INVESTIGATIONS INTO ORTHODONTIC TOOTH MOVEMENT RATE

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INVESTIGATIONS INTO ORTHODONTIC TOOTH MOVEMENT RATE

Thesis submitted for the Degree of
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By

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Abstract

Orthodontic treatment is a lengthy process determined primarily by rate of tooth movement, which is controlled by the biological response to orthodontic force. An understanding of the clinical parameters and biological processes underlying orthodontic tooth movement (OTM) is an important foundation for investigations of orthodontic treatment time.

This thesis has systematically reviewed the evidence on rate of OTM and duration of treatment during the alignment and canine retraction phases of orthodontic treatment with fixed appliances; characterised the salivary peptidome and protease profile during the alignment stage of orthodontic treatment with fixed appliances; investigated the effect of adipokines on inflammation and extracellular matrix (ECM) remodelling biomarkers in compressed human periodontal and gingival fibroblasts in the presence or absence of inflammation; and assessed the effect of appointment interval (2 weeks versus 8 weeks) on duration and rate of orthodontic tooth alignment.

The systematic review and meta-analysis demonstrated that pooled duration for complete alignment of the mandibular dentition was 263.0 days, initial alignment of the mandibular incisor teeth was 100.7 days, and pooled average incisor irregularity changes were 2.9 mm (month 1), 1.5 mm (months 1-2), 0.7 mm (months 2-3), 0.3 mm (months 3-4), 0.3 mm (months 4-5), and 0.2 mm (months 5-6). These data were obtained from 35 randomised controlled trials (RCTs). Moreover, the estimated average pooled duration to achieve canine retraction was 4.98 months, pooled average canine retraction was 0.97 mm at months 0-1, 1.83 mm at months 0-2, 2.44 mm at months 0-3, 3.49 mm at months 0-4, and 4.25 mm at months 0-5. The data were obtained from 50 RCTs.

In the retrospective study, a total of 2852 naturally-occurring peptides were detected, deriving from 436 different proteins. Proteasix predicted 73 proteases potentially involved in generating these peptides, including metalloproteinases, cathepsins, and calpains. The tissue

culture study demonstrated that leptin showed pro-inflammatory properties by selectively enhancing IL-1α-induced expression of IL-6, IL-8, MMP-1, MMP-3, and MMP-8 in human periodontal and gingival fibroblasts in the presence or absence of compressive force. However, AdipoRon exhibited anti-inflammatory properties by attenuating these biomarkers under similar conditions.

The prospective RCT showed that patients reviewed every 2 weeks needed a mean of 168.5 days less than the 8-week group to achieve complete alignment. Moreover, a faster rate of OTM was observed within the 2-week group, with an overall rate of 0.07 mm per day compared to 0.03 mm per day in the 8-week group.

Patient- and treatment-related characteristics were associated with the reported rates of OTM. Surgically-assisted orthodontics was associated with a reduced duration of initial alignment and greater canine retraction. In addition, shorter intervals between appointments significantly reduced the time required to achieve complete alignment. Moreover, the rate of OTM depends on the extent of periodontal and gingival fibroblast-mediated ECM remodelling, which relies, in part, on the combination of orthodontic forces and inflammation. Finally, protease prediction from peptidome data demonstrates a potential tool for identifying novel biomarkers and discriminating between different approaches proposed to accelerate OTM.

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List of publications and presentations

Publications

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Fidaa Wazwaz, Hayder Saloom, Jack W Houghton., Martyn T. Cobourne, Guy H. Carpenter. 2023. Salivary peptidome analysis and protease prediction during orthodontic treatment with fixed appliances. Scientific Reports, 13, 677.

Presentations

Oral Presentation, 22nd Annual Postgraduate Research Day King's College London, 2nd June 2020, London, UK. The effect of adipokines on the secretion of matrix metalloproteinase-1 in human gingival fibroblasts.

