TNFα (10 mM) to induce an inflammatory state.

An equal volume of graft material was used (3 cm^3) within each sinus cavity. Deep and superficial fasciae of the masseter muscle were reapposed with 3–0 vicryl in a simple continuous pattern. Subcutaneous tissue and the skin were closed separately in a similar manner with 3-0 vicryl. (Figure 13)

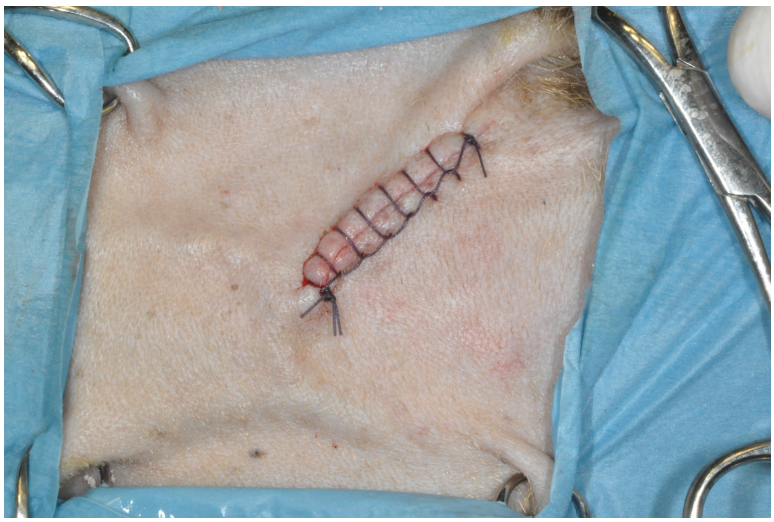


Figure 13: Subcutaneous tissue and the skin were closed separately in a simple continuous pattern

The animals were treated i.v. with 15 mg/kg of ampicillin (Vetamplius®, Fatro) every 12 h for 3 days and and tramadol 2 mg/kg. Surgical wounds were inspected daily. Groups of animals were euthanized at 15 and 30 days by applying an overdose of thiopental (Pentothal Sodium, Intervet) 10-15 mg/kg IV and embutramide (Tanax®). The head were removed, and individual bone blocks containing the augmented sinus and the surrounding hard tissues were fixed.

Histological preparation

The cellularized scaffolds were fixed for one night in 3.7% formalin at 4°C, decalcified for 20 days with a solution based on formalin and formic acid, and included in paraffin. The inclusion in paraffin was carried out with the following passages:

- two 30-minute passages in 50% ethanol;
- three 30-minute passages in 70% ethanol;
- two 30-minute passages in 95% ethanol;
- two 30-minute passages in 100% ethanol;
- xylene until clearing;
- two one-hour passages in liquid paraffin in a heater at 60°C;
- inclusion.

Serial 7-mm sections were cut perpendicular to the osseous defects and surrounding bone (Reichert-Jung 2050, Nussloch, Germany).

The bone sections were stained with haematoxylin and eosin staining and Masson's trichrome (MT) and observed under a light microscope.

Semi-quantitative analysis of cells

In order to analyze the cellular response to treatments, masked microscopic examinations were performed on sections. Cells were identified by: haematoxylin and eosin staining, for: inflammatory cells, endothelial cells and fibroblasts;
by Masson's trichrome (MT) for the detection bone structure (type I collagen, mature bone, woven bone). Briefly, two investigators analyzed in a masked fashion at least 3 slides for each experiment by light microscopy using as the initial magnification.

In vivo dog model

Six Labrador dogs were used for the experiment. During surgical procedures, the animals were pre-anaesthetized with atropina 0.04 mg/kg + medetomidina 0.04 mg/kg + ketamina 5 mg/kg and sedated with isoflurano 1,5 a 3% CAM + O_2 al 95%.

All mandibular premolars and the first molars were extracted bilaterally. Three months after tooth extractions, a crestal incision was performed in the premolar-molar region in both side of the mandible. Full-thickness mucoperiosteal flaps were elevated, and six experimental sites were identified in the edentulous alveolar ridges, each side of the mandible. The surgical preparation of the sites was performed according to the manual of the implant system (Sweden & Martina, Due Carrare, Padova, Italy). Twist drills were used to prepare each recipient site for implants, 10 mm long and 3.3 mm in diameter (Premium™, Sweden & Martina).

Subsequently, especially designed step drills were used to widen the marginal 5 mm of the implant bed to 5.4 mm. (Figure 14)

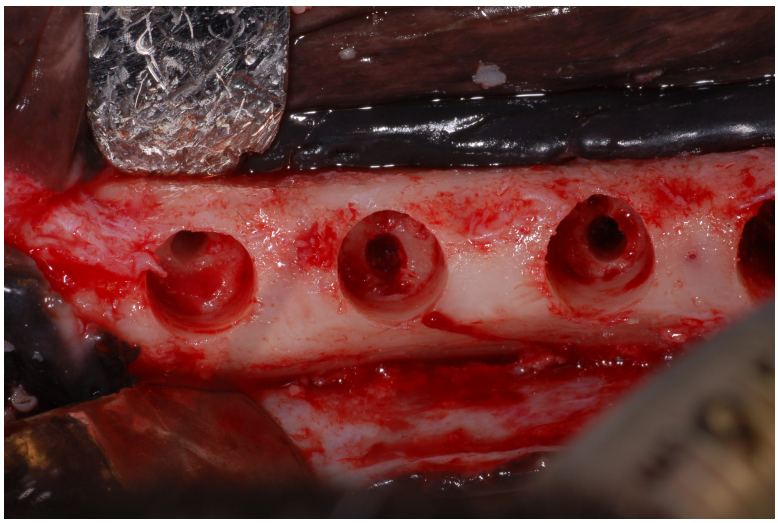


Figure 14: Surgical preparation of the sites. Especially designed step drills were used to widen the marginal 5 mm of the implant bed to 5.4 mm

Implants were subsequently installed with their margin flush to the bone crest and healing caps were screwed on the implants.

Following installation, a marginal gap occurred around the implants. (Figure 15)

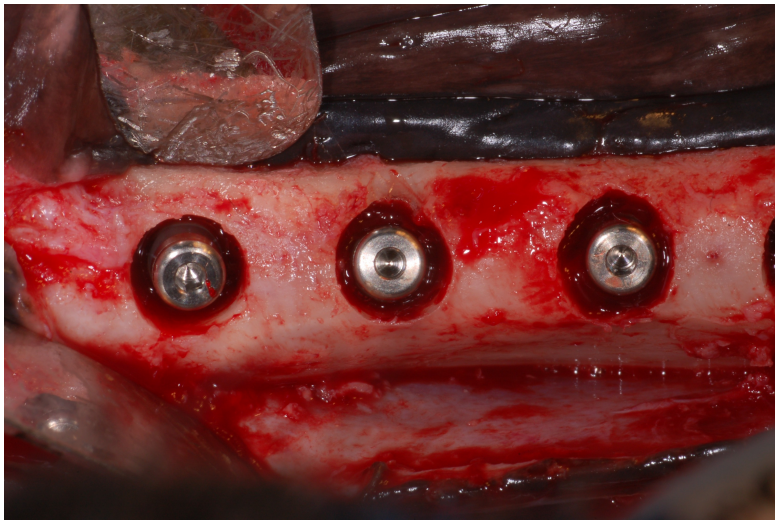


Figure 15: Implants were installed with their margin flush to the bone crest. A marginal gap occurred around the implants

The marginal defects in the right side of the mandible were filled with hydroxyapatite based granular scaffolds biomaterial (Bio-Oss®; spongious granule, particle size 0.25-1 mm; Geistlich Pharmaceutical, Wolhusen, Switzerland) mixed

with a concentrate of stem cells (ADSc). The defects in the left side of the mandible were filled with hydroxyapatite based granular scaffolds biomaterial (Bio-Oss®; spongious granule, particle size 0.25-1 mm; Geistlich Pharmaceutical, Wolhusen, Switzerland) alone. (Figure 16)

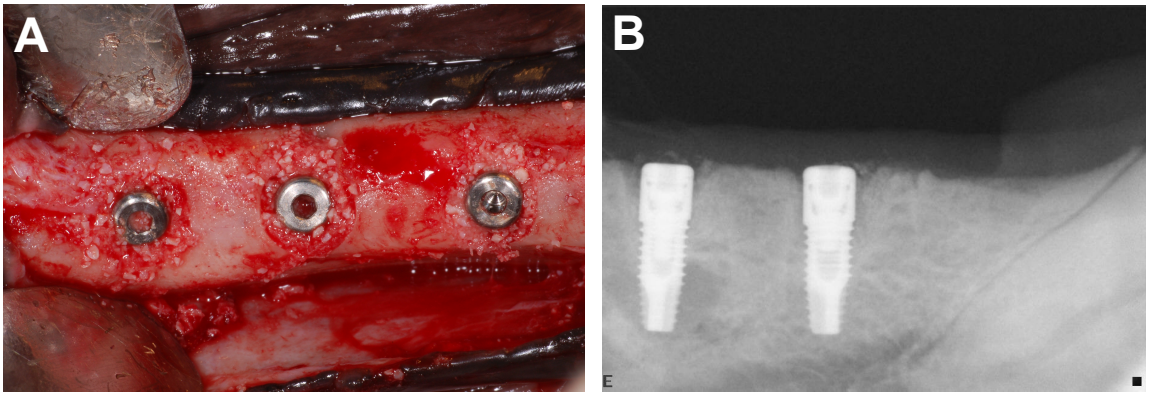


Figure 16: A: marginal defects in the right side of the mandible were filled with biomaterial mixed with a concentrate of stem cells (ADSc). B: postoperative x-ray showing implants with marginal defect.

The flaps were sutured allowing a fully-submerge healing.

The sacrifice of the animals were planned after 1 month of healing.

After the surgeries the animals received antibiotic for 8 days (enrofloxacina 3 ml/48 kg) and tramadol 2 mg/kg.

The animals were kept in kennels and on concrete runs at the university's field laboratory with free access to water and fed with moistened balanced dogs' chow.

Postoperatively, the wounds were inspected daily for clinical signs of complications. Check-ups were performed on regular basis throughout the experiment. The animals were euthanatized according the following protocol: eparina 1000 UI + ketamine 10 mg/kg + xilacina 1mg/kg + succinilcolina 0.2 mg/kg and KCl 25 meq.

Histological preparation

The mandibles were removed, and individual bone blocks containing the implant and the surrounding soft and hard tissues were fixed in 4% formaldehyde solution. The specimens were dehydrated in a series of graded ethanol solutions, and finally embedded in resin.

The blocks were cut in a bucco-lingual plane following the long axis of the

implants, using a diamond band saw fitted in a precision slicing machine. A central section were harvested and then hand-polished and thinned to about 50 µm using a grinding machine. The histological slides were stained with Stevenel's blue with alizarin red and examined under a standard light microscope for histological analysis. (Figure 17)

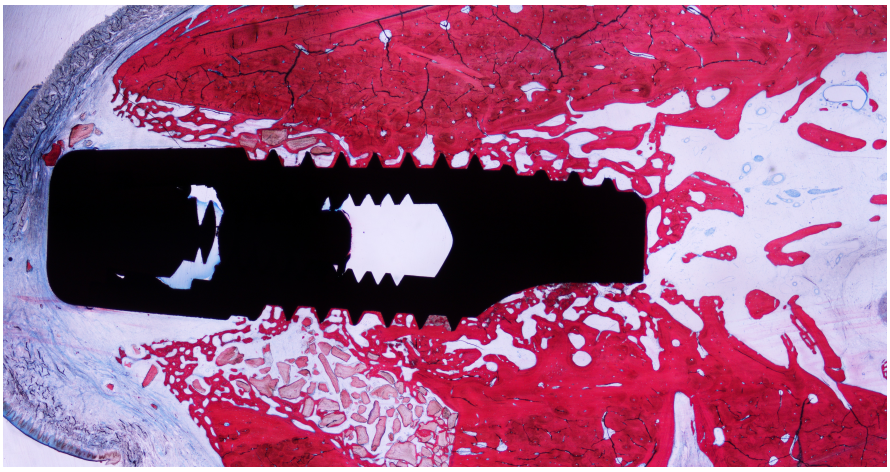


Figure 17: histological slides were thinned to about 50 µm and stained with Stevenel's blue with alizarin red

With a light microscope for histological analysis (20x), all histological slides were observed in order to detect: PMNs, phagocytic and non phagocytic cells, fibroblasts, endothelial cells, collagen type I and new bone formation. Neoangiogenesis was investigated to see if in the first time of healing neoangiogenesis is accelerated by stem cells.

Results

In Vitro

Culture of adult stem cells

Two different types of adult stem cells were isolated from their tissues of origin:

- Adipose derived Stem Cells (ADSCs) from the adipose tissue;
- Dental Pulp Stem Cells (DPSCs) from the dental pulp.

The cell suspensions obtained by enzymatic digestion were inoculated on standard cell culture plates. After three days of culture, with the optical microscope one can see the presence of cells with fibroblastoid morphology adhering to the plastic (Figure 18).

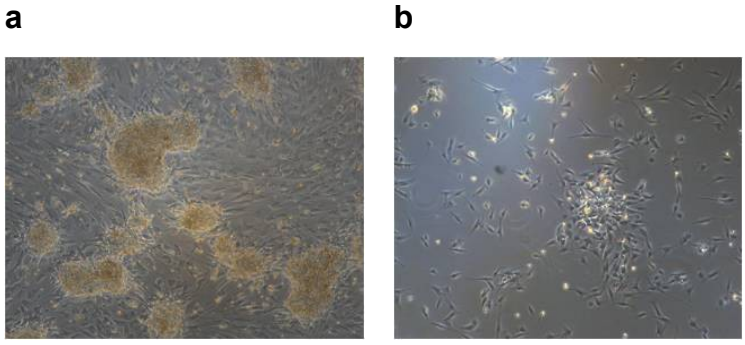


Figure 18: Optical microscopiy analysis of the primary culture of ADSCs (a) and DPSCs (b). 10X enlargements.

Before being inoculated on the biomaterial, the adult stem cells were analyzed phenotypically by immunofluorescence (Figure 19) for the purpose of assessing the presence of the following markers on the cell surface:

- CD 73: also known as ecto-5'-nucleotidase. It is a 69 kDa protein anchored to the plasma membrane, where it catalyzes the conversion of extracellular nucleotides into nucleotides permeable to the cell membrane. It was found in hematocytes, in bone marrow cells and in thymic, renal and liver epitheli-al cells.

- CD 90: also known as Thy-1 (THYmocyte differentiation antigen 1) due to its first identification in thymocytes. It was also found on the surface of neuronal cells, endothelial cells, mesenchymal stem cells and hematopoietic stem cells.
- CD 105: also known as endoglin. It is a 180 kDa homodimeric transmembrane glycoprotein whose expression was found in endothelial cells and in embryonic stem cells, mesenchymal stem cells and bone marrow cells

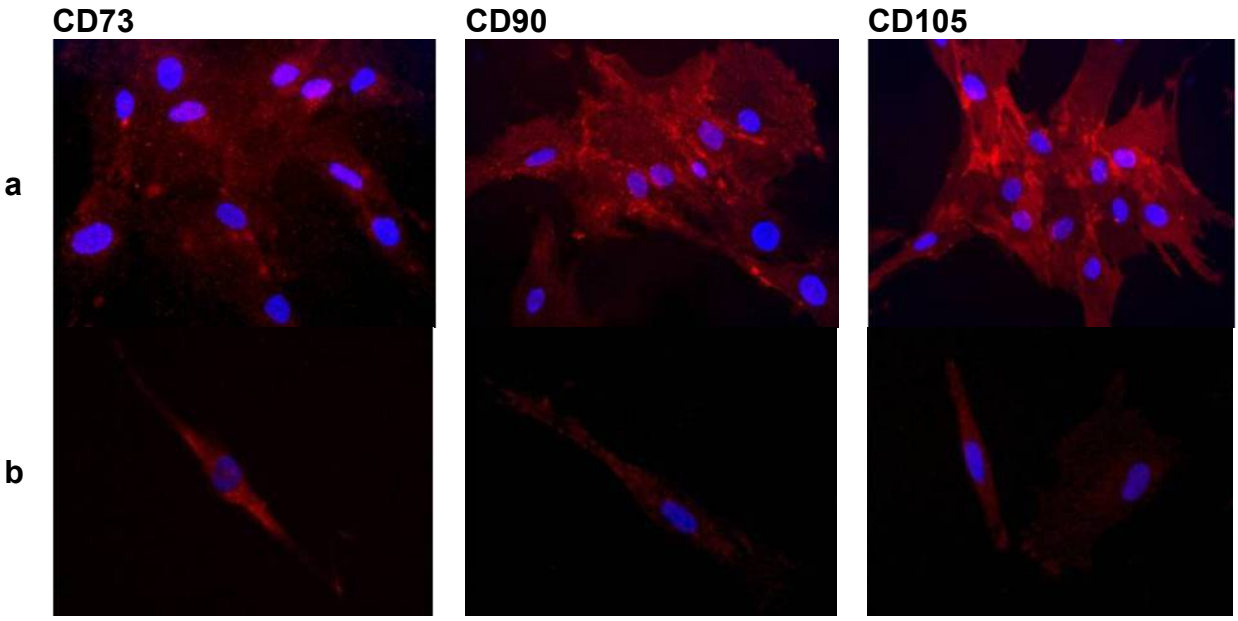


Figure 19: Fluorescence microscopy analysis of the primary culture of ADSCs (a) and DPSCs (b). Expression of CD73, CD90 and CD105 markers. 40X enlargements.

In vitro hard tissue reconstruction

ADSC and DPSC adult stem cells were used in combination with suitable biomaterials and differentiation factors in order to reconstruct hard tissue *in vitro*. In particular, the following tissue was reconstructed:

- vascularised bone tissue from ADSCs/DPSC inoculated on hydroxyapatite-based scaffolds:

Differentiation of ADSCs on a hydroxyapatite-based support

By means of enzymatic digestion, the adipose tissue-derived mesenchymal stem cells (ADSCs) were isolated and then amplified in complete DMEM medium for two weeks. Afterwards, the ADSCs were inoculated with a density of 10^6 cells/cm^2 on hydroxyapatite-based matrices, previously treated with fibronectin to favour cell adhesion. The matrices inoculated with the ADSCs were cultured in the presence of differentiation medium made up of osteogenic and endothelial factors. Differentiation was prolonged for 21 days and morphological analyses, proliferation tests, gene expression and molecular cytogenetic analyses were carried out at intervals of 7 days.

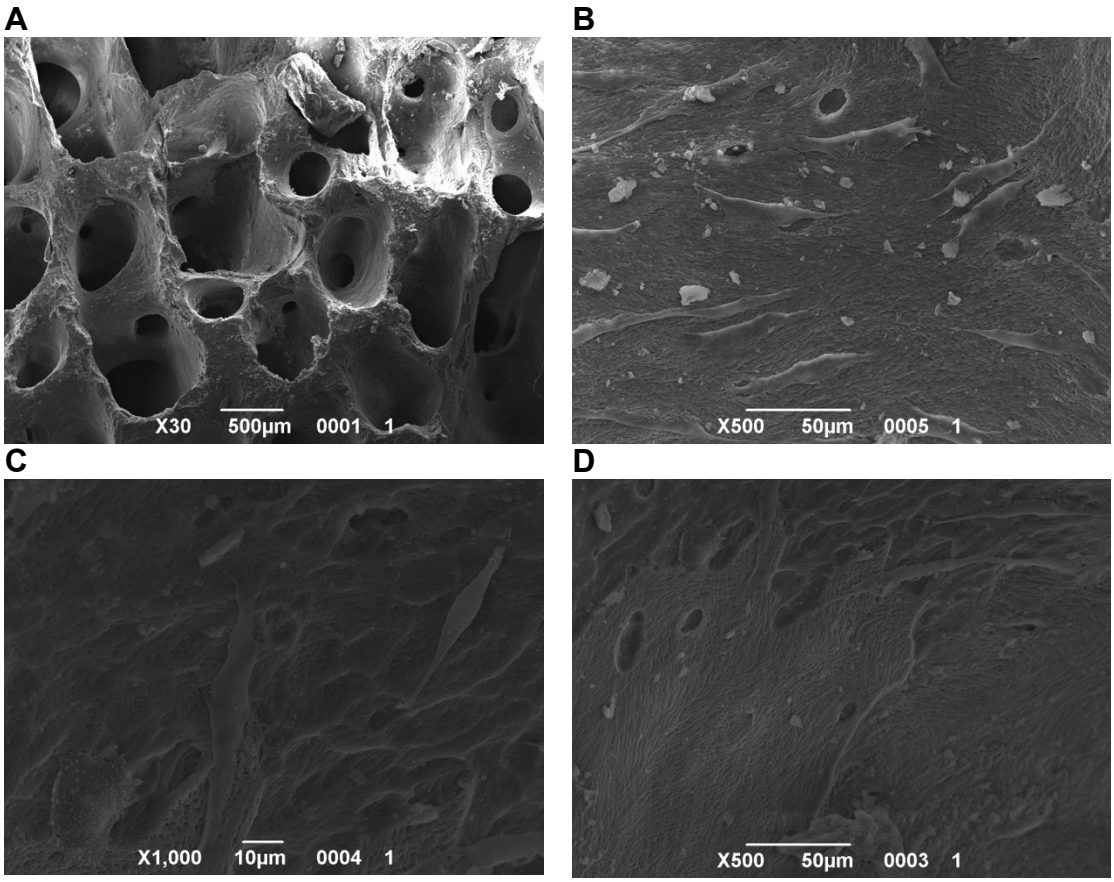


Figure 20: Scanning electron microscopy (SEM) analysis of the construct based on hydroxyapatite (A) and inoculated with ADSCs after 7 (B), 14 (C) and 21 (D) days of culture in osteo-endothelial differentiation medium.