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Abbreviations

µl:	microliter
°C:	Degrees centigrade
AKT:	Protein kinase B
ANOVA:	One-way analysis of variance
BCA:	Bicinchoninic acid
BMI:	body mass index
cDNA:	Complementary deoxyribose nucleic acid
CI:	Confidence interval
CGRP:	Calcitonin gene-related peptide
CLB:	Conventionally ligated bracket
DMEM	Dulbecco's modified eagles medium
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DTT:	Dithiothreitol
ECM:	Extra cellular matrix
EDTA:	Ethylenediaminetetraacetic Acid
ELISA:	Enzyme-linked immunosorbent assay
eNOS:	Endothelial nitric oxide synthase
EX:	Extraction
FBS:	Fetal bovine serum
GCF:	Gingival crevicular fluid
GRADE:	Grades of Recommendations, Assessment, Development and Evaluation
hGF:	Human gingival fibroblast
HIV:	Human immunodeficiency virus
hPDLF:	Human periodontal fibroblasts
IL:	Interleukin
iNOS:	Inducible nitric oxide synthase
KDa:	Kilo Dalton
LAFC:	Laser-assisted flapless corticotomy
LC-MS/MS:	Liquid chromatography tandem mass spectrometry
LDH:	Lactate dehydrogenase
LDS:	Lithium dodecyl sulphate

LII:	Little's irregularity index
LLLT:	Low level laser therapy
mA:	Milliamp
MD:	Mean difference
mg:	Milligram
ml:	Milliliter
mm:	Millimeter
mM:	Millimolar
MMP:	Matrix metalloproteinase
MOP:	Micro-osteoperforation
MTT:	3-(4,5- dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide
mRNA:	Messenger ribonucleic acid
mTOR:	Mammalian target of rapamycin
mTORC:	Mammalian target of rapamycin complex
NiTi:	Nickel titanium
nM:	Nanomolar
NO:	Nitric oxide
NSAID:	Non-steroidal anti-inflammatory drug
OPG:	Osteoprotegerin
OTM:	Orthodontic tooth movement
PBS:	Phosphate buffered saline
PDL:	Periodontal ligament
pg:	Picogram
PI3K:	Phosphoinositide 3-kinase
PMN:	Polymorphonuclear
RANK:	Receptor activator of nuclear factor kappa B
RANKL:	Receptor activator of nuclear factor kappa B ligand
RAP:	Regional acceleratory phenomenon
RCTs:	Randomised controlled trials
RNA:	Ribonucleic acid
RT-qPCR:	Quantitative real-time polymerase chain reaction
S6K1:	S6 kinase1
SD:	Standard Deviation
SDS:	Sodium dodecyl sulphate

SDS-PAGE:	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SS:	Stainless steel
TAD:	Temporary anchorage device
TBST:	Tris Buffered Saline with Tween
TIMP1:	Tissue inhibitor of metalloproteinase
TGF-β:	Transforming growth factor- β
TNF-α:	Tumour necrosis factor-α
TPA:	Transpalatal arch
VEGF:	Vascular endothelial growth factor
WMS:	Whole mouth saliva
4E-BP1:	Eukaryotic translation initiation factor 4E-binding protein 1

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Chapter 1 Introduction

Orthodontics is the field of dentistry concerned with growth and development of the jaws, face and dentition, the occlusion, and the diagnosis, interception, and treatment of occlusal anomalies. Orthodontic treatment aims to enhance dentofacial aesthetics and function of the teeth and jaws, improving a person's quality of life and self-esteem. It entails the treatment of malocclusion, caused by genetic and environmental factors or some combination of the two. This is accomplished by achieving ideal occlusion within the context of appropriate function and physiologic adaptation, acceptable dentofacial aesthetics and self-image, and stability. Fixed and removable appliances are used in conventional orthodontic treatment to accomplish a predetermined treatment plan (Turner et al., 2021, Graber et al., 2011).

Comprehensive orthodontic treatment with fixed orthodontic appliances generally takes less than two years to be completed (Tsichlaki et al., 2016), with a variety of factors impacting the outcome and duration. Accelerating OTM has long been desired by orthodontists and patients, to facilitate shorter treatment duration and reduced adverse effects such as pain, discomfort, periodontal diseases, dental caries, and iatrogenic damage such as root resorption. Therefore, clinicians have been increasingly pursuing innovative methods and approaches to accelerate OTM and potentially reduce treatment duration (Huang et al., 2014, Krishnan and Davidovitch, 2006, Mavreas and Athanasiou, 2008, Zainal Ariffin et al., 2011).