The morphological analysis of the constructs was carried out by SEM after 7, 14 and 21 days of culture in osteo-endothelial medium (Figure 20). The images acquired by SEM show that the ADSCs progressively colonized the biomaterial,

also proliferating inside the porous structure, until constituting, after 21 days of culture, a continuous layer of cells (Figure 20 D).

The proliferation of the ADSCs inoculated on the hydroxyapatite-based scaffolds was also assessed by MTT assay (Figure 21 A) and by quantification of the cells adhering to the matrices (Figure 21 B) after 7, 14 and 21 days of culture in osteo-endothelial differentiation medium. The quantification of the adhering cells was determined indirectly through a previously built calibration curve that correlates the amount of DNA extracted from the ADSCs to the number of cells subjected to extraction. The data collected show an increase in the proliferation time and in the number of ADSCs inoculated on the scaffolds.

A **B**

Figure 21: MTT assay (A) and quantification of the cells adhering (B) to the hydroxyapatite-based supports after 7, 14 and 21 days of culture in osteo-endothelial differentiation medium.

The differentiation of the ADSCs inoculated on the hydroxyapatite-based matrices was assessed by genic expression analysis with Real-Time PCR (Figure 22).

To this purpose, three different differentiation conditions were tested:

- Three-dimensional cultures of ADSCs in the presence of osteogenic factors;
- Three-dimensional cultures of ADSCs in the presence of endothelial factors;
- Three-dimensional cultures of ADSCs in the presence of both factors.

Osteogenic differentiation was determined by assessing the expression of some bone matrix markers, such as Osteopontin, Osteonectin, Osteocalcin and Type I Collagen [Ma et al. 2010]. On the other hand, endothelial differentiation was assessed by determining the expression of the marker CD 31 (also known as

Platelet Endothelial Cell Adhesion Molecule - PECAM-1), of the von Willebrand Factor (vW) [Marino et al. 2012] and of the Vascular Endothelial Growth Factor (VEGF). Furthermore, the expression of transcriptional factors was determined: Runt-related transcription factor 2 (RUNX2), essential for osteogenic differentiation, and Peroxisome Proliferator-Activated Receptors gamma (PPAR•‿, necessary for the regulation of adipocyte differentiation [Kawai et al. 2009].

A

B

C

Figure 22: Genic expression analysis by Real-Time PCR of ADSCs in the presence of osteogenic factors (A), endothelial factors (B), and osteogenic and endothelial factors (C) after a treatment of 7 (black bars), 14 (white bars) and 21 (grey bars) days.

In the presence of osteogenic factors only (Figure 22 A) there was an increase in the time of expression of the markers Osteopontin, Osteonectin, Osteocalcin and Type I Collagen. Over time, then, there was an increase in the expression of the osteogenic transcriptional factor RUNX2 and a contemporary reduction in the expression of the transcriptional factor PPAR•, specific for adipocyte differentiation. In this differentiation condition there was no expression of the endothelial markers

CD 31, vW and VEGF.

The association of endothelial factors with osteogenic factors (Figure 22 C), on the other hand, showed over time a greater increase in the expression of osteogenic markers. The same increase was also observed for RUNX2 associated with a greater reduction in the expression of PPAR•. Furthermore, the presence of endothelial factors determined the expression of the markers CD 31, vW and VEGF. For the latter, there was an increase in the expression over time like in the differentiation condition with endothelial factors only (Figure 22 B).

Genetic analysis of the construct

In order to ensure the genetic stability of the construct based on hydroxyapatite and differentiated ADSCs, Comparative Genomic Hybridization (CGH) arrays were performed to identify genomic deletions and amplifications.

The DNA extracted from the ADSCs inoculated on hydroxyapatite-based supports and treated with osteogenic and endothelial differentiation factors for 7, 14 and 21 days was analysed by CGH array (Figure 23). This analysis showed the absence of genomic imbalances, namely amplifications or deletions of regions of the genome, confirming that long-term culture in the presence of differentiation factors cannot induce DNA structural alterations. Figure 23 shows two regions as an example of the whole genome of the analyzed samples. In particular, Figure 23 A shows the DNA region related to the MYC (myelocytomatosis viral oncogene homolog) gene, while Figure 23 B the DNA region related to the RB1 (retinoblastoma 1) gene of the constructs after 7, 14 and 21 days. Both show no alterations, which are clearly evident in the same genomic regions of the positive control, represented by the DNA extracted from actinic keratosis cells (Figure 23 B and D).

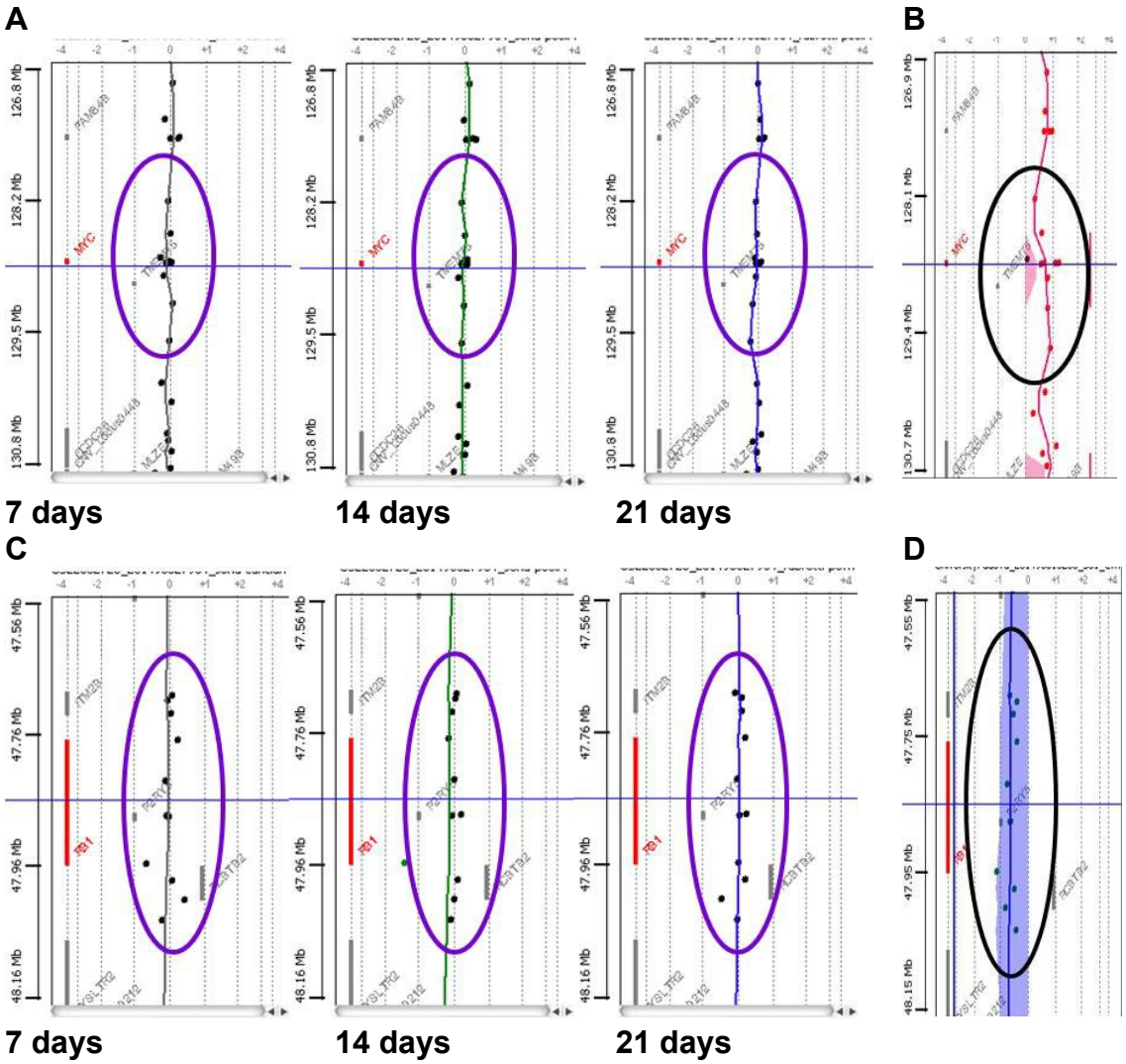


Figure 23: CGH array analysis. Two genic regions of the hydroxyapatite-based scaffold inoculated with ADSCs in osteo-endothelial differentiation medium after 7, 14 and 21 days (A, C) compared with the same regions of actinic keratosis (B, D). In (B) an amplification and in (D) a deletion.

Donor age-related biological properties of human dental pulp stem cells

Stemness of DPSCs

The isolation of DPSCs from human dental pulp and their *in vitro* differentiation capacity into various mesenchymal tissues were first established by using standard protocols. Under these conditions (using specific differentiation media), the classical adipogenic, osteogenic, and chondrogenic media were highly efficient in causing specific differentiation into the expected cell lineages, as confirmed by molecular hallmarks (Figure 24).

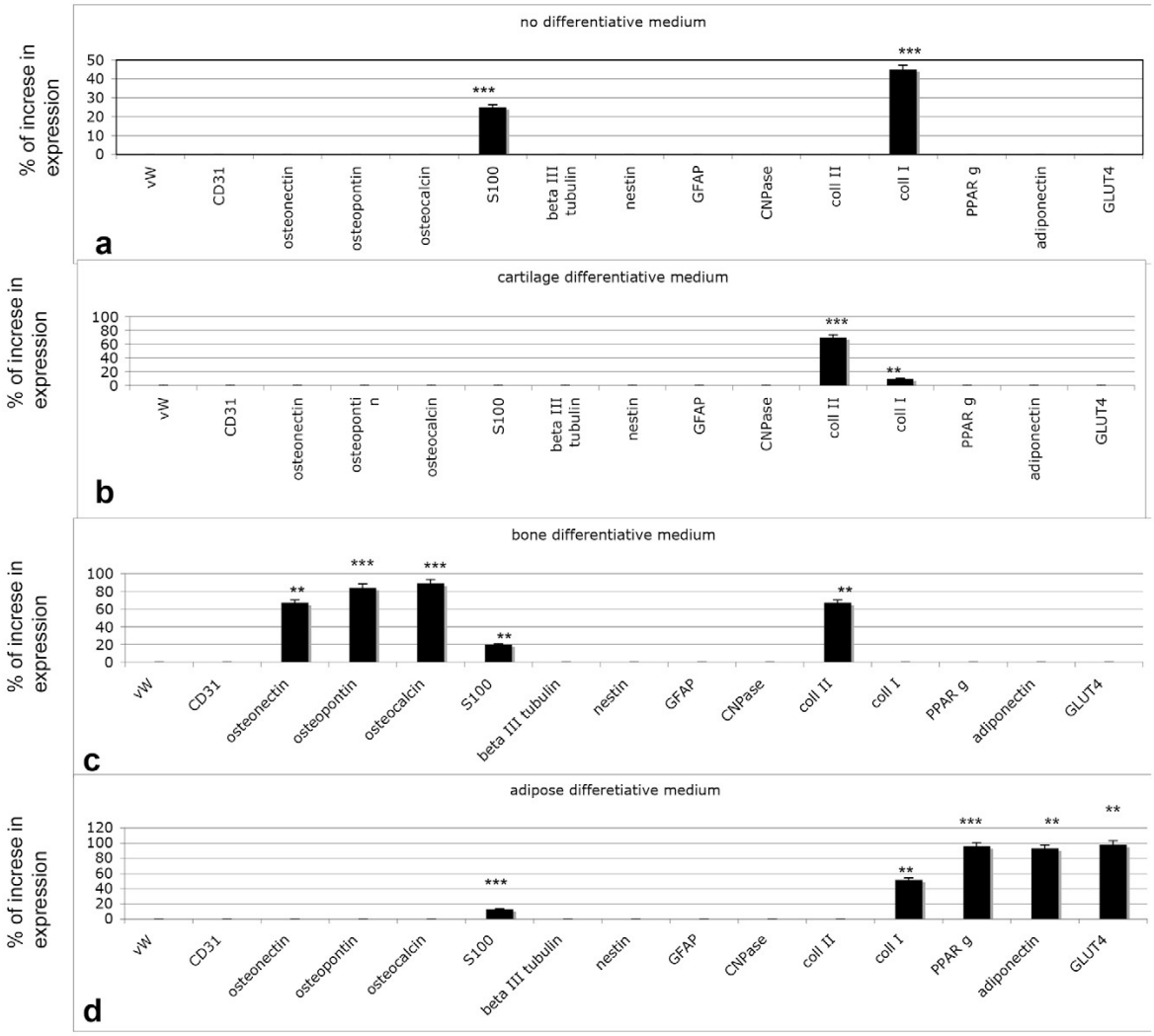


Figure 24: Gene expression according to real-time PCR on undifferentiated DPSCs in basal culture medium (A) and in media that induce differentiation: chondrogenic (B), osteogenic (C) or adipogenic (D). One-way analysis of variance (ANOVA) was used for data analyses. The Levene ́is test was used to demonstrate the equal variances of the variables. Repeated-measures ANOVA with a post-hoc analysis using Bonferroni's multiple comparison. T tests were used to determine significant differences (p,0.05). * p,0,05; * * p,0,01; * * * p,0,001. Repeatability was calculated as the standard deviation of the difference between measurements.

Moreover, we analyzed the commitment to these lineages versus the expression of endothelial, neuronal and glial like features. First, to confirm that cells isolated from dental pulp with our protocols were dental pulp stem cells (DPSCs), we cultured the cells in the presence of basal culture medium for undifferentiated DPSCs (Figure 24a), and we performed gene expression analyses to detect their phenotypes. After 2 days of culture in basal culture medium for undifferentiated

DPSCs, real-time PCR was performed. The genes selected for this screening were the following: Endothelial commitment: von Willebrand factor (vWF) and CD31. As reported in Figure 24a, no expression for these markers was detectable.
Bone commitment: osteopontin, osteonectin, and osteocalcin. No expression of these markers was detectable (Figure 24).
Neuronal commitment: S100, βIII tubulin, and nestin. Low expression of S100 was detectable, whereas no expression of other markers was found.
Glial commitment: GFAP and CNPase. Neither of these was detectable.
Adipogenic commitment: adiponectin, GLUT4, PPARγ. No expression was found.
Fibroblastic commitment: collagen type I was detected at 45% of the expression level of the control.
Twin cultures were then performed for 21 days in different media: chondrogenic (Figure 24b), osteogenic (Figure 24c), and adipogenic (Figure 24d). The gene expression patterns of the cultures confirmed the correct commitment. Indeed, in chondrogenic medium, only collagen type II was detectable, and no endothelial or neuronal markers were present. In osteogenic medium, collagen type I, osteopontin, osteonectin, and osteocalcin were abundant, whereas no traces of collagen type II or adipogenic genes were observable. In adipogenic medium, no neuronal, endothelial, bone or cartilage markers were detected. PPARγ, adiponectin and GLUT4 (specific for adipogenesis) were observed (Figure 24d).

Proliferative activity

Population doubling time (PDT) is used to evaluate the ability of the cell to duplicate in number and is therefore a direct marker of the proliferative ability of the cell. In this experiment, we analyzed the PDT of DPSCs cultured in non-differentiative medium. PDT was evaluated at 3 different in vitro passages (p) of the cultures:

- p2: early passage
- p5: medium-term culture
- p8: long-term culture

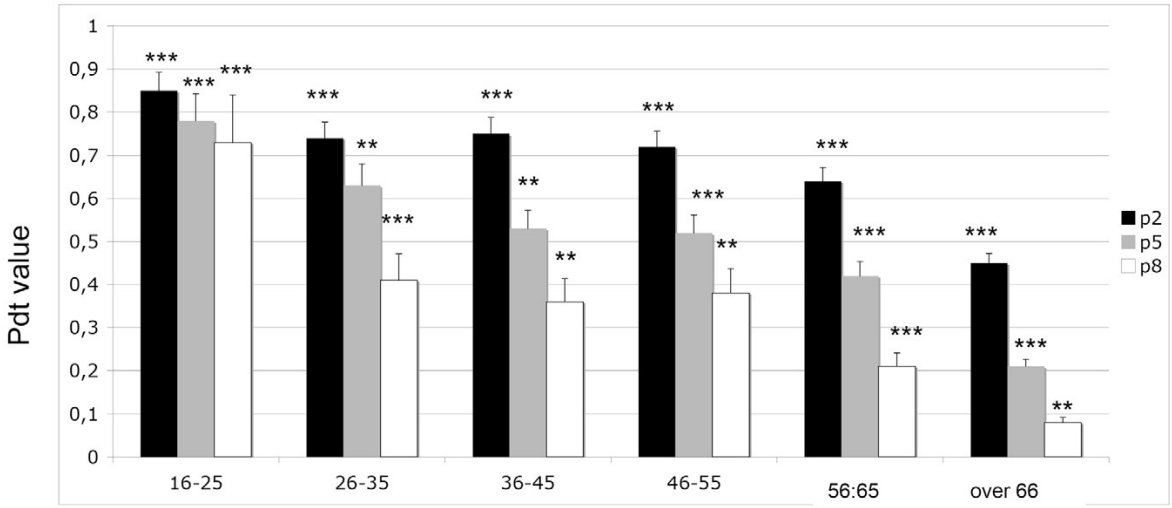


Figure 25: Population doubling time (PDT) of DPSCs cultured in the presence of basal culture medium for undifferentiated DPSCs. The graph shows in vitro passage (starting from p2 for young cells (black bar) to p8 (white bar)) and the age of the donor (from 16 to over 67). T tests were used to determine significant differences (p,0.05). * p,0,05; * * p,0,01; * * * p,0,001

As reported in Figure 25, we observed well-defined cell growth for each passage in each age class. For the aged group (up to 67 years), proliferative ability decreased in time and during in vitro aging. This property was not evident for stem cells derived from young donors (up to 25 years), as a high PDT value was maintained in all cell passages. These data confirm the good proliferative ability of stem cells. After age 25, this ability decreased in proportion to the in vitro passage. Interestingly, up to age 56, a high level of proliferation was detectable at p2.

Qualitative analyses of lineage commitment

Qualitative and quantitative analyses of stemness were performed, starting from the data reported in the literature [Zhang et al. 2008; Volponi et al. 2010; Yan et al. 2011; Yang et al. 2010; Huo et al. 2010; Nakashima et al. 2009; Sloan and Waddington 2009]. The commitment ability of DPSCs was tested by culturing them in the presence of differentiation media. Markers for endothelium, bone, and nervous system were detected by immunostaining (Figure 26 and Figure 27 for negative controls) and real-time PCR (Figure 28).

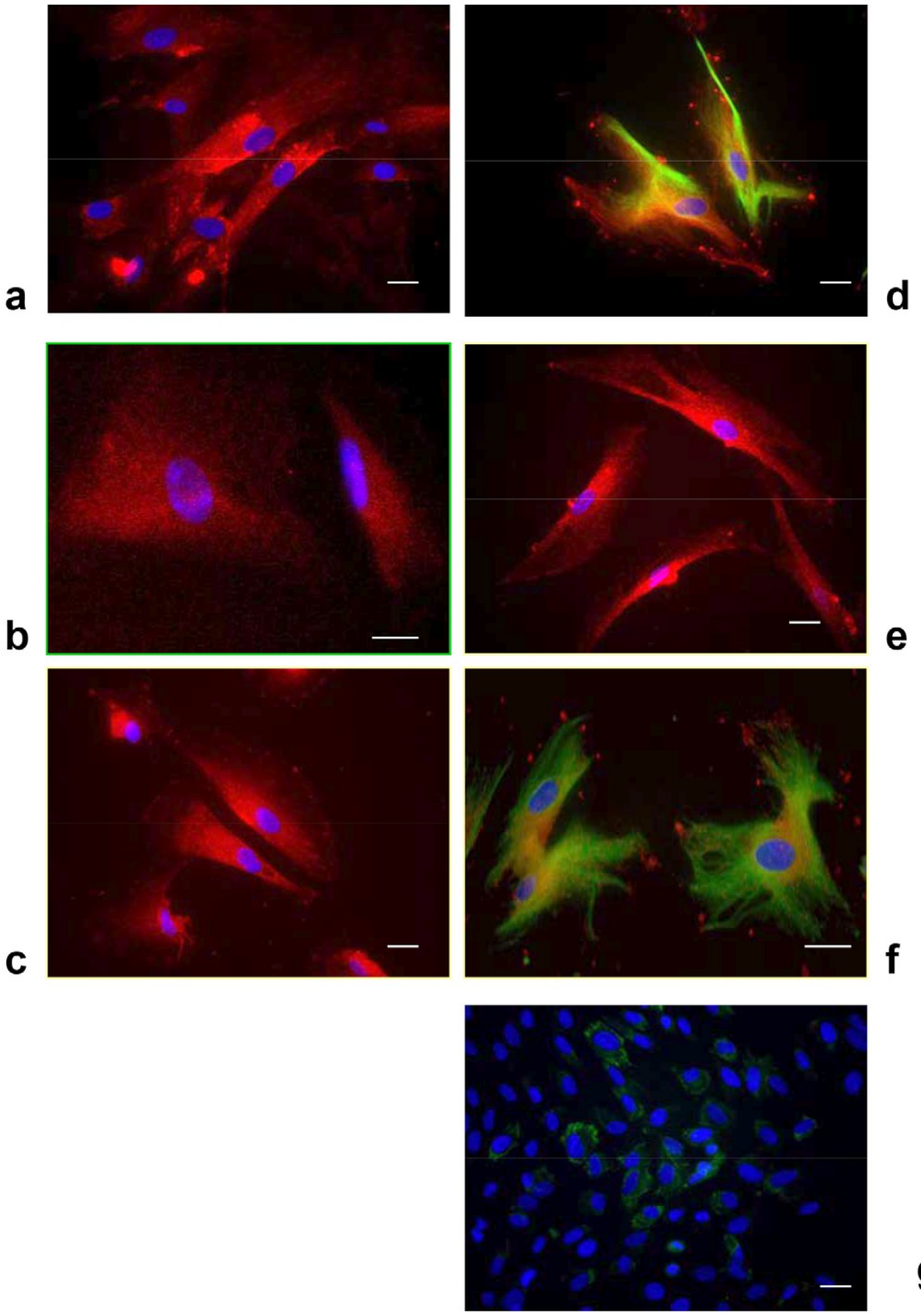


Figure 26: Immunofluorescence analyses of DPSCs committed to several cell lineages. (A) CD31 for endothelial cells (red cells). (B) Osteonectin for osteogenic commitment. (C) S100 (red cells, for neurogenic commitment), (D) nestin (red fibers) and bIII tubulin (green), (E) GFAP (red staining), (F) CNPase (green) and nestin (red) (406) for glial-like commitment. (G) Negative control: DPSCs cultured in non-differentiative medium and stained with primary Ab against GFAP (no red-positive cell are detectable) and primary Ab against CNPase (no green-positive cells are detectable). Bar: 30 mm.