OTM represents the basis of orthodontic treatment and is triggered by orthodontic forces that induce stress and strain in the periodontal ligament, resulting in remodelling of the periodontal tissues and ultimately, tooth movement (Isola et al., 2016, Lindauer and Denis Britto, 2000).

1.1 Orthodontic tooth movement

Orthodontic tooth movement (OTM) denotes movement of the tooth within the alveolar bone as the result of biological reactions to an external stimulus that disrupts the physiologic equilibrium of the paradental tissues. This is accomplished by applying controlled mechanical forces to the teeth via orthodontic appliances leading to the remodelling of alveolar bone and the periodontal ligament (PDL), which ultimately leads to translocation and change of tooth position (Krishnan and Davidovitch, 2006). Tissue responses to orthodontic forces are best viewed as an exaggerated form of normal physiological remodelling associated with foci of tissue repair rather than a pathological condition (Meikle, 2006).

OTM comprises two connected processes: deflection of the alveolar bone and remodelling of the dental pulp, PDL, gingiva, and alveolar bone. The applied force compresses the alveolar bone and PDL on one side, while stretching the PDL on the opposing side (Dolce et al., 1996). Mechanical load is transmitted from the tooth to the alveolar bone through the PDL, causing immediate minor reversible injury to the paradental tissues and related vascular network (Davidovitch et al., 1988, Mostafa et al., 1991, Li et al., 2018, Wise and King, 2008). This leads to the release of numerous cytokines, growth factors, neurotransmitters, colony-stimulating factors and other molecules; these molecules produced elicit biological reactions in the numerous cell types in and around the tooth, creating a favourable milieu for tissue deposition or resorption (Krishnan and Davidovitch, 2006).

Typically, OTM occurs in four distinct phases: initial, lag, acceleration, and linear phases (Pilon et al., 1996). In the initial phase, the tooth's immediate and rapid displacement in the PDL space and tooth socket occurs, reducing PDL width on the compression side. This initial phase lasts twenty-four to forty-eight hours after orthodontic force application (Krishnan and Davidovitch, 2006, Zainal Ariffin et al., 2011). The lag phase follows, in which tooth

movement is halted for twenty to thirty days due to hyalinization of the PDL in regions of compression, and no further movement occurs until all necrotic tissues are removed by macrophages and multinucleated cells (Feller et al., 2015a, Zainal Ariffin et al., 2011). The movement then continues with a constantly increased rate in the acceleration phase through the alveolar bone in a continuous cycle of bone and PDL remodelling until the maximum capacity of these biological processes is reached. At this point, the linear phase follows, during which the rate of movement becomes constant and no longer depends on the magnitude of force applied (Pilon et al., 1996).

1.1.1 Biological response to orthodontic forces

Following orthodontic force application, two distinct areas in the PDL and associated alveolar bone have been identified. The orthodontic forces cause instantaneous strain in the periodontium, resulting in compression and tension zones of the PDL and leading to bone resorption and formation in these areas, respectively, which eventually results in tooth movement (Feller et al., 2015a, Davidovitch et al., 1988) (Figures 1.1 and 1.2).

1.1.1.1 Compression region

In the region of compression, disarrangement of adjacent tissues and gradual compression of the blood vessels occur. Compression of the vasculature results in localised ischemia, reduced blood flow and hypoxia, which influence the metabolic activity of PDL cells. Under these hypoxic conditions, the cells rely on anaerobic glycolysis, with many metabolic enzymes, such as lactate dehydrogenase, increasing during anaerobic metabolism (Kitase et al., 2009, Ren et al., 2008, Zainal Ariffin et al., 2011, Feller et al., 2015b).

Inflammation is induced by constriction of the PDL microvasculature, causing localised necrosis (hyalinisation) and compensatory hyperaemia in the surrounding PDL and vessels of the pulp. Hyalinisation is characterised by cell-free areas of the PDL, with collagen's normal tissue architecture and staining properties being lost in these sites. In hyalinisation zones, there are many cell pieces (debris), regions of degraded matrix interspersed between the intact collagen fibrils, and in certain cases, pyknotic nuclei (von Böhl et al., 2004, Zainal Ariffin et al., 2011, Bonafe-Oliveira et al., 2003, Krishnan and Davidovitch, 2006). These necrotic tissues release chemo-attractants, attracting giant phagocytic multinucleated cells, macrophages, and osteoclasts activated from the adjacent bone marrow spaces to the periphery of necrotic PDL, which resorb the hyalinised tissue of the PDL (Wise and King, 2008, Miyagawa et al., 2009, Ingman et al., 2012, Krishnan and Davidovitch, 2006).