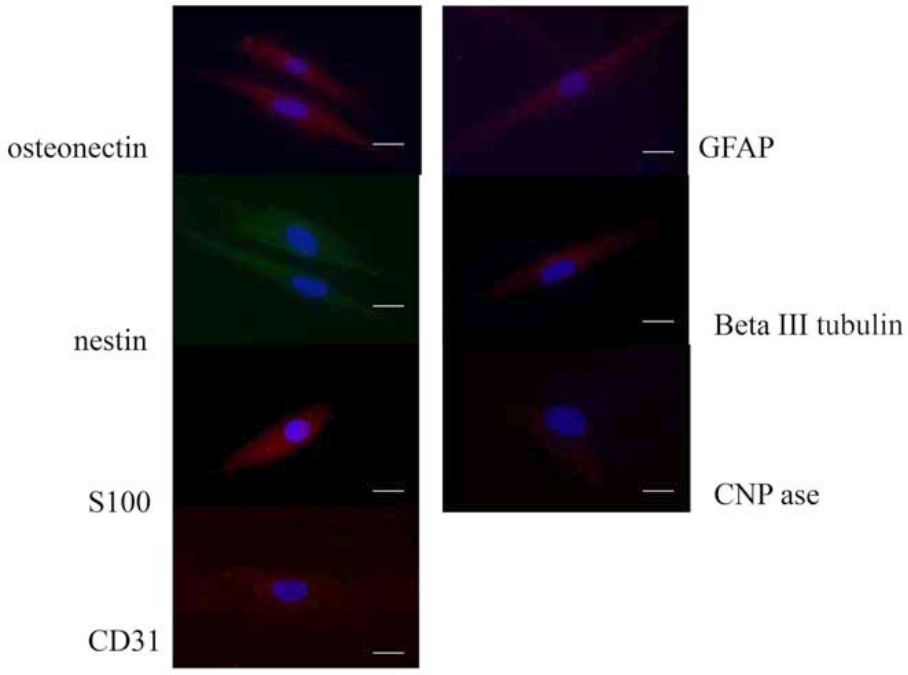


Figure 27: Immunofluorescence analyses of human fibroblasts (used as control). Bar: 30 mm.

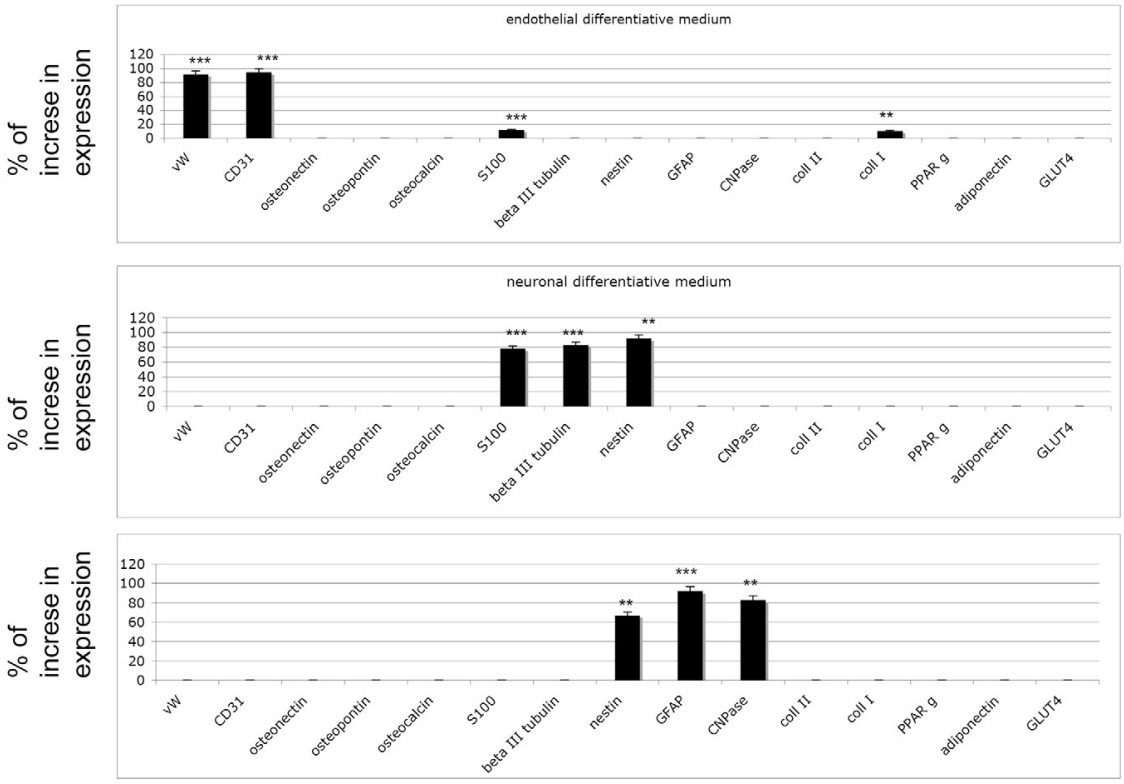


Figure 28: Gene expression by real-time PCR on DPSCs in endothelial, neuronal or glial differentiation medium. One-way analysis of variance was used for data analyses. T tests were used to determine significant differences (p,0.05). * p,0,05; * * p,0,01; * * * p,0,001. Repeatability was calculated as the standard deviation of the difference between measurements.

As control, DPSCs cultured in basal culture medium for undifferentiated DPSCs was. To test the specificity of the antibodies used for immunostaining, fibroblast cells were used as control. To analyze the results, we determined whether DPSCs always expressed the correct specific markers in the presence of a given differentiation medium. If DPSCs expressed the expected markers, we referred to them as maintaining that differentiation phenotype, and if they did not, we referred to them as losing this ability.

Endothelial markers

Specific markers for endothelial commitment, CD31 and vWF, were selected. DPSCs cultured in the presence of endothelial differentiation medium expressed CD31, as indicated by the well-defined red staining (Figure 27 a). Thus, the DPSCs correctly committed to the endothelial phenotype. Corroborating the immunocytochemistry results, the gene expression (Figure 28) of CD31 and vWF also indicated endothelial differentiation.

Bone markers

Osteonectin, a bone-specific protein that binds selectively to both hydroxyapatite and collagen, was selected as a marker of commitment to bone cells. Osteonectin links the bone mineral and collagen phases, perhaps initiating active mineralization in normal skeletal tissue. In the osteogenic medium, the red positive reaction (Figure 26 b) indicated the novel bone phenotype acquired by the cells. This result was supported by the expression of osteopontin, osteocalcin and collagen type I (Figure 28).

Neuronal markers

To analyze neuronal commitment capacity, we detected S100 (Figure 26 c), a calcium-binding protein normally present in cells derived from the neural crest (Schwann cells and glial cells), in mesenchymal-derived cells such as chondrocytes and adipocytes, and in dendritic cells. In Figure 26 c, it is evident that DPSCs expressed S100, as confirmed by the red fluorescent staining. Nestin, a type VI intermediate filament (IF) protein, is a protein marker for neural stem cells because it is mostly expressed in nerve cells. In our monolayer cell cultures, DPSCs in the presence of neuronal medium expressed nestin (Figure 26 d, red staining), further indicating neuronal commitment. The last protein tested for the neuronal phenotype was βIII tubulin, a protein abundant in the central and peripheral nervous systems, where it is prominently expressed during fetal and

post-natal development. All of the DPSCs cultured in neuronal differentiation medium showed a positive expression of this neuronal protein (Figure 26 d, green staining; Figure 27, negative control). The gene expression detected with real-time PCR (Figure 28) confirmed the immunocytochemistry results.

Glial markers

During gliogenesis, nestin is replaced by other IF proteins, such as glial fibrillary acidic protein (GFAP). In our cultures, indeed, no cells expressed GFAP if cultured in neuronal medium (data not shown), whereas in the presence of glial medium, a well-defined cytoskeletal structure positive for GFAP was evident (Figure 25 e, red staining). Another important signal related to glial phenotypes was the co-expression of nestin and CNPase (Figure 25 f). CNPase (2',3'-cyclic nucleotide 3'-phosphodiesterase) is expressed at high levels by oligodendrocytes in the central nervous system and by Schwann cells in the peripheral nervous system (Figure 25f, green staining). Its co-expression with nestin (Figure 25f, red staining) confirmed the commitment of DPSCs to the correct phenotype. In Figure 28, the gene expression levels of these proteins are shown.

Quantitative analyses

We analyzed the percent of positive cells for each marker and compared their lineage commitment abilities. These analyses were performed at the 3 most significant in vitro passages of DPSCs (P2, P5, P8) for each age group (Figure 29) to test if the stemness of DPSCs is related to the *in vitro* (passage) and *in vivo* (age of donor) aging of the cells.

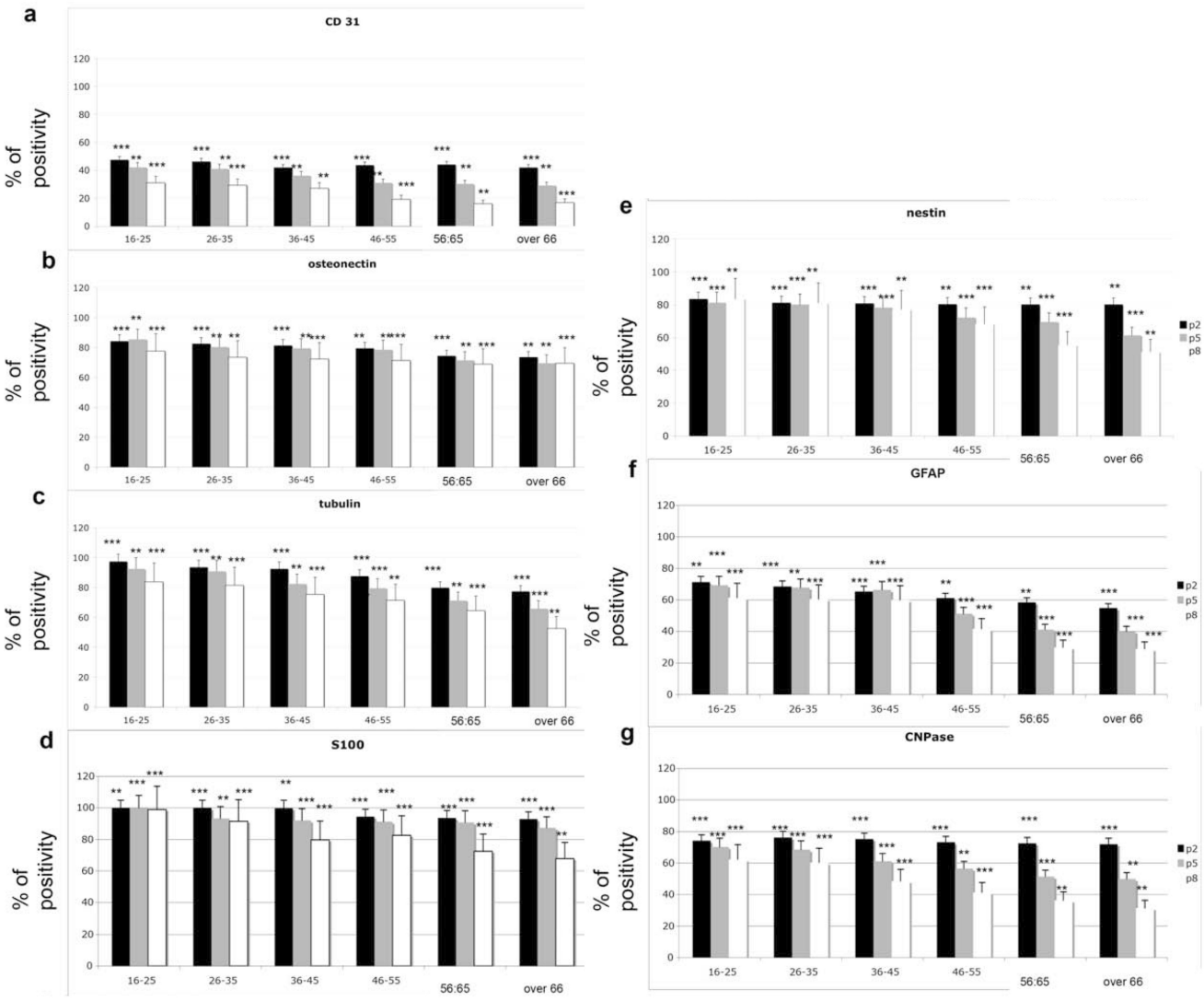


Figure 29: Quantitative analyses of lineage commitment were performed by analyzing the percent of positive cells for each marker. The commitment was detected in all donor age and in vitro passage groups. T tests were used to determine significant differences (p,0.05). * p,0,05; * * p,0,01; * * * p,0,001. Repeatability was calculated as the standard deviation of the difference between measurements.

Endothelial commitment

The commitment into endothelial cells was detected in all groups analyzed. Specifically, young in vitro cultures (namely, p2) showed good commitment (approximately 40% of total cells) into endothelial cells for all age groups analyzed (Figure 29a, black bar). This behavior was maintained as cells aged: the cells at p5 (gray bar) showed a similar percentage of endothelial commitment as p2 for all age groups. When cells aged in vitro (p8, white bar), they lost this ability in proportion to the age of the donor.

Bone commitment

A marked in vitro commitment of DPSCs was detectable for each in vitro passage and for each age. Figure 29b shows that approximately 80% of DPSCs from both p2 and p5 acquired a bone phenotype when they were derived from patient of less of 55 years old. After this age, a strong commitment (approximately 70%) was still evident up to p8.

Neuronal commitment

Nestin- (Figure 29c) and S100-positive cells (Figure 29d) were present at higher levels (100% of the cell population) during p2 (black bars) from donors up to 36 years old. In cells from donors up to 45 years old, Nestin (Figure 29e) was detectable in a smaller percentage of cell the population (80%), but its expression endured during the *in vitro* aging (from p2 to p8). Cells from senior donors showed a decrease in this marker over time *in vitro*.

GFAP (Figure 29f) and CNPase (Figure 29g) were present in approximately 70% of the cells in all p2 cultures for all ages. Starting from 45 years old, their presence dramatically decreased at p5 (gray bar) and at p8 (white bar).

3D cultures

Cells of the most representative groups, the 16-25 age group and the senior age group (over 66) at p5, were seeded onto HA granules, and their osteogenic potential was evaluated. The marker expression in monolayer conditions versus 3D conditions were then compared. As shown in Figure 30, the presence of HA considerably improved the osteogenic population: osteopontin, osteonectin, osteocalcin, collagen type I, collagen type III, cathepsin B (CTSB), cathepsin D CTSD, distal-less homeobox 1 (DLX1), DLX5, dental sialoprotein (DSPP), fibroblast growth factor 8 (FGF8), transforming growth factor β1 (TGF-β1), and RANK were more strongly expressed when cells where mixed with HA than in monolayer conditions from both donor age groups.

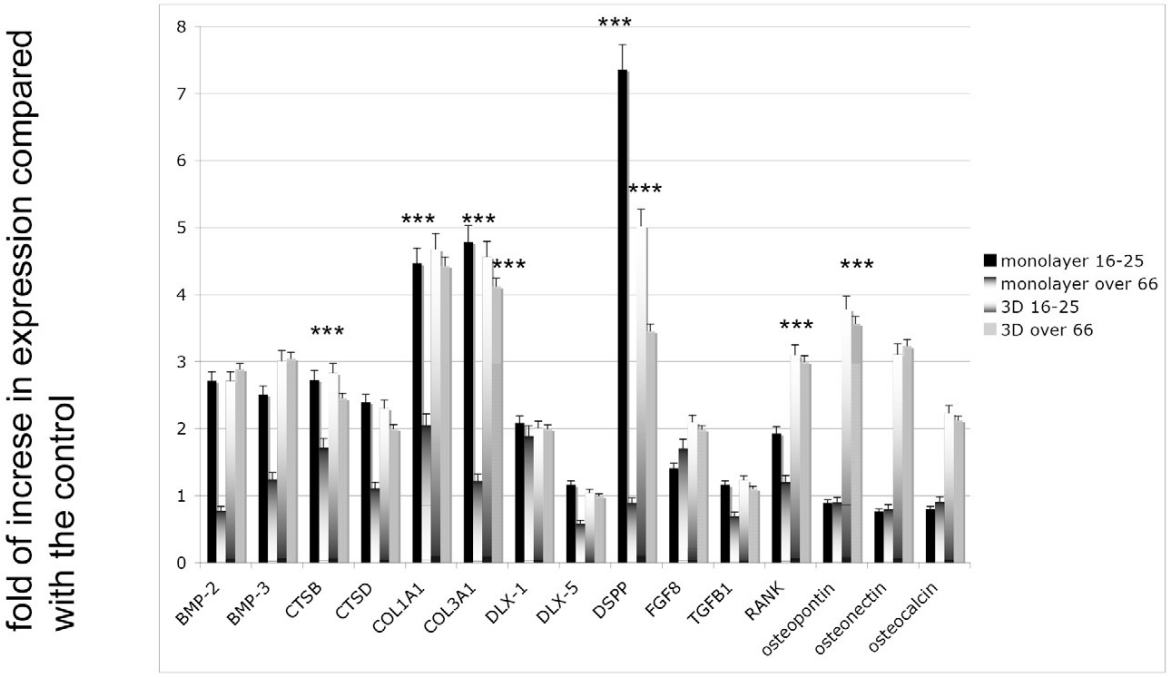


Figure 30: Gene expression by real-time PCR on DPSCs derived from the younger donor group (16–25) and from the senior group (over 66) in monolayers and in nanostructured scaffolds. Markers selected were bone morphogenic protein (BMP) 2, BMP-3, cathepsin B (CTSB), CTSD, collagen type I (Col1A1), collagen type III (Col3A1), Distal-less homeobox (DLX) 1, DLX5, fibroblast growth factor (FGF), transforming growth factor b1 (TGFb1), receptor activator of nuclear factor kappa-B (RANK), osteopontin, osteonectin, and osteocalcin. The results for each experiment are from quadruplicate experiments. Values are expressed as the mean 6 SD. T tests were used to determine significant differences (p,0.05). * p,0,05; * * p,0,01; * * * p,0,001.

Perimplantitis and CGH analysis: chromosomal aberration

The results we obtained from this preliminary study of 20 patients with peri-implantitis are the following

CGH array has been performed on DNA derived from blood sample collected from 20 patients affected with perimplantitis.

The result obtained, reported in fig.17 show for all the patient affected by perim-plantitis, the presence of a duplication in omozygosis (red square) of a genome portion corresponding to the position at the chromosome 6, with starting point at 43846044 pb position, and stop point position at 43862079 pb, for a total of 16 kb ca.

DNA derived from 5 patient with implants and not affected by perimplantitis has been also analised by CGH array and this duplication has not been detected (Fig-

ure 31b). A detailed analyses of this genomic portion revealed that this position correspond to the exon 8 of VEGF gene.

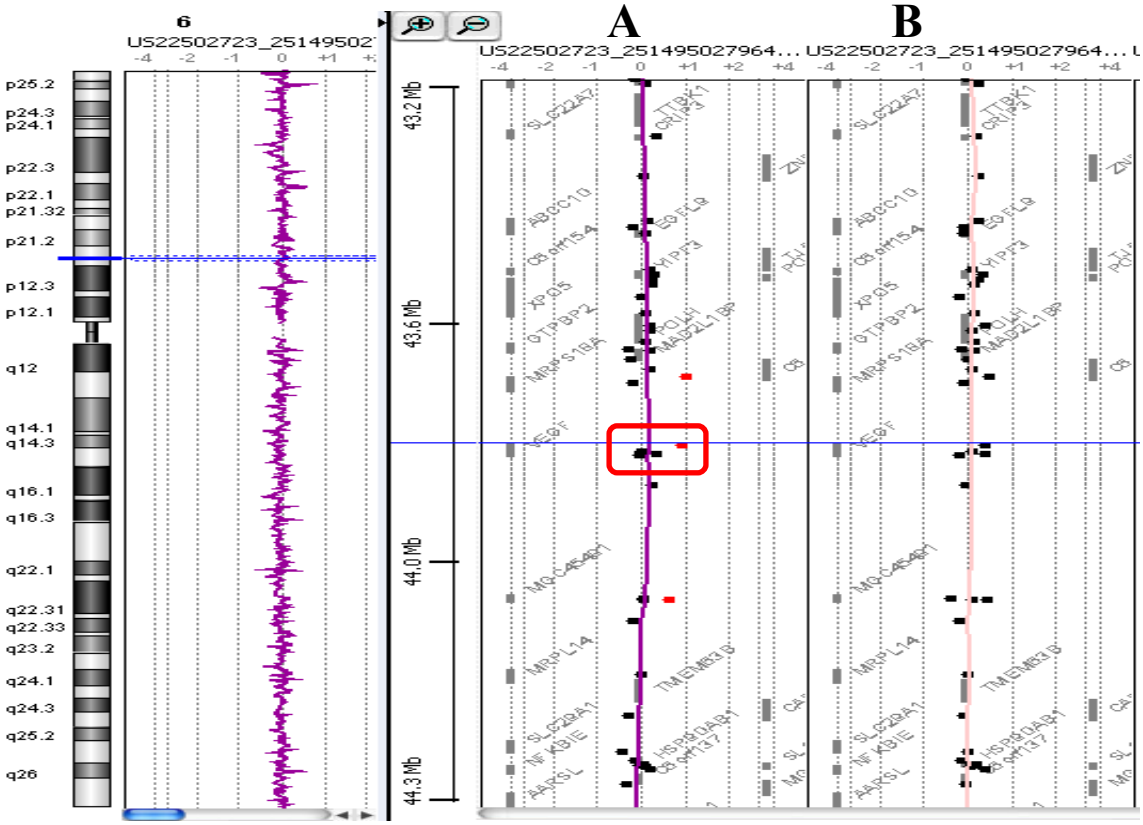


Figure 31: A: presence of a duplication in omozygosis (red square) of a genome portion corresponding to the position at the chromosome 6. B: duplication has not been detected

In Vivo

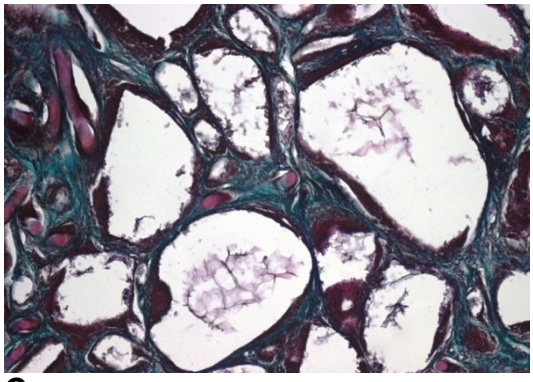
In vivo rat implantation of the construct with ADSCs

For the purpose of testing the osteo-regenerative capacilities of the ADSCs, *in vivo* experiments were carried out on immunocompromised Wistar rats. The experiments of implantation on the animals were preceded by the *in vitro* preparation of the constructs: the ADSCs were inoculated on the hydroxyapatite-based matrices with a density of 10^6 cells/cm^2 and were later incubated for 7 days with osteo-endothelial medium. These constructs were then implanted in

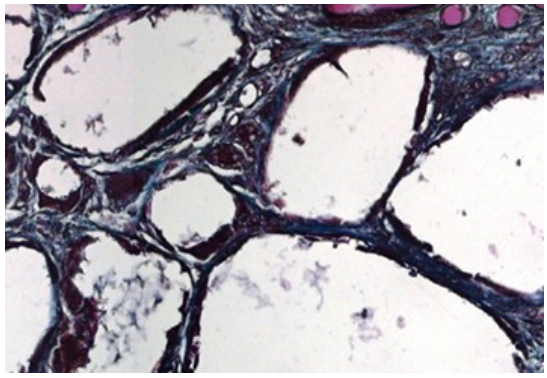
correspondence of lesions of the flat bones of the skull of immunocompromised rats. The experiments were carried out by comparing the constructs previously prepared *in vitro* (Figure 32b) with a control situation, that is to say, the hydroxyapatite-based matrices with no cells (Figure 32a).

Figure 32: *In vivo* experiments. Implantation of the hydroxyapatite-based matrix

A



B



C

with no cells (A) and of the hydroxyapatite-based matrix with ADSCs (B) in the flat bones of the rat skull. Goldner trichrome staining; 20x enlargements. In (C) genic expression analysis by Real-Time PCR of the hydroxyapatite-based matrix with no cells (white bars) and of the hydroxyapatite-based matrix with ADSCs (black bars).

Goldner trichrome staining performed on two different implants shows that both scaffolds are infiltrated by cells with the deposition of extracellular matrix, in particular of Type I Collagen. What distinguishes the two implants is the presence of vessels in correspondence of the hydroxyapatite-based support inoculated with ADSCs.

Genic expression experiments were carried out on the implants by Real-Time PCR to confirm the histological analysis (Figure 32c). The data collected show that the presence of ADSCs in the construct guarantees the presence of a population of cells from the osteogenic phenotype characterized by the expression of the

markers Osteopontin, Osteonectin, Osteocalcin, Type I Collagen and RUNX2 and of a population of endothelial cells that express the markers CD 32, vW and VEGF.

In vivo rat implantation of the construct with DPSCs

The bone regeneration activity of the DPSCs was assessed in vivo using a rat calvarial defect model (Figure 33a; c).

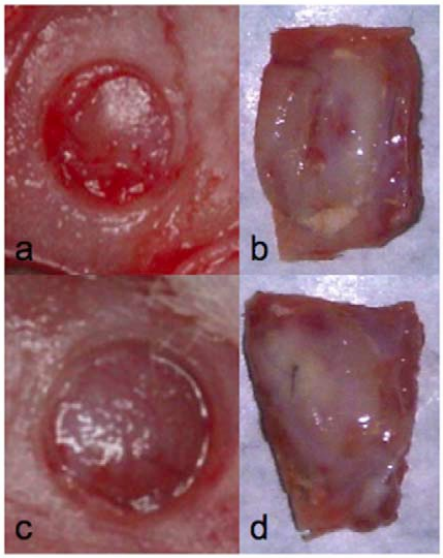


Figure 33: Critical size defect (A, C) before the treatment with DPSCs. Defect after treatment with DPSCs from the younger group (B) and older group (D).

We considered 2 different groups to ascertain the influence of undifferentiated DPSCs on new bone formation: DPSCs from the 16-25-year donor group (Figure 33b) and from the over-66 group at p5 (Figure 33d). As controls, HA granules without cells were used.

Concerning the cell populations filling the implants, it was evident that no inflammatory reaction around or inside the implant containing stem cells (Figure 34) from either donor age group occurred (Figure 34a from the younger donor group; Figure 34b from the senior donor group).

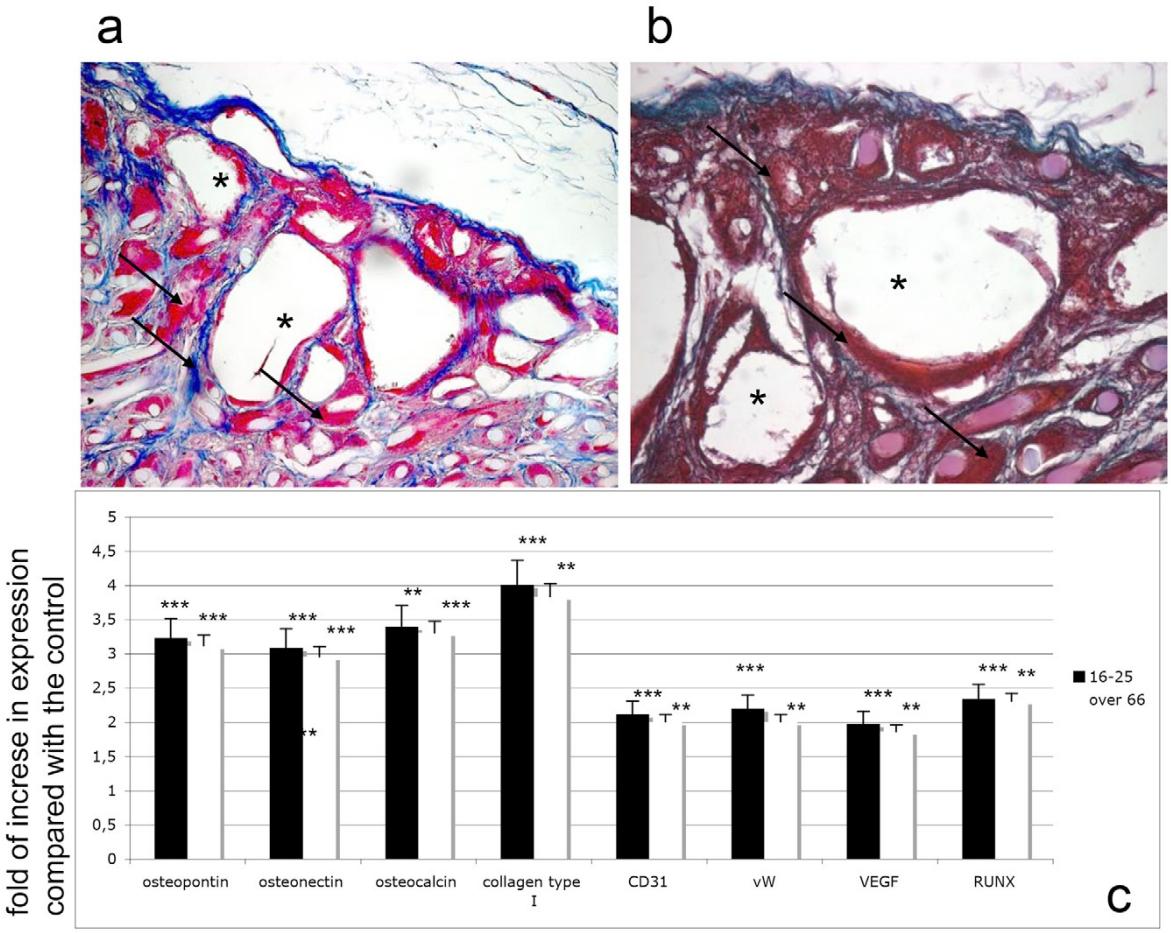


Figure 34: In vivo engraftment of HA nanostructured scaffolds (van Gieson staining, 206). (A) DPSCs from the younger group (16–25). (B) DPSCs from the senior group (.66). *, HA granules; black arrows, extracellular matrix. (C) Real-time PCR. Time course of osteogenic (osteopontin, osteonectin, osteocalcin, collagen type I, Runx2) and vasculogenic (CD31, vWF, VEGF) mRNA expression analyzed by semi-quantitative real-time PCR of HA nanostructured scaffolds in vivo embedded with DPSCs after 21 days. The results for each experiment are from quadruplicate experiments. Values are expressed as the mean 6 SD. T tests were used to determine significant differences (p,0.05). * p,0,05; * * p,0,01; * * * p,0,001.

In both implants enriched with stem cells, the HA nanostructured granules (Figure 34c *) were fully embedded with osteoblast-like cells capable of producing a good extracellular matrix consisting mainly of collagen type I, as revealed by van Gieson staining (Figure 34c, black arrows). A more detailed analysis of the cell population was performed using real-time PCR for osteogenic markers. As shown in Figure 33c, the presence of HA considerably improved the osteogenic population: the markers for osteopontin, osteonectin, osteocalcin, collagen type I, RUNX, VEGF, CD31, vWF, and vascular endothelial growth factor (VEGF) were more strongly expressed when cells where mixed with HA than in monolayer conditions from both donor age groups.

Sheep cellular response to scaffolds

Cellular events involved in bone regeneration are summarised in Table 6.

In normal regenerated tissue, the sites treated with HA and with HA with stem cells show at 15 days no infiltration of inflammatory cells population. There are no polymorphic nuclear cells (i.e. granulocytes), phagocytic cells (include macrophages and monocyte-derived giant cells) and non-phagocytic cells, plasma cells and mast cells. It should be noted that absolute numbers of PMNs and non-phagocytic cells are lower than for phagocytic cells. Even if the sites without stem cells show a presence of fibroblasts, endothelial cells and type I collagen, sites treated with stem cells exhibit a greater number of these cells.

At day 30 with HA scaffolds alone a moderate presence of fibroblast, endothelial cells and collagen fibers is observable, while higher presence of all cells types and of a extracellular matrix well structured is revealed in presence of stem cells. (Table 6)

Table 6: Cells were scored from not present (-) to abundantly present (+++)

Days after implantation	PMNs a	Phagocytic cellsb	Non-phagocytic cells	Fibroblasts	Endothelial cells	Collagen type I
15 days without stem cells	-	-	-	+	+	+
30 days without stem cells	-	-	-	++	++	++
15 days with stem cells	-	-	-	++	++	++
30 days with stem cells	-	-	-	+++	+++	+++

a PMNs = polymorphic nuclear cells, i.e. granulocytes
b Phagocytic cells include macrophages and monocyte-derived giant cells
c Non-phagocytic cells include lymphocytes, plasma cells and mast cells

At day 15, the presence of an inflammatory state created using TNFα (10 mM), without stem cells, showed high infiltration of granulocytes and macrophages. Scarce fibroblast, endtothelial cells and collagen fibers were observable in any site. Treatment with stem cells in presence of inflammed tissue showed a big

presence of inflammatory cells such as granulocytes and macrophages, scarce presence of fibroblast, and moderate quantity of endothelial cells and collagene type I fibers.

At day 30 the sites without stem cells showed a moderate amount of polymorphic nuclear cells (granulocytes), phagocytic cells (macrophages) and some non-phagocytic cells. There is also a moderate presence of fibroblasts, endothelial cells and collagen type I cells.

At day 30 the cellular response at the treatment with stem cells included a significative amount of endothelial cells, collagen type I cells and fibroblasts. Polymorphic nuclear cells, phagocytic cells and non-phagocytic cells were scarce. Collagen fibers were present overall in stem cells-treated tissue. (Table 7)

Table 7: Cells were scored from not present (-) to abundantly present (+++)
HA scaffold treated with TNFα (10 mM)

Days after implantation	PMNsa	Phagocytic cellsb	Non-phagocytic cells	Fibroblasts	Endothelial cells	Collagen type I
15 days without stem cells	+++	+++	++	+	+	+
30 days without stem cells	++	++	+	++	++	++
15 days with stem cells	+++	+++	++	+	++	++
30 days with stem cells	+	+	+	+++	+++	+++

In the site enriched with stem cells the HA granules were fully embedded with fibroblast-like cells. These cells are capable of producing a good extracellular matrix consisting mainly of collagen type I, as revealed by Alzan Mallory staining in blue (black arrows). Also significantly vessel (yellow arrows) could be found inside the scaffolds. (Figure 35, Figure 36) The presence of extracellular matrix and neo angiogenesis is crucial in the process of healing.

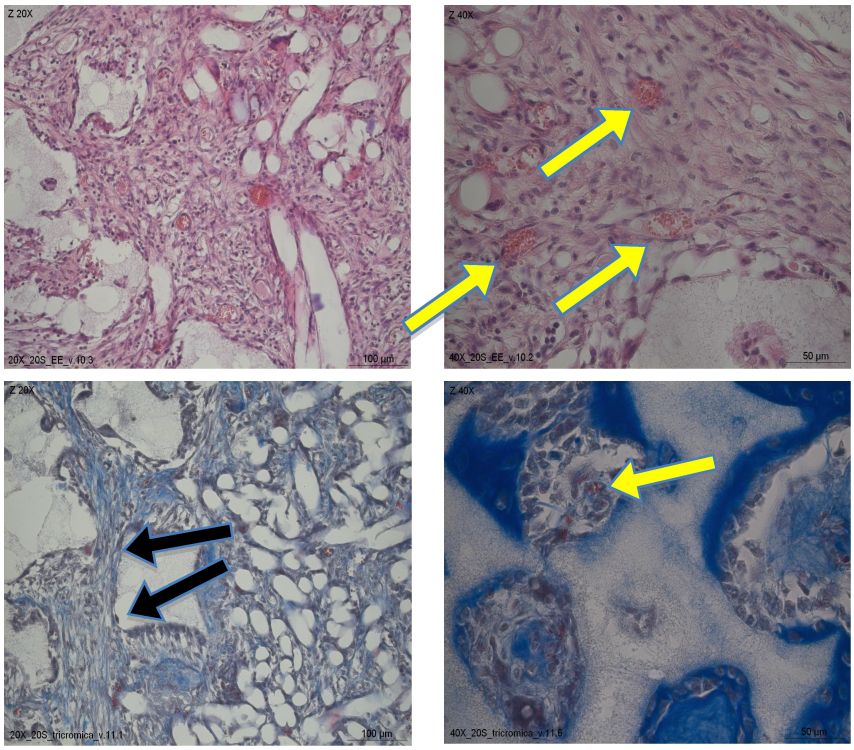


Figure 35: 15 days of healing. Site with HA scaffold treated with TNFα and stem cells. Upper images are stained with EE, lower with Alzan Mallory. Yellow arrows indicate vessels, black ones fibroblasts. In EE staining, dark dots represents inflammatory cells.

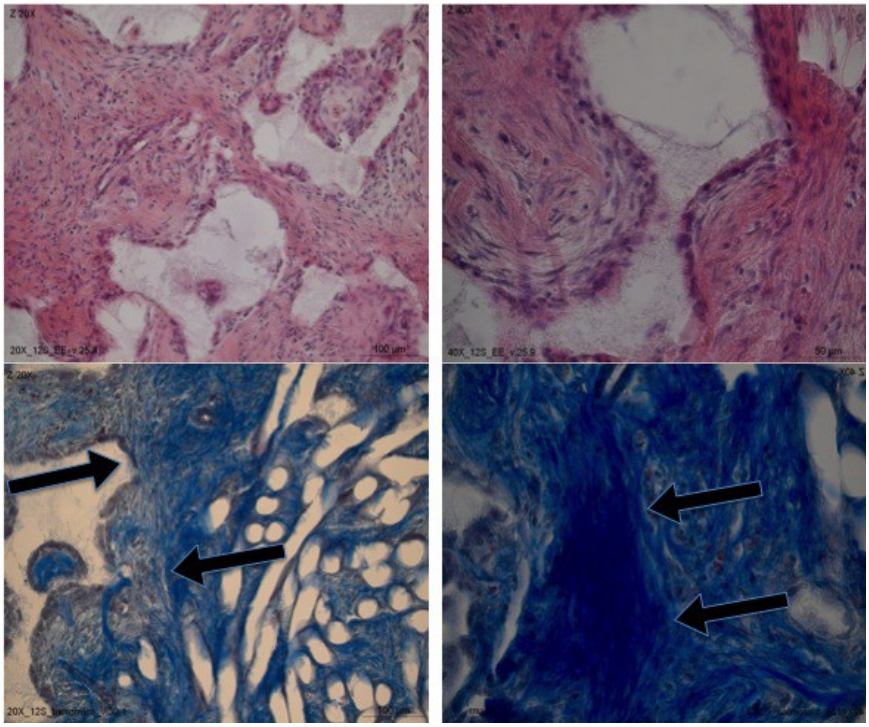


Figure 36: 30 days of healing. Site with HA scaffold treated with TNFα and stem cells. Upper images are stained with EE, lower with Alzan Mallory. Black arrows indicate extracellular matrix.

Dogs cellular response to scaffolds

Cellular events involved in bone regeneration have been analyzed with Stevenel's blue and alizarin red. These solutions stein in blue collagen fibers and in red mineralized tissues. (Figure 37)

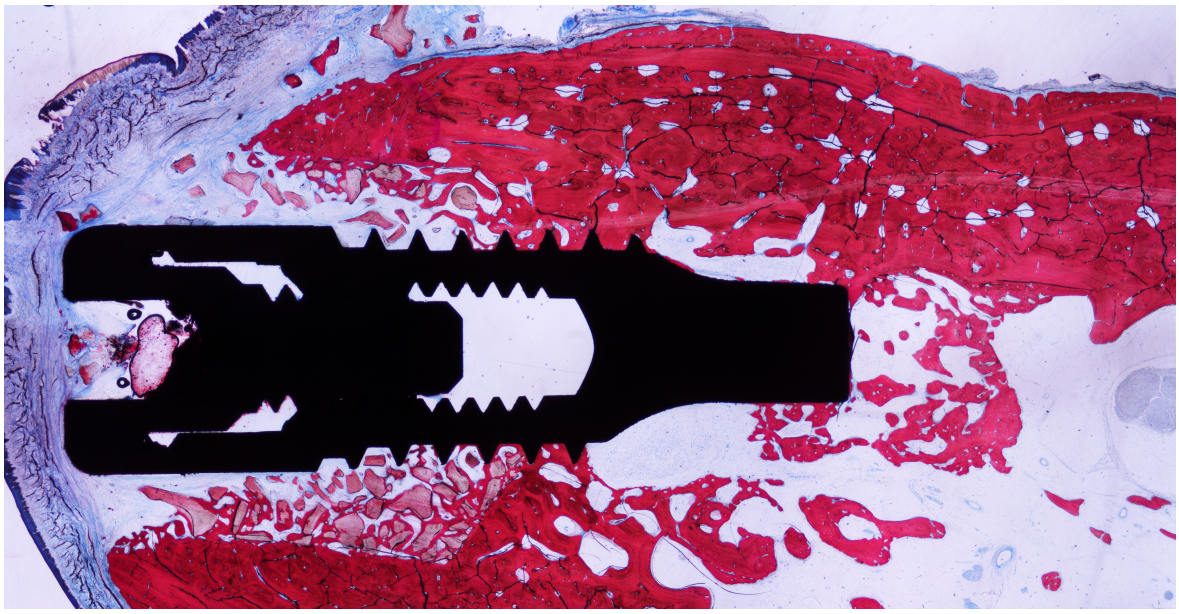


Figure 37: Specimen 30 days after dental implant insertion. This preparation was obtained with Stevenel's blue and alizarin red staining. Note the marginal gap occurred around the implant filled with HA scaffold.