Alveolar bone resorption occurs in the compression side, where osteoclasts attack the adjacent lamina dura, and frontal resorption of alveolar bone occurs, leading to tooth movement within two days following force application. This occurs when the applied orthodontic forces are light and within the accepted therapeutic range, resulting in negligible necrosis. Whereas, if orthodontic forces are excessive, osteoclasts remove the underside of the lamina dura of the alveolar bone adjacent to the hyalinized PDL via undermining resorption. This undermining resorption delays tooth movement and can cause root resorption. However, clinically, it is impossible to avoid PDL hyalinization; therefore, hyalinization is always present to some extent, and OTM is the result of both undermining and frontal resorption (Proffit et al., 2019, Isola et al., 2016, Miyagawa et al., 2009, Ingman et al., 2012, Li et al., 2018). Eventually, when the magnitude of force decreases, the compressed PDL returns to its original width, and neovascularization and regeneration of the injured PDL and alveolar bone ensue (Miyagawa et al., 2009, Feller et al., 2015b, Zainal Ariffin et al., 2011).

Pro-inflammatory cytokines that induce tissue resorption are released in compression areas. For example, prostaglandins, IL-1, IL-6, TNF-α, and receptor activator of nuclear factor kappa B ligand (RANKL) are all increased in the PDL during tooth movement (Yamaguchi and Kasai, 2005). In addition, rises in lysosomal enzymes, acid phosphatase, tartrate-resistant acid phosphatase, and cathepsin B are also found at compression sites, implying that they may play essential roles in the process of hard and soft tissue degradation by increased numbers of macrophage and dendritic-like cells during OTM (Yamaguchi et al., 2004, Keeling et al., 1993, Vandevska-Radunovic et al., 1997).

1.1.1.2 Tension region

On the tension side, where tooth displacement stretches the PDL fibres that attach the tooth to bone in the direction opposite to tooth movement, blood vessels are distended, and blood flow is maintained or increased, causing increased oxygen levels. These chemical changes lead to the differentiation of osteoblasts from local precursor cells (mesenchymal stem cells). Mature osteoblasts deposit osteoid tissue that subsequently undergoes mineralization. Bone deposition continues until the width of the PDL space has been restored to its normal limits (Zainal Ariffin et al., 2011, Ingman et al., 2012, Feller et al., 2015b, Li et al., 2018).

Several biomarkers have been shown to mediate bone formation in the tension sites, such as endothelial nitric oxide synthase (eNOS) and alkaline phosphatase; thereby, these biomarkers could help assess osteoblastic activity (Ariffin et al., 2010, Tan et al., 2009, Sprogar et al., 2008). Furthermore, tension forces considerably increase the expression of the osteogenic transcription factor Runx2 as well as the bone matrix proteins osteocalcin and osteopontin (Garlet et al., 2008, Garlet et al., 2007).

Tension sites have often been described as predominantly osteogenic, with no major inflammatory component. Nevertheless, there is evidence that inflammatory responses to tensile forces may depend on the stain magnitude. Low-magnitude tensile forces exhibit anti-inflammatory properties and induce magnitude-dependent anabolic signals in PDL cells (Long et al., 2001). On the other hand, high-magnitude tensile forces show pro-inflammatory properties, demonstrated by increasing the production of inflammatory cytokines (Long et al., 2002).