Histological specimen represented in (Figure 38 Figure 39) is a sample of healing 30 days after fixture insertion. The site was regenerated with HA scaffold with stem cells. It can be noticed in black a portion of fixture, in pink the HA scaffold, in red the mineralized tissues and in blue collagen fibers.

In the site enriched with stem cells the HA granules were fully embedded with fibroblast-like cells capable of producing a good extracellular matrix consisting mainly of collagen type I, and properly mineralized tissue. No inflammatory infiltrates are presents in the tissue. Significantly vessel (yellow arrows) could be found inside the scaffolds. Dental implant is well integrated with a good contact between new bone and implant surface. Where a large bone-to-implant contact

(BIC) is present, implant show a good stability and is considered osteointegrated.

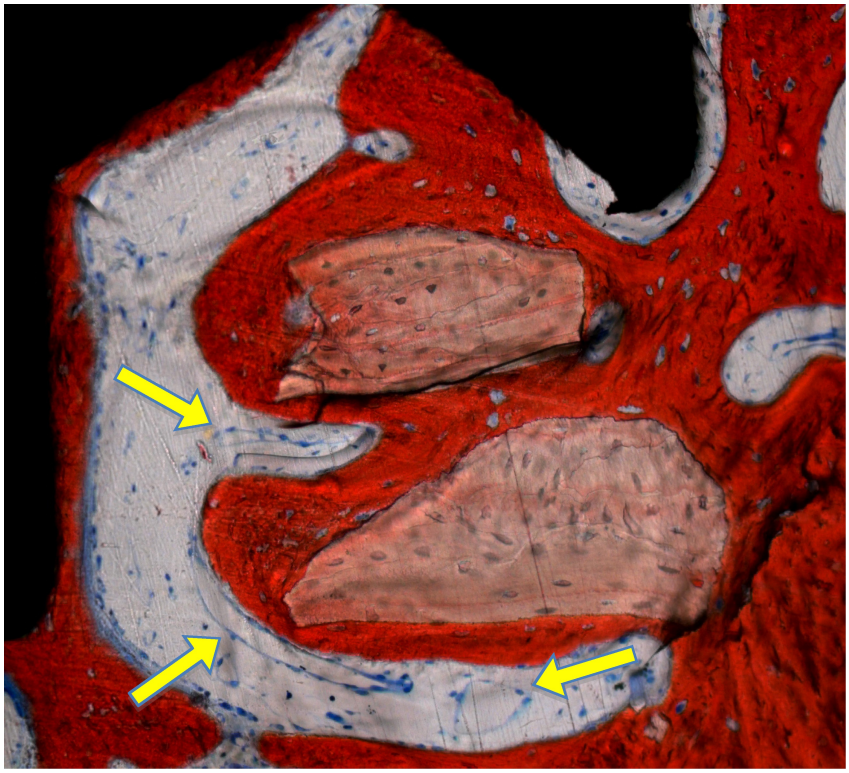


Figure 38: 30 days of healing. Stevenel's blue and alizarin red staining. The site was regenerated with HA scaffold with stem cells. Pink staining indicate the HA scaffold, red the mineralized tissues and in blue collagen fibers. Yellow arrows indicate vessels.

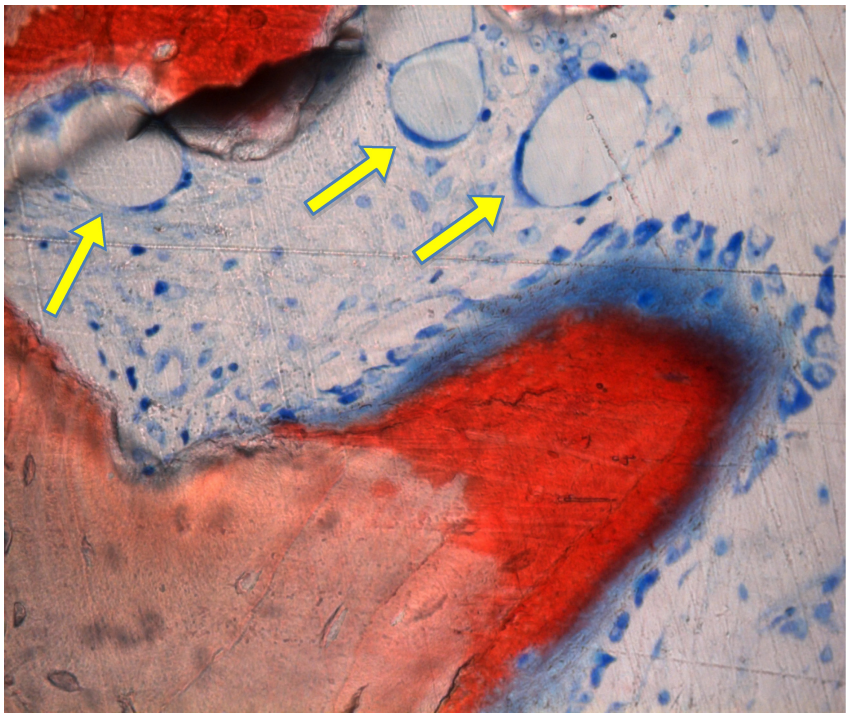


Figure 39: 30 days of healing. Stevenel's blue and alizarin red staining. The site was regenerated with HA scaffold with stem cells. Pink staining indicate the HA scaffold, red the mineralized tissues and in blue collagen fibers. Yellow arrows indicate vessels. Note the new bone formation.

Figure 39 shows a higher magnification (40x) the deposition of new bone matrix. There are numerous vessels that indicate a process of neo-angiogenesis and the scaffold of HA stained in pink closer with osteoblasts stained in blue. The red portion represent the new bone that is placed by osteoblasts directly in contact with the surface of the HA scaffolds.

At day 30 after surgery both sites with or without stem cells do not show polymorphic nuclear cells (i.e. granulocytes), phagocytic cells (include macrophages and monocyte-derived giant cells) and non-phagocytic cells, plasma cells and mast cells. It should be noted that absolute numbers of PMNs and non-phagocytic cells are lower than for phagocytic cells.

Site without stem cells show a scarce amount of fibroblasts, endothelial cells and collagen type I cells. Also the new bone formation is scarce. In site treated with HA scaffold with stem cells there is larger amount of endothelial cells, collagen type I cells and the new bone formation is more evident. (Table 8)

Table 8: Cells were scored from not present (-) to abundantly present (+++)

Days after implantation	PMNsa	Phagocytic cellsb	Non-phagocytic cellsc	Fibroblasts	Endothelial cells	Collagen type I	New bone
30 days without stem cells	-	-	-	+	+	+	+
30 days with stem cells	-	-	-	+	++	++	++

a PMNs = polymorphic nuclear cells, i.e. granulocytes
b Phagocytic cells include macrophages and monocyte-derived giant cells
c Non-phagocytic cells include lymphocytes, plasma cells and mast cells

Discussion

Injuries caused by trauma, edentulism condition, tumor or cyst resection, infectious diseases, extreme atrophy after tooth extractions may result into serious functional, aesthetical and psychological sequelae [Cohen 1995; Hunt and Hobar 2003].

In such situations, absence of hard and soft tissues can be disfiguring and often compromise basic functions such as : mastication, speech and also psychological conditions [Davis and Telischi 1994; Kadota et al. 2008; Curtis et al. 1997; Urken et al. 1991]. The progression of certain oral conditions may also result in craniofacial defects of difficult resolution. (Figure 40)

Periodontitis is a chronic inflammatory disease of bacterial etiology, characterized by the loss of support around teeth, including alveolar bone resorption and soft tissue alterations [Genco 1992; Kinane and Bartold 2007; Feng and Weinberg 2006].

Dental implant tooth replacements, one of the most popular therapies for total or partial edentulism, may be affected by a similar condition known as periimplantitis [Misch 2008]. Achieving predictable regeneration in the treatment of craniofacial defects is remarkably challenging in most clinical scenarios given the loss of structural support and different embryologic origins of the affected tissues, among other factors.

Autogenous tissues have been widely used and are still considered as the gold standard to which all other biomaterials are compared [Dimitriou et al. 2011a].

Nevertheless, even the most advanced reconstructive techniques using autologous materials are often insufficient to restore extensive or complex maxillofacial defects [Susarla et al. 2011].

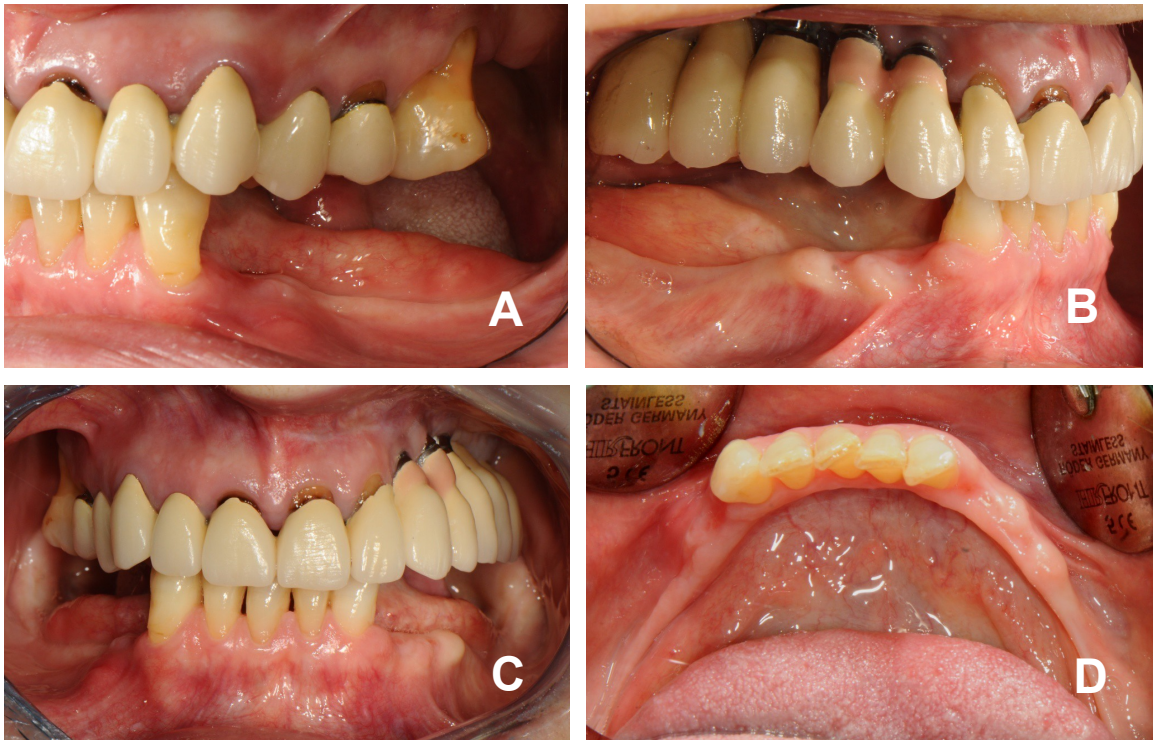


Figure 40: Severe atrophy in the posterior mandibular. Lateral view (A, B); frontal view (C); occlusal view (D)

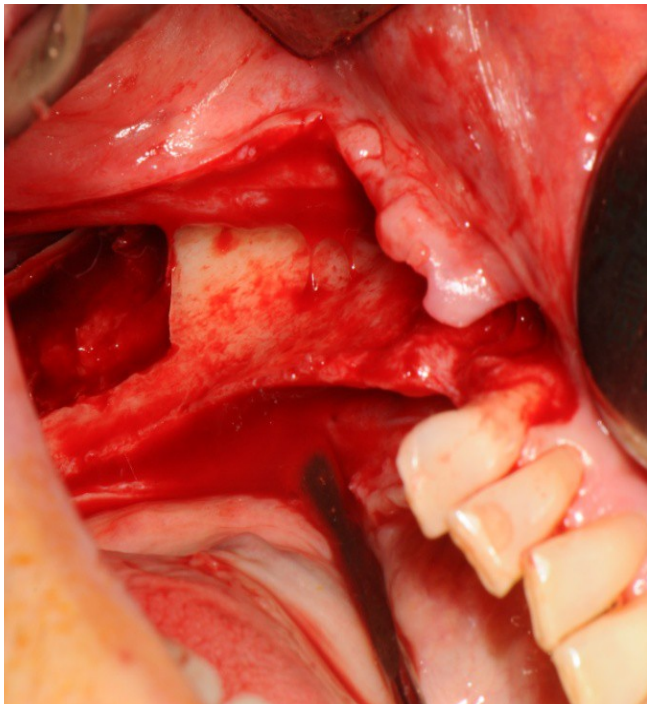


Figure 41: sample taking of bone from retromolar zone

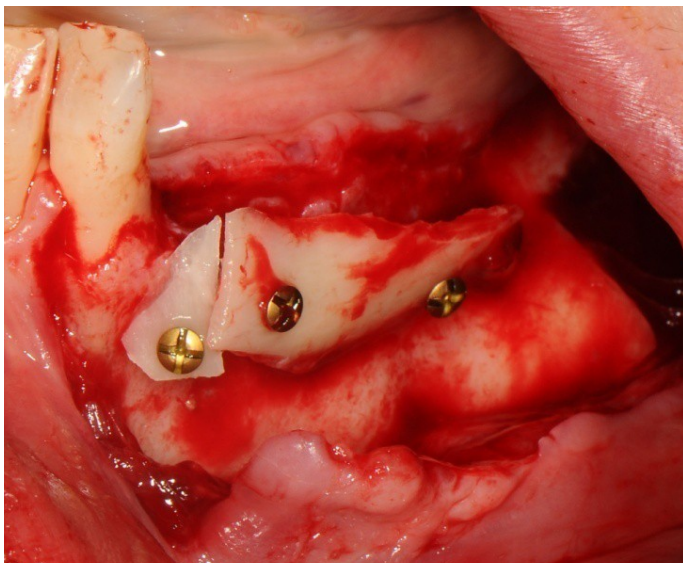


Figure 42: reconstructive techniques using autologous materials

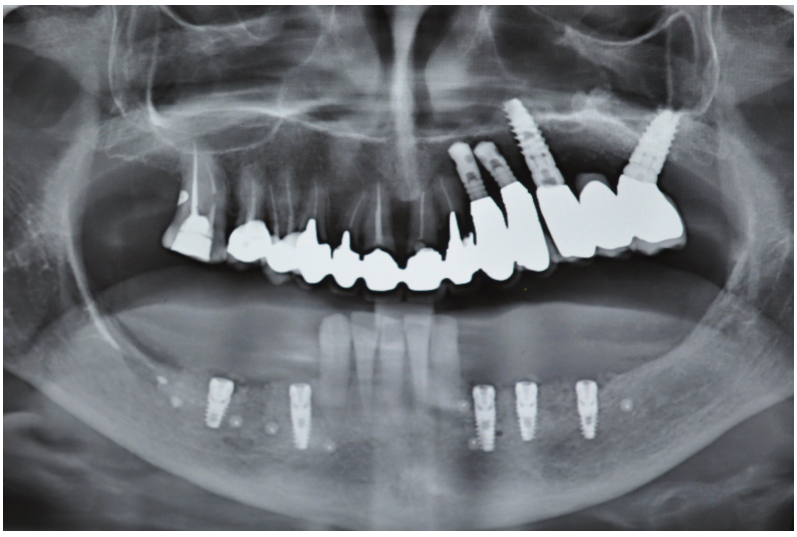


Figure 43: panoramic radiograph showing good bone regeneration and implant placement

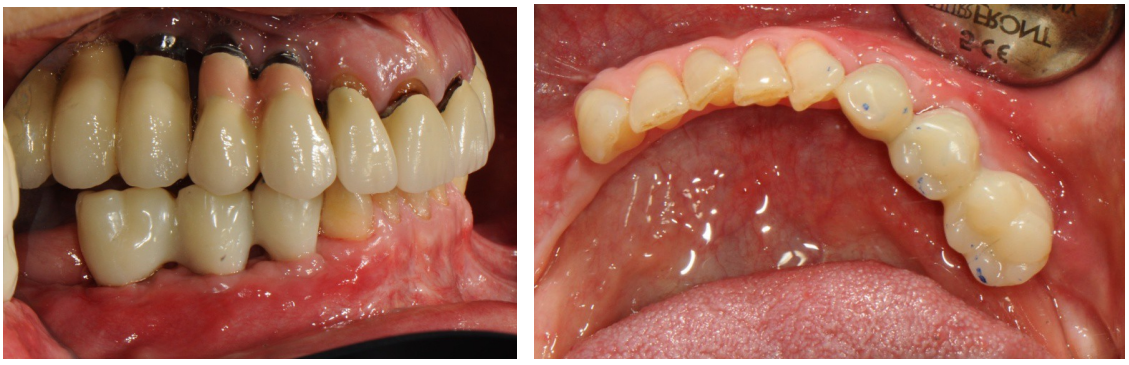


Figure 44: Implant prosthetic rehabilitation in the posterior region

Autografts contain all of the basic elements necessary to induce effective tissue regeneration, provided cells, extracellular matrix and cytokines [Pape et al. 2010; Khan et al. 2005]. However, the use of autogenous tissue involves the need of harvesting it from a donor site, with the consequent drawbacks in terms of costs, procedure time, patient discomfort and possible complications. (Figure 41,Figure 42,Figure 43, Figure 44)

Additionally, oftentimes the volume of harvested tissues is not sufficient to fill or cover a defect, given the limited availability of autogenous tissues [Dimitriou et al. 2011b; Zouhary 2010]. To overcome these limitations, a variety of exogenous substitute materials, including allografts, xenografts and alloplasts, have been introduced in clinical practice over the last three decades [Bauer and Muschler 2000; De Long et al. 2007]. These materials primarily act as scaffolds, supporting the migration of cells from the periphery of the grafted area. Substitutes are indicated in the treatment of cases where the application of autografts alone may not be possible [Finkemeier 2002]. Unfortunately, when comparing these biomaterials to autografts other limitations emerge. The presence of cellular populations, orchestrate the release of growth factors, maintenance of a stable scaffold, and stimulate angiogenesis and are key for successful tissue regeneration as they play a fundamental role on the healing process [Taba et al. 2005]. Controlling the dynamics of these elements allows for a more predictable treatment of challenging craniofacial defect.

Novel tissue engineering therapies aimed at enabling clinicians to achieve predictable regeneration have been recently developed.

The idea of using stem cells for therapeutic purposes has become genuinely feasible, though it is clear from the scientific evidence accumulated to date that more research is needed first because there are still numerous problems to solve. In particular, although embryonic stem cells have an unlimited potential for differentiation, their use is restricted for various reasons. There are not only ethical issues to consider that hinder their transplantation, but also rejection phenomena and the risk of generating teratomas [Yu and Thomson 2008].

These are some of the reasons why adult mesenchymal stem cells (MSC) have been used in the field of tissue engineering. MSCs form a population of stromal cells contained in the bone marrow and the majority of postnatal connective tissues. They are capable of differentiating not only into cells of mesenchymal derivation, but also into non-mesenchymal cell lines. In addition, cultured MSCs

have a marked proliferative capacity as well as retaining their potential for multilinear differentiation, and this makes them interesting candidates for tissue regeneration purposes [Augello and De Bari 2010]. Another favorable feature of MSCs lies in that they are readily available in postnatal tissues such as adipose tissue, and also – of more interest in dentistry – in dental pulp.

Having the chance to harvest stem cells from teeth and then use them for bone regeneration therapies or to control inflammatory phenomena such as peri-implantitis in the same patient is undeniably fascinating. This would enable us biologically to 'come full circle', so that patients can use their own cells to treat themselves.

However about the capacity of regeneration of bone tissue, it has an innate capacity.

It is generally by no means easy for it to heal spontaneously after major resections, severe trauma, fractures or tumors and, when it comes to the world of dentistry, tooth extractions are always followed by some degree of physiological alveolar bone resorption. Extracting a tooth entails the loss of the periodontal ligament and, with it, an associated loss of most of the blood flow to the bone tissue. Studies in vitro (Pietrusson) and on animal models (Lindhe), and clinical studies too (Lindhe, Caneva) have all demonstrated shrinkage of the hard and soft tissues after a tooth has been extracted. The resulting condition of bone atrophy can also be exacerbated by the presence of movable prosthetics that come to bear directly on the alveolar mucosa, giving rise to severe bone resorption. Dental implants are a good therapeutic solution for edentulous patients because they can be used to support movable prostheses, and by transferring the masticatory load directly to the bone, they reduce bone resorption phenomena. The amount of bone available is not always sufficient for the clinician to insert an implant, however, in which case it becomes necessary to resort to grafts of autologous or allogeneic bone tissue. Autotransplants and allotransplants have produced promising results, but their use is limited for various reasons, including a limited availability, donor site morbidity, long recovery times, and the risk of transmitting disease [De Long et al. 2007]

In recent years, the adoption of bone substitutes bio-engineered with mesenchymal stem cells (MSC) has developed into a promising alternative approach for the treatment of large bone defects without any of the side-effects associated with the more conventional therapies. The use of cellularized bone

substitutes is also restricted, however, because of their limited osteointegration, and this is due to the graft being poorly vascularized.

The cells in natural tissues are distributed so that they are never more than 200 nm away from a capillary, and this distance suffices to ensure the diffusion of oxygen, nutritional substances and waste. Once implanted, the cells of tissues engineered in the laboratory should likewise come to be no further away from the nearest capillary in order to guarantee their survival [Lovett et al. 2009]. Generally speaking, when bone substitutes are transplanted into the host bone they become vascularized as a consequence of the inflammatory response needed for the lesion to heal. The implanted cells' state of hypoxia can also facilitate the graft's vascularization through the release of angiogenic growth factors. This spontaneous vascularization is very limited, however, and the process is too slow to reach the innermost cells of the implant. The vascularization of bone replacement tissues thus remains a major obstacle to overcome before satisfactory clinical results can be obtained.

In the light of these considerations, an in vitro model of vascularized bone tissue was developed starting from MSCs harvested from adipose tissue (ADSCs). Adipose tissue is an interesting source of MSCs because it is available in abundance and readily accessible. In addition, it has been possible to obtain large numbers of cells in vitro in just a few culture steps, avoiding the risk of senescence and the onset of chromosomal anomalies. The multi-potency of ADSCs, thanks to their mesenchymal origin, has been amply demonstrated, and so has their capacity for osteogenic differentiation [Zuk et al. 2002]. ADSCs are also characterized by a particular plasticity towards the endothelial phenotype, a feature relating to the origin and physiology of adipose tissue. In fact, the development of adipose tissue correlates closely with the development of the capillary network essential to its maintenance: angiogenesis and adipogenesis are coordinated in time and space [Casteilla et al. 2011].

Given the above considerations, the properties of ADSCs have been exploited in vitro to reconstruct bone tissue with vascular elements for the purpose of facilitating cell survival after the graft has been implanted. ADSCs were seeded on hydroxyapatite-based matrices and grown in the presence of osteogenic and endothelial factors for 21 days. Morphological analyses, proliferation tests, gene expression analysis and molecular cytogenetic tests were completed every 7 days. Using SEM, the morphological analyses demonstrated a gradual proliferation of

the ADSCs also within the cavities in the biomaterial. After 21 days of culture in osteo-endothelial differentiating medium, the cells had formed a thin continuous layer over the surface of the biomaterial. The cells' proliferation on the scaffold was also confirmed by means of the MTT-dye viability test and quantification of the cells adhering to the matrices. The osteogenic and endothelial differentiation of the ADSCs was established by means of gene expression analyses using real-time PCR. Three differentiation methods were tested:

(a) Three-dimensional culture of ADSCs in the presence of osteogenic factors;

(b) Three-dimensional culture of ADSCs in the presence of endothelial factors;

(c) Three-dimensional culture of ADSCs in the presence of both factors.

Already after a week of culture on the three-dimensional support, osteogenic differentiation had given rise to the expression of the bone matrix components osteopontin, osteonectin, and osteocalcin that are fundamental to the interaction between the extracellular matrix and the cells, and essential for the mineralization of the matrix. The appropriate composition of the extracellular matrix was also confirmed by the expression of collagen type I, which is essential to the formation and maturation of hydroxyapatite crystals. Osteogenic differentiation was also ascertained by assessing the expression of the transcription factors RUNX2 and PPAR•. The differentiation of MSCs is governed by the expression and/or activation of these transcription factors: RUNX2 determines their differentiation into osteoblasts, while PPAR• prompts their differentiation into adipocytes. PPAR• also has an important role in bone metabolism [Viccica et al. 2010]. It facilitates adipogenesis at the expense of osteogenesis, particularly by inhibiting RUNX2 function and reducing the number of osteoblasts in the bone marrow. Vice versa, an increase in RUNX2 expression inhibits adipogenesis and favors osteogenesis [Kawai et al. 2009]. In the presence of osteogenic factors, three-dimensional cultures of ADSCs reveal a gradual increase in RUNX2 expression coinciding with a marked reduction in the expression of PPAR•, confirming that osteogenic differentiation occurs at the expense of adipogenic differentiation. On the other hand, the osteogenic and endothelial co-differentiation of the three-dimensional cultures of ADSCs prompted a faster increase in the expression of all the above-mentioned osteogenic markers, associated with a prolonged increase in the expression of the endothelial surface marker CD31, and of the soluble endothelial factors vW and VEGF. The three-dimensional co-differentiation thus led to the in vitro reconstruction of a cellularized bone substitute comprising an osseous

component and an endothelial component essential to the bone substitute's preservation and growth. After these co-differentiated constructs had been transplanted in vivo into immunocompromised rats, there was evidence of a proper capillary network having formed inside the material.

Scaffolds with and without osteogenic and endothelial differentiated cells were grafted on lesions prepared on flat cranial bones and, here again, morphological and molecular analyses conducted on the grafts 3 weeks after their implantation in vivo revealed the presence of both endothelial osteogenic cell lines within the grafts containing the stem cells.

For some years now, any transplantation of tissues bio-engineered in the laboratory in humans has been subject to validation to ensure that they are safe in terms of the absence of any genetic alterations. Their safety in this sense could be ascertained by validating their chromosomal stability by means of a karyotype analysis. This is one of the diagnostic tests most commonly used to identify chromosomal anomalies in the fields of oncology, gynecology and pediatrics. The method is based on the culture of a monolayer of cells and the subsequent analysis under the microscope of the cells in metaphase after treating them with a mitotic spindle blocker. This means that karyotyping is not applicable to three-dimensional cultures, and that is why the genetic stability of the osteo-endothelial constructs was analyzed using an innovative approach, called comparative genomic hybridization (CGH). This is a molecular cytogenetic technique based on the extraction of the cells' DNA and the subsequent identification of any variations in the number of gene pairs distributed throughout the genome. This method enables deletions and amplifications in the gene to be quantified with a greater resolution than karyotyping, accurately identifying the domains and the points where the regions involved in any imbalance start and end. CGH arrays demonstrated the genetic stability of the three-dimensional constructs, confirming that the long-term culture in the presence of differentiation factors is unable to induce structural DNA changes.

In conclusion, vascularized bone tissue was reconstructed in vitro by combining ADSCs with a hydroxyapatite scaffold. Tests demonstrated that the ADSCs simultaneously differentiated into endothelial cells and osteogenic cells inside the same three-dimensional support, facilitating angiogenesis in the construct after its in vivo implantation. The development of new vessels inside the graft is a process essential to the long-term survival of the implanted cells and to the

osteointegration of bio-engineered bone substitutes. CGH array analysis enabled us to establish the genetic safety of the construct for potential implantation in humans.

In the light of the findings of this study on bone reconstruction in vitro, the feasibility of bone regeneration and/or post-extraction alveolar preservation with the aid of stem cells and biomaterials would pave the way to new clinical strategies in the field of dentistry. From a social standpoint, this would be a great revolution for all the previously-mentioned biological reasons. More patients would be able to benefit from bone regeneration techniques, eliminating the problems relating to edentulism, and also those relating to bone harvesting from intra- or extra-oral sites and the associated morbidity. Last but not least, there would be advantages for both clinician and patient in terms of the timing and consequent costs of such treatments.

Regarding teeth istead, although they have complex structures that make them hard and strong, they are vulnerable to trauma and bacterial infections. When damaged teeth are still repairable, regenerating some parts of their structure may prevent or delay the loss of the whole tooth. This is important because tooth loss has more than just aesthetic consequences; it also affects the basal functions of the mouth and quality of life [Huang 2009]. How tooth loss caused by trauma, caries or periodontal disease can be regenerated depends on the compartment involved because a tooth consists of tissues rich in cells, such as the periodontal ligament, cement and pulp, and also noncellular tissues like the enamel and dentin. Of all the structural components of a tooth, only the enamel is incapable of regeneration; all the other tissues have a more or less marked capacity for regeneration, depending on a number of factors [Inanç and Elçin 2011].

When the structure of a tooth is damaged, the pulp has an important role in its regeneration, participating in a process called the reparative dentinogenesis. When the dental pulp is exposed due to the enamel and the overlying dentin being lost, coating it with a material containing calcium oxide enables the pulp to generate new dentin. The healthy pulp cells migrate to the site of the damage where they proliferate thanks to the presence of growth factors released into the surrounding dentin matrix, and they form osteodentin over the necrotic layer. The cells in the osteodentin subsequently differentiate into odontoblasts that synthesize the reparative dentin. This mineralized tissue serves two important purposes: it keeps the pulp intact and it acts as a protective barrier [Nakashima

2005].

Dentin regeneration is no longer possible, however, in cases of severe trauma, root fractures, or destructive caries, when it becomes necessary to either devitalize or extract the tooth. Since a vital dental pulp is fundamental to a tooth's homeostasis and longevity, in cases where destructive caries have led to dental pulp necrosis, the ideal treatment would be a regenerative approach involving the removal of the diseased tissues and necrotic pulp and their replacement with new pulp tissue in order to revitalize the tooth. The goal of such regenerative therapy on the dental pulp is to reconstitute healthy pulp tissue for placing in the area adjacent to the dentin in order to prompt a process of reparative dentinogenesis. Depending on the patient's clinical conditions, two types of dental pulp regeneration can be performed, i.e. a partial pulp regeneration in situ, or a total pulp replacement with de novo synthesized pulp [Sun et al. 2011].

Sadly, dental pulp tissue regeneration and engineering is still difficult to achieve. The tissue of an engineered pulp needs to be fully functionalized: it should be vascularized and innervated; it should have a cell density and extracellular matrix structurally similar to those of natural pulp; and it should be capable of generating new odontoblasts and new dentin. The first step in dental pulp regeneration involves the isolation and amplification of cells capable of differentiating and producing the above-mentioned structures. The use of dental pulp stem cells (DPSCs) has been suggested for this purpose, not only because they are the physiological constituents of dental pulp, but also for their mesenchymal origin and capacity to differentiate into odontoblasts [Gronthos et al. 2000] and neurons [d'Aquino et al. 2009].

In our study, as illustrated in the Materials and Methods section, and in the Results, we succeeded in isolating stem cells from dental pulp harvested from lower third molars. By means of enzymatic digestion, we isolated DPSCs from third molars extracted from donors of various ages to establish which age group enables large quantities of stem cells to be harvested in the shortest time possible. For this purpose, at various culture steps we calculated the population doubling time (PDT) for cells obtained from donors of various ages in the presence of non-differentiating medium.

The data obtained indicate that the DPSCs proliferate rapidly in the first culture steps irrespective of the donor's age, while there was evidence of the PDT values dropping with increasing amplification steps. Only the DPSCs isolated from donors

who were between 16 and 25 years old followed a different trend, maintaining a constant proliferation rate irrespective of the number of culture steps. Given this feature, DPSCs deriving from individuals belonging to this age group were used for the reconstruction in vitro of tooth-like tissue. Before making the construct, preliminary immunofluorescence and gene expression tests were conducted on monolayers of cells to assess the DPSCs' capacity for differentiation. They underwent glial, neuronal and endothelial differentiation, demonstrating the expression of the following markers: nestin, S100, CNPase and GFAP in the case of glial differentiation; nestin and ↕III tubulin in the case of neuronal differentiation; and CD31 and vW in the case of endothelial differentiation. Having ascertained the DPSCs' capacity for differentiation, we prepared three-dimensional constructs using a biomaterial obtained from the complete esterification of hyaluronic acid in order to mimic the fiber-rich structure of the extracellular matrix of dental pulp. These scaffolds were steeped in a mixture of glial and neuronal factors, then DPSCs were seeded on them in the presence of endothelial differentiating medium. After 14 days of differentiation, the constructs were analyzed using immunofluorescence after staining with hematoxylin and eosin, which revealed cells infiltrating the three-dimensional scaffold, the deposition of extracellular matrix (and collagen type I in particular), and the expression of GFAP, ↕ III tubulin and vW, which are markers of glial, neuronal and endothelial differentiation, respectively.

These results thus enabled us to identify a suitable source for obtaining large quantities of DPSCs within a short amount of time. Seeding these cells on a three-dimensional scaffold consisting of hyaluronic acid in the form of the nonwoven fabric - similar to the extracellular matrix of dental pulp - could provide a model for dental pulp regeneration. Moreover, the DPSCs' glial, neuronal and endothelial differentiation on these scaffolds makes the use of such substitutes promising for the regeneration of the nerve and vessel components of dental pulp.

These findings are not enough for any considerations on the efficacy of the model, however. Further analyses will need to be conducted to establish the utility of such a construct in the regeneration of dental pulp. Having said that, an important aspect to emphasize of our study conducted on dental pulp is that we succeeded in demonstrating that stem cells can be isolated not only from the pulp of patients under thirty, but also from that of adults between 60 and 70 years of age. We found the proliferative capacity of the latter's DPSCs lower than in the case of

younger individuals, but it is important to know that even older adults can obtain stem cells from their own dental pulp for bone regeneration purposes.

Another issue to bear in mind when it comes to isolating stem cells from dental pulp is the type of tooth involved. The most suitable teeth for this purpose are those chosen for the present study, i.e. third molars (whether they have erupted or not), because the amount of pulp that can be extracted from an incisor or premolar tooth is not enough to enable the isolation of an adequate quantity of stem cells.

The studies carried out on animal models have shown the potential of mesenchymal stem cells in the field of bone regeneration and of inflammation. Both in experiments on sheep and dogs, the presence of new blood vessels and extracellular matrix allows us to state how stem cells, with an appropriate scaffold, are able to promote bone regeneration faster.

From a clinical point of view the advantages are manifold such as fewer surgeries, elimination of sample taking from the donor site, and a faster regeneration.

From a point of view of inflammation, mesenchymal stem cells (MSCs) are known to migrate to tissue injury sites to participate in immune modulation, tissue remodeling and wound healing.

Indeed in dentistry, tissue engineering could be consider a new frontier in the regeneration of missing oral tissues/organs [Koyano 2012; Kaigler and Mooney 2001].

Our results are comparable with other reserch where stem cell-based tissue engineering has already been applied to clinical trials with demonstrated efficacy in orofacial bone tissue regeneration [Yamada et al. 2006; Ueda et al. 2008; Yamada et al. 2008; Kaigler et al. 2013].

Pieri et al. [Pieri et al. 2010] demonstrated that the transplantation of autologous ASCs with an inorganic bovine bone scaffold (Bio-Oss1) enhanced new bone formation and implant osseointegration following vertical bone augmentation of the calvarial bone of rabbits, which suggests that ASCs may be useful for vertical alveolar bone augmentation for implant treatment. [Wen et al. 2011].

Moreover, Ishizaka et al. [Ishizaka et al. 2012] demonstrated that ASC transplantation induced pulp regeneration in the root canal after pulpectomy in dogs, and Hung et al. [Hung et al. 2011] demonstrated that ASCs implants were able to grow self assembled new teeth containing dentin, periodontal ligament and alveolar bone in adult rabbit extraction sockets with a high success rate.

In addition to tissue repair and regeneration, immunomodulatory properties have

also recently been identified for MSCs in animals and humans that may be related to therapeutic effects such as angiogenesis, anti-inflammation and antiapoptosis [Nauta and Fibbe 2007]. Furthermore, recent reports suggest that MSCs have low inherent immunogenicity [Rasmusson et al. 2007]. Therefore, the immunomodulatory properties of MSCs may make them more attractive than other types of stem cells for some applications in cell transplantation.

In a recent review Ren et al. [Ren et al. 2012] underline that therapeutic mechanisms of MSCs include their homing efficiency to the tissue injury sites, their differentiation potential, their capability to produce a large amount of trophic factors, and their immunomodulatory effect. Because tissue damage sites are complicated milieus with distinct types of inflammatory cells and factors, available data have demonstrated that the properties of MSCs could be fundamentally influenced by the inflammatory elements.

The results obtained with the analysis of cgh for peri-implantitis are preliminary but are encouraging. We found a very interesting correspondence between duplication-DNA and the presence of peri-implantitis. Unfortunately, we know little about the early diagnosis of peri-implantitis. However, this might be the right way to find those predisposed to the peri-implant disease. More studies are needed to confirm our results.

In conclusion, research on all available stem cells in dentistry should be continued to permit their manipulation for the regeneration of oral tissues. Based on the accumulated knowledge, the type of stem cell to be used for a given application will be decided by considering a balance of the differentiation capacity with accessibility/availability, which may vary on a case-by-case basis.

Research efforts on adult stem cells and pluripotent stem cells should be concomitantly performed with cross-communication to permit the development of new and effective strategies for regenerative dentistry.

Bibliography

AA, S. 2002. The vertical heights of maxillary and mandibular bones in panoramic radiographs of dentate and edentulous subjects. *Quintessence international (Berlin, Germany : 1985) 33*, 6, 433–438.

Akizuki, T., Oda, S., Komaki, M., et al. 2005. Application of periodontal ligament cell sheet for periodontal regeneration: a pilot study in beagle dogs. *Journal of periodontal research 40*, 3, 245–251.

Araujo, M.G. and Lindhe, J. 2005. Dimensional ridge alterations following tooth extraction. An experimental study in the dog. *Journal of Clinical Periodontology 32*, 2, 212–218.

Augello, A. and De Bari, C. 2010. The regulation of differentiation in mesenchymal stem cells. *Human gene therapy 21*, 10, 1226–1238.

BA, D., S, T., Smith, V., et al. 2007. Trends in oral health status: United States, 1988-1994 and 1999-2004. *Vital and health statistics. Series 11, Data from the national health survey* 248, 1–92.

Balasundaram, G. and Webster, T.J. 2007. An overview of nano-polymers for orthopedic applications. *Macromolecular bioscience 7*, 5, 635–642.

Bauer, T.W. and Muschler, G.F. 2000. Bone graft materials. An overview of the basic science. *Clinical orthopaedics and related research* 371, 10–27.

Bei, M. and Maas, R. 1998. FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. *Development (Cambridge, England) 125*, 21, 4325–4333.

Bluteau, G., Luder, H.U., De Bari, C., and Mitsiadis, T.A. 2008. Stem cells for tooth engineering. *Eur Cell Mater*.

Boskey, A.L. 1998. Biomineralization: conflicts, challenges, and opportunities. *Journal of cellular biochemistry. Supplement 30-31*, 83–91.

Boskey, A.L. 2003. Biomineralization: an overview. *Connective tissue research 44 Suppl 1*, 5–9.

Brånemark, P.I., zarb, G.A., and Albrektsson, T. 1985. *Tissue integrated prostheses*. Quintessence Publishing (IL), Chicago.

Brennan, D.S., Spencer, A.J., and Roberts-Thomson, K.F. 2008. Tooth loss, chewing ability and quality of life. *Quality of life research : an international journal of quality of life aspects of treatment, care and rehabilitation 17*, 2, 227–235.

Bressan, E., Favero, V., Gardin, C., Ferroni, L., and Iacobellis, L. 2011. Biopolymers for hard and soft engineered tissues: application in odontoiatric and plastic surgery field. *Polymers*.

Bressan, E., Ferroni, L., Gardin, C., et al. 2012. Donor age-related biological properties of human dental pulp stem cells change in nanostructured scaffolds. *PloS one 7*, 11, e49146.

Brito, C., Tenenbaum, H.C., Wong, B.K.C., Schmitt, C., and Nogueira-Filho, G. 2013. Is keratinized mucosa indispensable to maintain peri-implant health? A systematic review of the literature. *Journal of biomedical materials research. Part B, Applied biomaterials*.

Brittberg, M., Lindahl, A., Nilsson, A., Ohlsson, C., Isaksson, O., and Peterson, L. 1994. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *The New England journal of medicine 331*, 14, 889–895.

Capitani, S., Comoglio, P., Molinaro, M., and Nussdorfer, G. 1996. *Trattato di Istologia*. McGraw Hill Professional.

Carossa, S., Bucca, C., De Lillo, A., and Corsalini, M. 2000. Correlation between edentulism, sleep disorders and arterial hypertension. Preliminary research]. *Minerva stomatologica 49*, 399–404.

Casteilla, L., Planat-Benard, V., Laharrague, P., and Cousin, B. 2011. Adipose-derived stromal cells: Their identity and uses in clinical trials, an update. *World journal of stem cells 3*, 4, 25–33.

Cecchinato, D., Parpaiola, A., and Lindhe, J. 2013. Mucosal inflammation and incidence of crestal bone loss among implant patients: a 10-year study. *Clinical Oral Implants Research*.

Chen, V.J., Smith, L.A., and Ma, P.X. 2006. Bone regeneration on computer-designed nano-fibrous scaffolds. *Biomaterials 27*, 21, 3973–3979.

Cohen. 1995. Perspectives on craniofacial asymmetry. III. Common and/or well-known causes of asymmetry. *International Journal of Oral and Maxillofacial Surgery 24*, 2, 7–7.

Cunha-Cruz, J., Hujoel, P.P., and Nadanovsky, P. 2007. Secular Trends in Socio-economic Disparities in Edentulism: USA, 1972-2001. *Journal of Dental Research 86*, 2, 131–136.