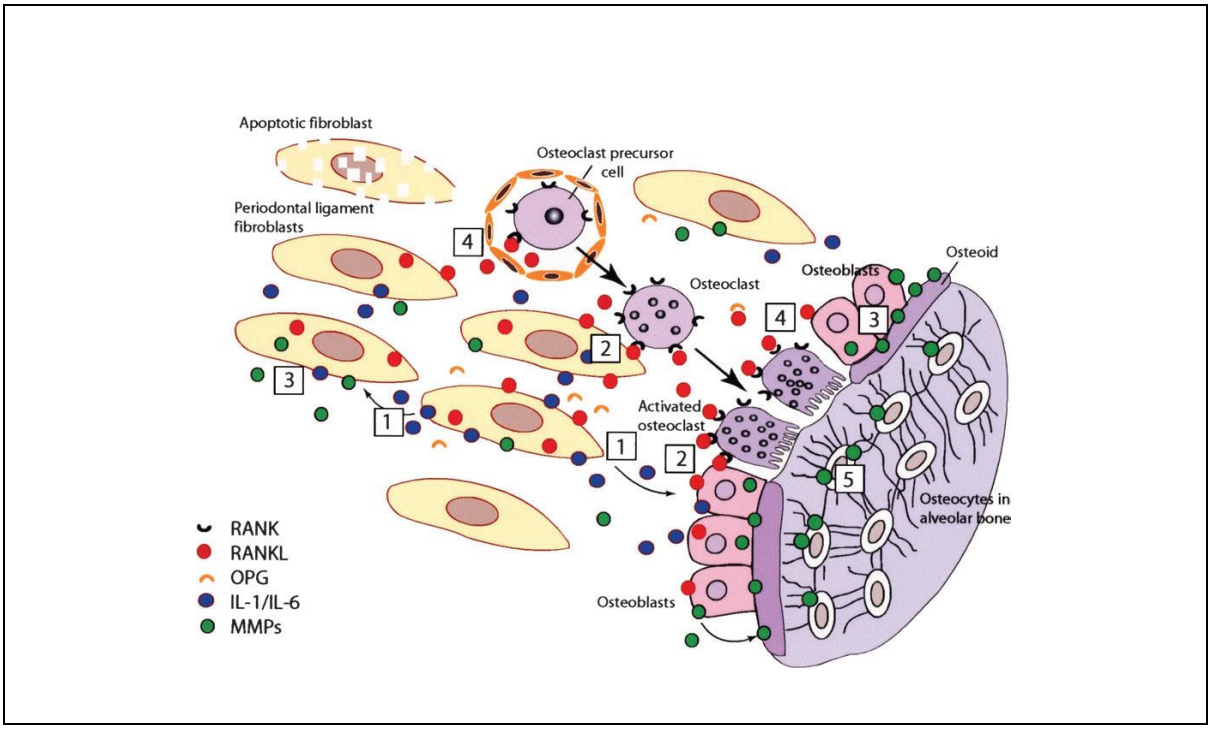

Figure 1.1 Biological response of the periodontium to orthodontic forces at compression region.

Periodontal ligament (PDL) cells produce interleukin-1 (IL-1) and IL-6 (1); IL-1 and IL-6 upregulate the production of receptor activator of nuclear factor kappa B ligand (RANKL) (2) and matrix metalloproteinases (MMPs) (3) by PDL cells and osteoblasts. RANKL stimulates the formation and activation of osteoclasts which degrade the mineralized part of alveolar bone (4). Osteocytes in alveolar bone express MMPs as a response to alveolar bone deformation (5). Adapted from (Meikle, 2006).