Curtis, D.A., Plesh, O., Miller, A.J., et al. 1997. A comparison of masticatory function in patients with or without reconstruction of the mandible. *Head & neck 19*, 4, 287–296.

d'Aquino, R., De Rosa, A., Laino, G., et al. 2009. Human dental pulp stem cells: from biology to clinical applications. *Journal of experimental zoology. Part B, Molecular and developmental evolution 312B*, 5, 408–415.

d'Aquino, R., Graziano, A., Sampaolesi, M., et al. 2007. Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. *Cell Death and Differentiation 14*, 6, 1162–1171.

Davis, R.E. and Telischi, F.F. 1994. Traumatic facial nerve injuries: review of diag-

nosis and treatment. *The Journal of cranio-maxillofacial trauma 1*, 3, 30–41.

De Long, W.G., Einhorn, T.A., Koval, K., et al. 2007. Bone grafts and bone graft substitutes in orthopaedic trauma surgery. A critical analysis. *The Journal of bone and joint surgery. American volume 89*, 3, 649–658.

Dennis, J.E., Carbillet, J.-P., Caplan, A.I., and Charbord, P. 2002. The STRO-1+ marrow cell population is multipotential. *Cells, tissues, organs 170*, 2-3, 73–82.

Dhandayuthapani, B., Krishnan, U.M., and Sethuraman, S. 2010. Fabrication and characterization of chitosan-gelatin blend nanofibers for skin tissue engineering. *Journal of Biomedical Materials Research*, n/a–n/a.

Dimitriou, R., Jones, E., McGonagle, D., and Giannoudis, P.V. 2011a. Bone regeneration: current concepts and future directions. *BMC medicine 9*, 66.

Dimitriou, R., Mataliotakis, G.I., Angoules, A.G., Kanakaris, N.K., and Giannoudis, P.V. 2011b. Complications following autologous bone graft harvesting from the iliac crest and using the RIA: A systematic review. *Injury 42*, S3–S15.

Dogan, A., Ozdemir, A., Kubar, A., and Oygür, T. 2002. Assessment of periodontal healing by seeding of fibroblast-like cells derived from regenerated periodontal ligament in artificial furcation defects in a dog: a pilot study. *Tissue Engineering (United States) 8*, 2, 273–282.

Dogan, A., Özdemir, A., Kubar, A., and Oygür, T. 2003. Healing of artificial fenestration defects by seeding of fibroblast-like cells derived from regenerated periodontal ligament in a dog: a preliminary study. *Tissue engineering*.

Dosunmu, O.O., Chase, G.G., Kataphinan, W., and Reneker, D.H. 2006. Electrospinning of polymer nanofibres from multiple jets on a porous tubular surface. *Nanotechnology 17*, 4, 1123–1127.

Douglass, C.W., Shih, A., and Ostry, L. 2002. Will there be a need for complete dentures in the United States in 2020? *The Journal of Prosthetic Dentistry 87*, 1, 5–8.

Dozin, B., Malpeli, M., Camardella, L., Cancedda, R., and Pietrangelo, A. 2002. Response of young, aged and osteoarthritic human articular chondrocytes to inflammatory cytokines: molecular and cellular aspects. *Matrix biology : journal of the International Society for Matrix Biology 21*, 5, 449–459.

Dozin, B., Malpeli, M., Cancedda, R., et al. 2005. Comparative evaluation of autologous chondrocyte implantation and mosaicplasty: a multicentered randomized clinical trial. *Clinical journal of sport medicine : official journal of the Canadian Academy of Sport Medicine 15*, 4, 220–226.

Elani, H.W., Harper, S., Allison, P.J., Bedos, C., and Kaufman, J.S. 2012. Socio-economic inequalities and oral health in Canada and the United States. *Journal of Dental Research 91*, 9, 865–870.

Elisseeff, J., McIntosh, W., Fu, K., Blunk, B.T., and Langer, R. 2001. Controlled-release of IGF-I and TGF-beta1 in a photopolymerizing hydrogel for cartilage tissue engineering. *Journal of orthopaedic research : official publication*

of the Orthopaedic Research Society 19, 6, 1098–1104.

Elsdale, T. and Bard, J. 1972. Collagen substrata for studies on cell behavior. *The Journal of cell biology 54*, 3, 626–637.

Elter, J.R., Offenbacher, S., Toole, J.F., and Beck, J.D. 2003. Relationship of periodontal disease and edentulism to stroke/TIA. *Journal of Dental Research 82*, 12, 998–1001.

Emingil, G., Buduneli, E., Aliyev, A., Akilli, A., and Atilla, G. 2000. Association between periodontal disease and acute myocardial infarction. *Journal of Periodontology 71*, 12, 1882–1886.

Esposito, M., Grusovin, M.G., and Worthington, H.V. 2011. Interventions for replacing missing teeth: treatment of peri-implantitis. *Cochrane database of systematic reviews 1*, CD004970–CD004970.

Esposito, M., Grusovin, M.G., Willings, M., Coulthard, P., and Worthington, H.V. 2007. The effectiveness of immediate, early, and conventional loading of dental implants: a Cochrane systematic review of randomized controlled clinical trials. *International Journal of Oral & Maxillofacial Implants 22*, 6, 893–904.

Feng, Z. and Weinberg, A. 2006. Role of bacteria in health and disease of periodontal tissues. *Periodontology 2000*.

Filho Cerruti, H., Kerkis, I., Kerkis, A., et al. 2007. Allogenous bone grafts improved by bone marrow stem cells and platelet growth factors: clinical case reports. *Artificial organs 31*, 4, 268–273.

Finkemeier, C.G. 2002. Bone-grafting and bone-graft substitutes. *The Journal of Bone & Joint Surgery*.

Fiske, J., Davis, D.M., Frances, C., and Gelbier, S. 1998. The emotional effects of tooth loss in edentulous people. *British Dental Journal 184*, 2, 90–93.

Foster, K., Greenhalgh, D., Gamelli, R.L., et al. 2008. Efficacy and safety of a fibrin sealant for adherence of autologous skin grafts to burn wounds: results of a phase 3 clinical study. *Journal of burn care & research : official publication of the American Burn Association 29*, 2, 293–303.

Francetti, L., Corbella, S., Taschieri, S., Cavalli, N., and Del Fabbro, M. 2013. Medium- and Long-Term Complications in Full-Arch Rehabilitations Supported by Upright and Tilted Implants. *Clinical Implant Dentistry and Related Research*, n/a–n/a.

Franco Lambert, A.P., Fraga Zandonai, A., Bonatto, D., Cantarelli Machado, D., and Pêgas Henriques, J.A. 2009. Differentiation of human adipose-derived adult stem cells into neuronal tissue: Does it work? *Differentiation 77*, 3, 221–228.

Friedenstein, A.J., Chailakhjan, R.K., and Lalykina, K.S. 1970. THE DEVELOPMENT OF FIBROBLAST COLONIES IN MONOLAYER CULTURES OF GUINEA-PIG BONE MARROW AND SPLEEN CELLS. *Cell Proliferation 3*, 4, 393–403.

Garcia-Godoy, F. and Murray, P.E. 2006. Status and potential commercial impact of stem cell-based treatments on dental and craniofacial regeneration. *Stem cells and development 15*, 6, 881–887.

Gardin, C., Bressan, E., Ferroni, L., et al. 2012a. In vitro concurrent endothelial and osteogenic commitment of adipose-derived stem cells and their genomical analyses through comparative genomic hybridization array: novel strategies to increase the successful engraftment of tissue-engineered bone grafts. *Stem cells and development 21*, 5, 767–777.

Gardin, C., Chiara, G., Ferroni, L., et al. 2012b. Nanostructured biomaterials for tissue engineered bone tissue reconstruction. *International journal of molecular sciences 13*, 1, 737–757.

Gardin, C., Vindigni, V., Bressan, E., and Ferroni, L. 2011. Hyaluronan and fibrin biomaterial as scaffolds for neuronal differentiation of adult stem cells derived from adipose tissue and skin. *International journal of ….*

Gault, P., Black, A., Romette, J.-L., et al. 2010. Tissue-engineered ligament: implant constructs for tooth replacement. *Journal of Clinical Periodontology 37*, 8, 750–758.

Genco, R.J. 1992. Host responses in periodontal diseases: current concepts. *Journal of Periodontology 63*, 4 Suppl, 338–355.

Genco, R.J. and Van Dyke, T.E. 2010. Prevention: Reducing the risk of CVD in patients with periodontitis. *Nature Reviews Cardiology 7*, 9, 479–480.

Gronthos, S., Mankani, M., Brahim, J., Robey, P.G., and Shi, S. 2000. 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 97*, 25, 13625–13630.

Gupte, M.J. and Ma, P.X. 2012. Nanofibrous scaffolds for dental and craniofacial applications. *Journal of Dental Research 91*, 3, 227–234.

Güler, A.U., Sumer, M., and Sumer, P. 2005. The evaluation of vertical heights of maxillary and mandibular bones and the location of anatomic landmarks in panoramic radiographs of edentulous patients for implant dentistry. *Journal of oral Rehabilitation 32*, 10, 741–746.

Hao, J., Ramachandran, A., and George, A. 2009. Temporal and spatial localization of the dentin matrix proteins during dentin biomineralization. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 57*, 3, 227–237.

Hay, E.D. 1991. Cell Biology of Extracellular Matrix.

Haynesworth, S.E., Baber, M.A., and Caplan, A.I. 1992. Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone 13*, 1, 69–80.

He, C., Xiao, G., Jin, X., Sun, C., and Ma, P.X. 2010. Electrodeposition on nanofibrous polymer scaffolds: Rapid mineralization, tunable calcium phosphate

composition and topography. *Advanced functional materials 20*, 20, 3568–3576.

HEALTH, S.C.F.N.P.F.O. 2001. *Oral health needs of Australians: national planning for oral health improvement, final report.* Australian Health Ministers' Advisory Council.

HELARY, C., ABED, A., MOSSER, G., LOUEDEC, L., MEDDAHI-PELLÉ, A., AND GIRAUD-GUILLE, M.M. 2011. Synthesis and in vivo integration of improved concentrated collagen hydrogels. *Journal of tissue engineering and regenerative medicine 5*, 3, 248–252.

HOTTA, T., YOKOO, S., TERASHI, H., AND KOMORI, T. 2007. Clinical and histopathological analysis of healing process of intraoral reconstruction with ex vivo produced oral mucosa equivalent. *The Kobe journal of medical sciences 53*, 1-2, 1–14.

HUANG, G. 2009. Pulp and dentin tissue engineering and regeneration: current progress. *Regenerative medicine*.

HUNG, C.-N., MAR, K., CHANG, H.-C., ET AL. 2011. A comparison between adipose tissue and dental pulp as sources of MSCs for tooth regeneration. *Biomaterials 32*, 29, 6995–7005.

HUNG, H.-C., JOSHIPURA, K.J., COLDITZ, G., ET AL. 2007. The Association Between Tooth Loss and Coronary Heart Disease in Men and Women. *Journal of Public Health Dentistry 64*, 4, 209–215.

HUNT, J.A. AND HOBAR, P.C. 2003. Common craniofacial anomalies: conditions of craniofacial atrophy/hypoplasia and neoplasia. *Plastic &amp Reconstructive Surgery 111*, 4, 1497–1410.

HUO, N., TANG, L., YANG, Z., ET AL. 2010. Differentiation of dermal multipotent cells into odontogenic lineage induced by embryonic and neonatal tooth germ cell-conditioned medium. *Stem cells and development 19*, 1, 93–104.

INANÇ, B. AND ELÇIN, Y.M. 2011. Stem cells in tooth tissue regeneration--challenges and limitations. *Stem cell reviews 7*, 3, 683–692.

IOHARA, K., ZHENG, L., ITO, M., TOMOKIYO, A., MATSUSHITA, K., AND NAKASHIMA, M. 2006. Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis. *Stem Cells 24*, 11, 2493–2503.

ISHIZAKA, R., IOHARA, K., MURAKAMI, M., FUKUTA, O., AND NAKASHIMA, M. 2012. Regeneration of dental pulp following pulpectomy by fractionated stem/progenitor cells from bone marrow and adipose tissue. *Biomaterials 33*, 7, 2109–2118.

IZUMI, K., FEINBERG, S.E., IIDA, A., AND YOSHIZAWA, M. 2003. Intraoral grafting of an ex vivo produced oral mucosa equivalent: a preliminary report. *International Journal of Oral and Maxillofacial Surgery 32*, 2, 188–197.

JAKOB, M., DÉMARTEAU, O., SCHÄFER, D., ET AL. 2001. Specific growth factors during the expansion and redifferentiation of adult human articular chondrocytes en-

hance chondrogenesis and cartilaginous tissue formation in vitro. *Journal of cellular biochemistry 81*, 2, 368–377.

Jia, H., Zhu, G., Vugrinovich, B., Kataphinan, W., Reneker, D.H., and Wang, P. 2002. Enzyme-carrying polymeric nanofibers prepared via electrospinning for use as unique biocatalysts. *Biotechnology progress 18*, 5, 1027–1032.

Jin, Q., Wei, G., Lin, Z., et al. 2008. Nanofibrous scaffolds incorporating PDGF-BB microspheres induce chemokine expression and tissue neogenesis in vivo. *PloS one 3*, 3, e1729.

Jin, Q.-M., Anusaksathien, O., Webb, S.A., Rutherford, R.B., and Giannobile, W.V. 2003a. Gene therapy of bone morphogenetic protein for periodontal tissue engineering. *Journal of Periodontology 74*, 2, 202–213.

Jin, Q.-M., Zhao, M., Webb, S.A., Berry, J.E., Somerman, M.J., and Giannobile, W.V. 2003b. Cementum engineering with three-dimensional polymer scaffolds. *Journal of biomedical materials research. Part A 67*, 1, 54–60.

Joshipura, K.J., Rimm, E.B., Douglass, C.W., Trichopoulos, D., Ascherio, A., and Willett, W.C. 1996a. Poor Oral Health and Coronary Heart Disease. *Journal of Dental Research 75*, 9, 1631–1636.

Joshipura, K.J., Willett, W.C., and Douglass, C.W. 1996b. The impact of edentulousness on food and nutrient intake. *Journal of the American Dental Association (1939) 127*, 4, 459–467.

Kadota, C., Sumita, Y.I., Wang, Y., et al. 2008. Comparison of food mixing ability among mandibulectomy patients. *Journal of oral Rehabilitation 35*, 6, 408–414.

Kahn, S.A., Beers, R.J., and Lentz, C.W. 2011. Use of acellular dermal replacement in reconstruction of nonhealing lower extremity wounds. *Journal of burn care & research : official publication of the American Burn Association 32*, 1, 124–128.

Kaigler, D. and Mooney, D. 2001. Tissue engineering's impact on dentistry. *Journal of dental education 65*, 5, 456–462.

Kaigler, D., Pagni, G., Galloro, A., Park, C.-H., Bartel, R.L., and Giannobile, W.V. 2010a. Acceleration of Human Oral Osseous Regeneration Using Bone Repair Cells.

Kaigler, D., Pagni, G., Park, C.-H., et al. 2013. Stem cell therapy for craniofacial bone regeneration: a randomized, controlled feasibility trial. *Cell transplantation 22*, 5, 767–777.

Kaigler, D., Pagni, G., Park, C.-H., Tarle, S.A., Bartel, R.L., and Giannobile, W.V. 2010b. Angiogenic and osteogenic potential of bone repair cells for craniofacial regeneration. *Tissue engineering. Part A 16*, 9, 2809–2820.

Kawaguchi, H., Hirachi, A., Hasegawa, N., et al. 2004. Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells. *Journal of Periodontology 75*, 9, 1281–1287.

Kawai, M., Devlin, M.J., and Rosen, C.J. 2009. Fat targets for skeletal health. *Nature Reviews: Rheumatology 5*, 7, 365–372.

Kelly, M., Steele, J., and Nuttall, N. 1998. Adult dental health survey. *Oral health in the United Kingdom 1998*.

Kern, S., Eichler, H., Stoeve, J., Klüter, H., and Bieback, K. 2006. Comparative Analysis of Mesenchymal Stem Cells from Bone Marrow, Umbilical Cord Blood, or Adipose Tissue. *Stem Cells 24*, 5, 1294–1301.

Kerr, E.N., Mealey, B.L., Noujeim, M.E., Lasho, D.J., Nummikoski, P.V., and Mellonig, J.T. 2008. The Effect of Ultrasound on Bone Dimensional Changes Following Extraction: A Pilot Study. *Journal of Periodontology 79*, 283–290.

Khan, S.N., Cammisa, F.P., Sandhu, H.S., Diwan, A.D., Girardi, F.P., and Lane, J.M. 2005. The biology of bone grafting. *Journal of the American Academy of Orthopaedic Surgeons*.

Kim, J.K., Baker, L.A., Seirawan, H., and Crimmins, E.M. 2012. Prevalence of oral health problems in U.S. adults, NHANES 1999-2004: exploring differences by age, education, and race/ethnicity. *Special Care in Dentistry 32*, 6, 234–241.

Kinane, D.F. and Bartold, P.M. 2007. Clinical relevance of the host responses of periodontitis. *Periodontology 2000 43*, 278–293.

Klinge, B., Meyle, J., and Meyle, J. 2012. Peri-implant tissue destruction. The Third EAO Consensus Conference 2012. *Clinical Oral Implants Research 23 Suppl 6*, 108–110.

Koyano, K. 2012. Toward a new era in prosthodontic medicine. *Journal of prosthodontic research 56*, 1, 1–2.

KRALL, E., HAYES, C., and GARCIA, R. 1998. How dentition status and masticatory function affect nutrient intake. *The Journal of the American Dental Association 129*, 1261–1269.

Krekeler, G., Schilli, W., and Diemer, J. 1985. Should the exit of the artificial abutment tooth be positioned in the region of the attached gingiva? *International Journal of Oral Surgery 14*, 6, 504–508.

Laino, G., d'Aquino, R., Graziano, A., et al. 2005. A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). *Journal of Bone and Mineral Research 20*, 8, 1394–1402.

Lang, N.P., Berglundh, T., Working Group 4 of Seventh European Workshop on Periodontology. 2011. Periimplant diseases: where are we now?--Consensus of the Seventh European Workshop on Periodontology. 178–181.

Lekic, P.C., Rajshankar, D., Chen, H., Tenenbaum, H., and McCulloch, C.A. 2001. Transplantation of labeled periodontal ligament cells promotes regeneration of alveolar bone. *The Anatomical record 262*, 2, 193–202.

Lévesque, J.-P., Takamatsu, Y., Nilsson, S.K., Haylock, D.N., and Simmons, P.J. 2001. Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil pro-

teases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor. *Blood 98*, 5, 1289–1297.

Li, H., Yan, F., Lei, L., Li, Y., and Xiao, Y. 2009. Application of autologous cryopreserved bone marrow mesenchymal stem cells for periodontal regeneration in dogs. *Cells, tissues, organs 190*, 2, 94–101.

Lindhe, J., Lang, N.P., and Karring, T. 2009a. Clinical periodontology and implant dentistry.

Lindhe, J., Lang, N.P., and Karring, T. 2009b. *Clinical Periodontology and Implant Dentistry*. John Wiley & Sons.

Liu, X. and Ma, P.X. 2004. Polymeric Scaffolds for Bone Tissue Engineering. *Annals of Biomedical Engineering 32*, 3, 477–486.

Liu, Y., Zheng, Y., Ding, G., et al. 2008. Periodontal ligament stem cell-mediated treatment for periodontitis in miniature swine. *Stem Cells 26*, 4, 1065–1073.

LOESCHE, W.J., SCHORK, A., TERPENNING, M.S., CHEN, Y.-M., DOMINGUEZ, B.L., and GROSSMAN, N. 1998. Assessing the Relationship Between Dental Disease and Coronary Heart Disease in Elderly U.S. Veterans. *The Journal of the American Dental Association 129*, 301–311.

Lops, D., Bressan, E., Pisoni, G., Cea, N., Corazza, B., and Romeo, E. 2012. Short implants in partially edentuolous maxillae and mandibles: a 10 to 20 years retrospective evaluation. *International journal of dentistry 2012*, 351793.

Lovett, M., Lee, K., Edwards, A., and Kaplan, D.L. 2009. Vascularization strategies for tissue engineering. *Tissue engineering. Part B, Reviews 15*, 3, 353–370.

Luan, X., Ito, Y., Dangaria, S., and Diekwisch, T.G.H. 2006. Dental follicle progenitor cell heterogeneity in the developing mouse periodontium. *Stem cells and development 15*, 4, 595–608.

Ma, J., He, X., and Jabbari, E. 2010. Osteogenic Differentiation of Marrow Stromal Cells on Random and Aligned Electrospun Poly(l-lactide) Nanofibers. *Annals of Biomedical Engineering 39*, 1, 14–25.

Ma, P.X. 2008. Biomimetic materials for tissue engineering. *Advanced Drug Delivery Reviews 60*, 2, 184–198.

Ma, P.X. and Zhang, R. 1999. Synthetic nano-scale fibrous extracellular matrix. *Journal of Biomedical Materials Research 46*, 1, 60–72.

Mao, J.J., Giannobile, W.V., Helms, J.A., et al. 2006. Craniofacial tissue engineering by stem cells. *Journal of Dental Research 85*, 11, 966–979.

Marcus, P.A., Joshi, A., Jones, J.A., and Morgano, S.M. 1996. Complete edentulism and denture use for elders in New England. *The Journal of Prosthetic Dentistry 76*, 3, 260–266.

Marei, M.K., Nouh, S.R., Saad, M.M., and Ismail, N.S. 2005. Preservation and regeneration of alveolar bone by tissue-engineered implants. *Tissue Engineering*

(United States) 11, 5-6, 751–767.

Marino, G., Rosso, F., Ferdinando, P., et al. 2012. Growth and endothelial differentiation of adipose stem cells on polycaprolactone. *Journal of biomedical materials research. Part A 100*, 3, 543–548.

Mariño, R., Albala, C., Sanchez, H., Cea, X., and Fuentes, A. 2013. Self-assessed oral-health status and quality of life of older Chilean. *Archives of gerontology and geriatrics 56*, 3, 513–517.

Matthews, J.A., Wnek, G.E., Simpson, D.G., and Bowlin, G.L. 2002. Electrospinning of collagen nanofibers. *Biomacromolecules 3*, 2, 232–238.

McGuire, M.K. and Nunn, M.E. 2005. Evaluation of the safety and efficacy of periodontal applications of a living tissue-engineered human fibroblast-derived dermal substitute. I. Comparison to the gingival autograft: a randomized controlled pilot study. *Journal of Periodontology 76*, 6, 867–880.

McGuire, M.K. and Scheyer, E.T. 2007. A Randomized, Double-Blind, Placebo-Controlled Study to Determine the Safety and Efficacy of Cultured and Expanded Autologous Fibroblast Injections for the Treatment of Interdental Papillary Insufficiency Associated With the Papilla Priming Procedure. *Journal of Periodontology 78*, 1, 4–17.

McGuire, M.K., Scheyer, E.T., Nevins, M.L., et al. 2011. Living Cellular Construct for Increasing the Width of Keratinized Gingiva: Results From a Randomized, Within-Patient, Controlled Trial. *Journal of Periodontology 82*, 10, 1414–1423.

McGuire, M.K., Scheyer, E.T., Nunn, M.E., and Lavin, P.T. 2008. A Pilot Study to Evaluate a Tissue-Engineered Bilayered Cell Therapy as an Alternative to Tissue From the Palate. *Journal of Periodontology 79*, 10, 1847–1856.

Min, S.-K., Lee, S.-C., Hong, S.-D., Chung, C.-P., Park, W.H., and Min, B.-M. 2010. The effect of a laminin-5-derived peptide coated onto chitin microfibers on re-epithelialization in early-stage wound healing. *Biomaterials 31*, 17, 4725–4730.

Misch, C.E. 2008. *Contemporary Implant Dentistry*. Mosby, St. Louis.

Mitchell, J., Bennett, K., and Brock-Martin, A. 2013. Edentulism in high poverty rural counties. *The Journal of rural health : official journal of the American Rural Health Association and the National Rural Health Care Association 29*, 1, 30–38.

Miura, M., Gronthos, S., Zhao, M., et al. 2003. SHED: stem cells from human exfoliated deciduous teeth. *Proceedings of the National Academy of Sciences of the United States of America 100*, 10, 5807–5812.

Mombelli, A. 1999. In vitro models of biological responses to implant microbiological models. *Advances in dental research 13*, 67–72.

Morelli, T., Neiva, R., Nevins, M.L., et al. 2011. Angiogenic biomarkers and healing of living cellular constructs. *Journal of Dental Research 90*, 4, 456–462.

Moroni, L., de Wijn, J.R., and van Blitterswijk, C.A. 2008. Integrating novel tech-

nologies to fabricate smart scaffolds. *Journal of biomaterials science. Polymer edition 19*, 5, 543–572.

Morsczeck, C., Götz, W., Schierholz, J., et al. 2005. Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biology 24*, 2, 155–165.

Moynihan, P. and Bradbury, J. 2001. Compromised dental function and nutrition. *Nutrition (Burbank, Los Angeles County, Calif.) 17*, 2, 177–178.

Musacchio, E., Perissinotto, E., Binotto, P., et al. 2007. Tooth loss in the elderly and its association with nutritional status, socio-economic and lifestyle factors. *Acta odontologica Scandinavica 65*, 2, 78–86.

Nakahara, T., Nakamura, T., Kobayashi, E., et al. 2004. In situ tissue engineering of periodontal tissues by seeding with periodontal ligament-derived cells. *Tissue Engineering (United States) 10*, 3-4, 537–544.

Nakanishi, Y., Izumi, K., Yoshizawa, M., Saito, C., Kawano, Y., and Maeda, T. 2007. The expression and production of vascular endothelial growth factor in oral mucosa equivalents. *International Journal of Oral and Maxillofacial Surgery 36*, 10, 928–933.

Nakashima, M. 2005. Bone morphogenetic proteins in dentin regeneration for potential use in endodontic therapy. *Cytokine & growth factor reviews 16*, 3, 369–376.

Nakashima, M., Iohara, K., and Sugiyama, M. 2009. Human dental pulp stem cells with highly angiogenic and neurogenic potential for possible use in pulp regeneration. *Cytokine & growth factor reviews 20*, 5-6, 435–440.

Nauta, A.J. and Fibbe, W.E. 2007. Immunomodulatory properties of mesenchymal stromal cells. *Blood 110*, 10, 3499–3506.

Oikarinen, K., Raustia, A.M., and Hartikainen, M. 1995. General and local contraindications for endosseal implants - an epidemiological panoramic radiograph study in 65-year-old subjects. *Community Dentistry and Oral Epidemiology 23*, 2, 114–118.

Paletta, J.R.J., Bockelmann, S., Walz, A., et al. 2009. RGD-functionalisation of PLLA nanofibers by surface coupling using plasma treatment: influence on stem cell differentiation. *J Mater Sci: Mater Med 21*, 4, 1363–1369.

Papadaki, E. and Anastassiadou, V. 2012. Elderly complete denture wearers: a social approach to tooth loss. *Gerodontology*.

Pape, H.C., Evans, A., and Kobbe, P. 2010. Autologous bone graft: properties and techniques. *Journal of orthopaedic trauma 24 Suppl 1*, S36–40.

Park, C.-H., Rios, H.F., Jin, Q., et al. 2012. Tissue engineering bone-ligament complexes using fiber-guiding scaffolds. *Biomaterials 33*, 1, 137–145.

Pieri, F., Lucarelli, E., Corinaldesi, G., et al. 2010. Dose-dependent effect of adipose-derived adult stem cells on vertical bone regeneration in rabbit calvarium.

Biomaterials 31, 13, 3527–3535.

Pontoriero, R., Tonelli, M.P., Carnevale, G., Mombelli, A., Nyman, S.R., and Lang, N.P. 1994. Experimentally induced peri-implant mucositis. A clinical study in humans. *Clinical Oral Implants Research 5*, 4, 254–259.

Pountos, I. and Giannoudis, P.V. 2005. Biology of mesenchymal stem cells. *Injury 36*, 3, S8–S12.

Pramstraller, M., Farina, R., Franceschetti, G., Pramstraller, C., and Trombelli, L. 2011. Ridge dimensions of the edentulous posterior maxilla: a retrospective analysis of a cohort of 127 patients using computerized tomography data. *Clinical Oral Implants Research 22*, 1, 54–61.

Quran, F., Clifford, T., Cooper, C., and Lamey, P.J. 2001. Influence of psychological factors on the acceptance of complete dentures - Quran - 2008 - Gerodontology - Wiley Online Library. *Gerodontology*.

Rasmusson, I., Uhlin, M., Le Blanc, K., and Levitsky, V. 2007. Mesenchymal stem cells fail to trigger effector functions of cytotoxic T lymphocytes. *Journal of leukocyte biology 82*, 4, 887–893.

Rastrelli, A., M, B., F, B., G, C., and A, P. 1990. Hyaluronic acid esters, a new class of semisynthetic biopolymer: chemical and physico-chemical properties. *Clinical Implant Material, Advances in Biomaterials 9*, 199–205.

Redford, M., Drury, T.F., Kingman, A., and Brown, L.J. 1996. Denture use and the technical quality of dental prostheses among persons 18-74 years of age: United States, 1988-1991. *Journal of Dental Research 75 Spec No*, 714–725.

Ren, G., Chen, X., Dong, F., et al. 2012. Concise review: mesenchymal stem cells and translational medicine: emerging issues. *Stem cells translational medicine 1*, 1, 51–58.

Rice, M.A., Dodson, B.T., Arthur, J.A., and Anseth, K.S. 2005. Cell-based therapies and tissue engineering. *Otolaryngologic clinics of North America 38*, 2, 199–214– v.

Roos-Jansåker, A.-M., Lindahl, C., Renvert, H., and Renvert, S. 2006. Nine- to fourteen-year follow-up of implant treatment. Part II: presence of peri-implant lesions. *Journal of Clinical Periodontology 33*, 4, 290–295.

Sanders, A.E., Slade, G.D., Carter, K.D., and Stewart, J.F. 2004. Trends in prevalence of complete tooth loss among Australians, 1979-2002. *Australian and New Zealand Journal of Public Health 28*, 6, 549–554.

Sánchez-Ayala, A., Lagravère, M.O., Gonçalves, T.M.S.V., Lucena, S.C., and Barbosa, C.M.R. 2010. Nutritional effects of implant therapy in edentulous patients--a systematic review. *Implant Dentistry 19*, 3, 196–207.

Schaefer, D., Martin, I., Jundt, G., et al. 2002. Tissue-engineered composites for the repair of large osteochondral defects. *Arthritis and rheumatism 46*, 9, 2524–2534.

Schropp, L.L., Wenzel, A.A., Kostopoulos, L.L., and Karring, T.T. 2003. Bone healing and soft tissue contour changes following single-tooth extraction: a clinical and radiographic 12-month prospective study. *The International journal of periodontics & restorative dentistry 23*, 4, 313–323.

Seo, B.-M., Miura, M., Gronthos, S., et al. 2004. Investigation of multipotent postnatal stem cells from human periodontal ligament. *The Lancet 364*, 9429, 149–155.

Sloan, A.J. and Waddington, R.J. 2009. Dental pulp stem cells: what, where, how? *International journal of paediatric dentistry / the British Paedodontic Society [and] the International Association of Dentistry for Children 19*, 1, 61–70.

Smith, I.O., Liu, X.H., Smith, L.A., and Ma, P.X. 2009. Nanostructured polymer scaffolds for tissue engineering and regenerative medicine. *Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology 1*, 2, 226–236.

Solchaga, L.A., Dennis, J.E., Goldberg, V.M., and Caplan, A.I. 1999. Hyaluronic acid-based polymers as cell carriers for tissue-engineered repair of bone and cartilage. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society 17*, 2, 205–213.

Soltan, M., Smiler, D., Prasad, H.S., and Rohrer, M.D. 2007. Bone block allograft impregnated with bone marrow aspirate. *Implant Dentistry 16*, 4, 329–339.

Song, J., Izumi, K., Lanigan, T., and Feinberg, S.E. 2004. Development and characterization of a canine oral mucosa equivalent in a serum-free environment. *Journal of biomedical materials research. Part A 71*, 1, 143–153.

Sonoyama, W., Liu, Y., Fang, D., et al. 2006. Mesenchymal Stem Cell-Mediated Functional Tooth Regeneration in Swine. *PloS one 1*, 1, e79.

Starr, J.M.J. and Hall, R.R. 2009. Predictors and correlates of edentulism in healthy older people. *Current Opinion in Clinical Nutrition and Metabolic Care 13*, 1, 19–23.

Sun, H.-H., Jin, T., Yu, Q., and Chen, F.-M. 2011. Biological approaches toward dental pulp regeneration by tissue engineering. *Journal of tissue engineering and regenerative medicine 5*, 4, e1–16.

Susarla, S.M.S., Swanson, E.E., and Gordon, C.R.C. 2011. Craniomaxillofacial reconstruction using allotransplantation and tissue engineering: challenges, opportunities, and potential synergy. *Annals of Plastic Surgery 67*, 6, 655–661.

Taba, M., Jin, Q., Sugai, J.V., and Giannobile, W.V. 2005. Current concepts in periodontal bioengineering. *Orthodontics and Craniofacial Research 8*, 4, 292–302.

Tallgren, A. 1972. The continuing reduction of the residual alveolar ridges in complete denture wearers: a mixed-longitudinal study covering 25 years. *The Journal of Prosthetic Dentistry 27*, 2, 120–132.

Thompson, D.F., Letassy, N.A., and Thompson, G.D. 1988. Fibrin glue: a review of its preparation, efficacy, and adverse effects as a topical hemostat. *Drug intelli-*

gence & clinical pharmacy 22, 12, 946–952.

THORSTENSSON, H. AND JOHANSSON, B. 2010. Why do some people lose teeth across their lifespan whereas others retain a functional dentition into very old age? *Gerodontology 27*, 1, 19–25.

TOBITA, M., UYSAL, A.C., OGAWA, R., HYAKUSOKU, H., AND MIZUNO, H. 2008. Periodontal tissue regeneration with adipose-derived stem cells. *Tissue engineering. Part A 14*, 6, 945–953.

TRAN, N. AND WEBSTER, T.J. 2009. Nanotechnology for bone materials. *Wiley Interdisciplinary Reviews - RNA 1*, 3, 336–351.

TSAKOS, G., HERRICK, K., SHEIHAM, A., AND WATT, R.G. 2010. Edentulism and fruit and vegetable intake in low-income adults. *Journal of Dental Research 89*, 5, 462–467.

UEDA, M., YAMADA, Y., KAGAMI, H., AND HIBI, H. 2008. Injectable bone applied for ridge augmentation and dental implant placement: human progress study. *Implant Dentistry 17*, 1, 82–90.

URKEN, M.L., BUCHBINDER, D., WEINBERG, H., ET AL. 1991. Functional evaluation following microvascular oromandibular reconstruction of the oral cancer patient: a comparative study of reconstructed and nonreconstructed patients. *The Laryngoscope 101*, 9, 935–950.

VICCICA, G., FRANCUCCI, C.M., AND MARCOCCI, C. 2010. The role of PPARγ for the osteoblastic differentiation. *Journal of endocrinological investigation 33*, 7 Suppl, 9–12.

VOLPONI, A.A., PANG, Y., AND SHARPE, P.T. 2010. Stem cell-based biological tooth repair and regeneration. *Trends in cell biology 20*, 12, 715–722.

WEBSTER, T.J., SCHADLER, L.S., SIEGEL, R.W., AND BIZIOS, R. 2001. Mechanisms of Enhanced Osteoblast Adhesion on Nanophase Alumina Involve Vitronectin. *Tissue engineering 7*, 3, 291–301.

WEI, G. AND MA, P.X. 2004. Structure and properties of nano-hydroxyapatite/polymer composite scaffolds for bone tissue engineering. *Biomaterials 25*, 19, 4749–4757.

WEI, G. AND MA, P.X. 2006. Macroporous and nanofibrous polymer scaffolds and polymer/bone-like apatite composite scaffolds generated by sugar spheres. *Journal of biomedical materials research. Part A 78*, 2, 306–315.

WEI, G., JIN, Q., GIANNOBILE, W.V., AND MA, P.X. 2007. The enhancement of osteogenesis by nano-fibrous scaffolds incorporating rhBMP-7 nanospheres. *Biomaterials 28*, 12, 2087–2096.

WEN, X., NIE, X., ZHANG, L., LIU, L., AND DENG, M. 2011. Adipose tissue-deprived stem cells acquire cementoblast features treated with dental follicle cell conditioned medium containing dentin non-collagenous proteins in vitro. *Biochemical and biophysical research communications 409*, 3, 583–589.

Whitesides, G.M., Mathias, J.P., and Seto, C.T. 1991. Molecular self-assembly and nanochemistry: a chemical strategy for the synthesis of nanostructures. *Science (New York, N.Y.) 254*, 5036, 1312–1319.

Woo, K.M., Chen, V.J., and Ma, P.X. 2003. Nano-fibrous scaffolding architecture selectively enhances protein adsorption contributing to cell attachment. *Journal of biomedical materials research. Part A 67*, 2, 531–537.

Woo, K.M., Jun, J.-H., Chen, V.J., et al. 2007. Nano-fibrous scaffolding promotes osteoblast differentiation and biomineralization. *Biomaterials 28*, 2, 335–343.

Wu, B., Liang, J., and Plassman, B.L. 2012. Edentulism trends among middle-aged and older adults in the United States: comparison of five racial/ethnic groups. *Community Dentistry and Oral Epidemiology 40*, 3, 145–153.

Xiaoxian Meng, Gilbert, G.H., Duncan, R.P., and Heft, M.W. 2007. Satisfaction With Dental Appearance Among Diverse Groups of Dentate Adults. *Journal of Aging and Health 19*, 5, 778–791.

Xie, J., Han, Z., Naito, M., et al. 2010. Articular cartilage tissue engineering based on a mechano-active scaffold made of poly(L-lactide-co-epsilon-caprolactone): In vivo performance in adult rabbits. *Journal of biomedical materials research. Part B, Applied biomaterials 94*, 1, 80–88.

Xie, Q., Närhi, T.O., Nevalainen, J.M., Wolf, J., and Ainamo, A. 1997. Oral status and prosthetic factors related to residual ridge resorption in elderly subjects. *Acta odontologica Scandinavica 55*, 5, 306–313.

Xu, Q., Izumi, K., Tobita, T., Nakanishi, Y., and Feinberg, S.E. 2009. Constitutive release of cytokines by human oral keratinocytes in an organotypic culture. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons 67*, 6, 1256–1264.

Yamada, Y., Nakamura, S., Ito, K., et al. 2008. Injectable tissue-engineered bone using autogenous bone marrow-derived stromal cells for maxillary sinus augmentation: clinical application report from a 2-6-year follow-up. *Tissue engineering. Part A 14*, 10, 1699–1707.

Yamada, Y., Ueda, M., Hibi, H., and Baba, S. 2006. A novel approach to periodontal tissue regeneration with mesenchymal stem cells and platelet-rich plasma using tissue engineering technology: A clinical case report. *The International journal of periodontics & restorative dentistry 26*, 4, 363–369.

Yamada, Y., Ueda, M., Hibi, H., and Nagasaka, T. 2004a. Translational research for injectable tissue-engineered bone regeneration using mesenchymal stem cells and platelet-rich plasma: from basic research to clinical case study. *Cell transplantation 13*, 4, 343–355.

Yamada, Y., Ueda, M., Naiki, T., and Nagasaka, T. 2004b. Tissue-engineered injectable bone regeneration for osseointegrated dental implants. *Clinical Oral Implants Research 15*, 5, 589–597.

Yamada, Y., Ueda, M., Naiki, T., Takahashi, M., Hata, K.-I., and Nagasaka, T. 2004c. Autogenous injectable bone for regeneration with mesenchymal stem cells and

platelet-rich plasma: tissue-engineered bone regeneration. *Tissue Engineering (United States) 10*, 5-6, 955–964.

YAN, M., YU, Y., ZHANG, G., TANG, C., AND YU, J. 2011. A journey from dental pulp stem cells to a bio-tooth. *Stem cell reviews 7*, 1, 161–171.

YANG, X., YANG, F., WALBOOMERS, X.F., BIAN, Z., FAN, M., AND JANSEN, J.A. 2010. The performance of dental pulp stem cells on nanofibrous PCL/gelatin/nHA scaffolds. *Journal of biomedical materials research. Part A 93*, 1, 247–257.

YEN, A.H.H. AND SHARPE, P.T. 2007. Stem cells and tooth tissue engineering. *Cell and Tissue Research 331*, 1, 359–372.

YOSHIBA, K., YOSHIBA, N., EJIRI, S., IWAKU, M., AND OZAWA, H. 2002. Odontoblast processes in human dentin revealed by fluorescence labeling and transmission electron microscopy. *Histochemistry and cell biology 118*, 3, 205–212.

YU, J. AND THOMSON, J.A. 2008. Pluripotent stem cell lines. *Genes & development 22*, 15, 1987–1997.

ZALLONE, A. 2007. *Il tessuto osseo. Il manuale di istologia*. Idelson-Gnocchi.

ZHANG, R. AND MA, P.X. 2000. Synthetic nano-fibrillar extracellular matrices with predesigned macroporous architectures. *Journal of Biomedical Materials Research 52*, 2, 430–438.

ZHANG, W., WALBOOMERS, X.F., VAN KUPPEVELT, T.H., ET AL. 2008. In vivo evaluation of human dental pulp stem cells differentiated towards multiple lineages. *Journal of tissue engineering and regenerative medicine 2*, 2-3, 117–125.

ZHAO, M., JIN, Q., BERRY, J.E., NOCITI, F.H., GIANNOBILE, W.V., AND SOMERMAN, M.J. 2004. Cementoblast delivery for periodontal tissue engineering. *Journal of Periodontology 75*, 1, 154–161.

ZHU, S., ZHANG, B., MAN, C., MA, Y., AND HU, J. 2011. NEL-like molecule-1-modified bone marrow mesenchymal stem cells/poly lactic-co-glycolic acid composite improves repair of large osteochondral defects in mandibular condyle. *Osteoarthritis and cartilage / OARS, Osteoarthritis Research Society 19*, 6, 743–750.

ZOUHARY, K.J. 2010. Bone graft harvesting from distant sites: concepts and techniques. *Oral and maxillofacial surgery clinics of North America 22*, 3, 301–16–v.

ZUK, P.A., ZHU, M., ASHJIAN, P., ET AL. 2002. Human Adipose Tissue Is a Source of Multipotent Stem Cells. *Molecular biology of the cell*.

ZUK, P.A., ZHU, M., MIZUNO, H., ET AL. 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Engineering (United States) 7*, 2, 211–228.