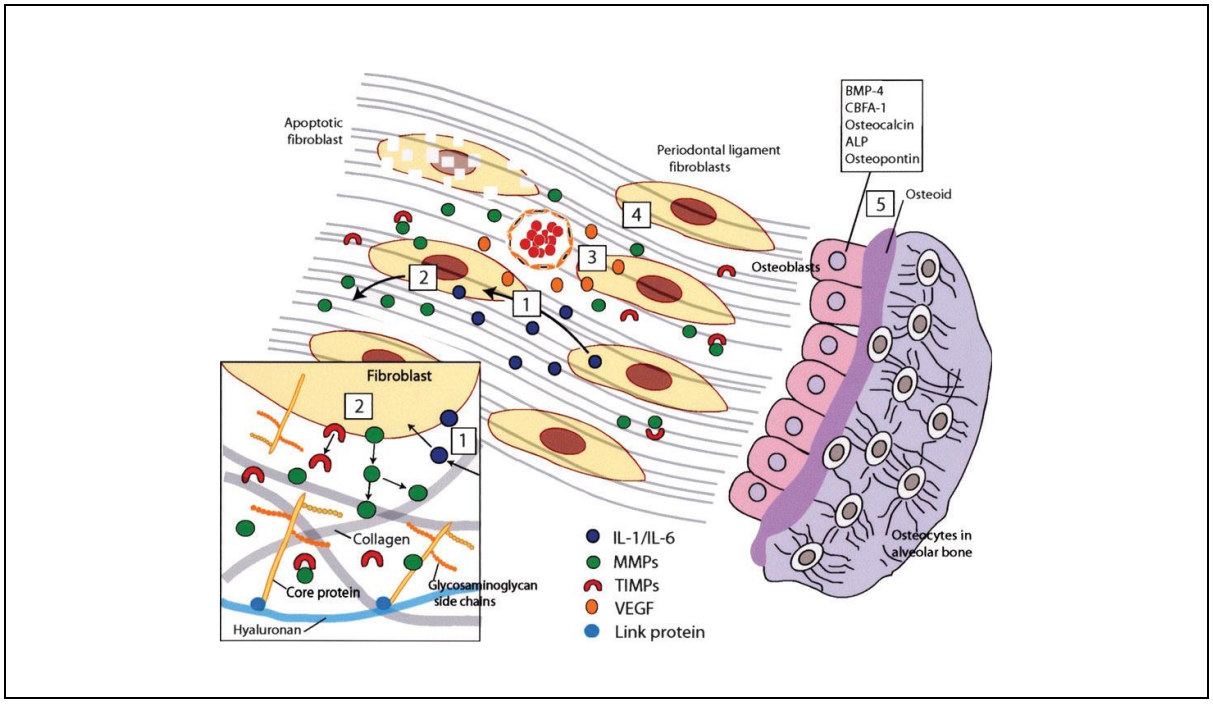

Figure 1.2 Biological response of the periodontium to orthodontic forces at the tension region.

PDL fibroblasts produce interleukin-1 (IL-1) and IL-6 (1); IL-1 and IL-6 induce matrix metalloproteinases (MMPs) and inhibit tissue inhibitor of metalloproteinases (TIMPs) production PDL cells (2); PDL fibroblasts synthesize vascular endothelial growth factor (VEGF) that stimulates angiogenesis (3); PDL cells (4), osteoblasts, and bone-lining cells undergo a biosynthetic phase in which structural and other matrix molecules are synthesised. Adapted from (Meikle, 2006).

1.1.2 The periodontium and OTM

The periodontium is thought to be an organ system that comprises four tissues, including the gingiva, PDL, cementum, and alveolar bone. Each of these tissues has distinctive characteristics and biochemical composition. The tissues together support and protect the teeth and keep them functioning properly (Bartold and Narayanan, 2006).

1.1.2.1 The periodontal ligament

The periodontal ligament (PDL), a highly vascular connective tissue, is interposed between the wall of the alveolar socket and the tooth root and is composed of a diverse cell population and

a fibrous ECM. These cell populations are comprised of fibroblasts, endothelial cells, undifferentiated mesenchymal, epithelial cells, cementoblasts, osteoblasts, osteoclasts, and others. The ECM consists mainly of a network of collagen fibres of type I, III, and V (Beertsen et al., 1997, Nojima et al., 1990, Bartold and Narayanan, 2006). Periodontal fibroblasts are the most predominant cell type and have been demonstrated to have osteogenic potential and to differentiate into osteoblasts in response to several stimuli (Basdra and Komposch, 1997, Nojima et al., 1990).

The PDL's crucial role in OTM has been demonstrated in implant and ankylosed tooth studies (both lack PDL), which were unresponsive to orthodontic forces (Wise and King, 2008). The PDL is the first tissue to respond to mechanical stimuli (Nakamura et al., 1996) and can quickly adapt to mechanical forces during OTM (Lekic and McCulloch, 1996). Orthodontic force loading results in a slight change in PDL thickness after one hour, whereas more significant alterations have been observed after six hours (Nakamura et al., 2008). OTM requires extensive remodelling and reorganization of the ECM of the PDL besides alveolar bone remodelling (Kawarizadeh et al., 2005). The collagen fibers in the ECM of the PDL are constantly remodelled to adapt to the positional changes of teeth, with their highest turnover rate in the PDL than in any other tissue (Ten Cate et al., 1976). Periodontal fibroblasts have a crucial role in PDL haemostasis in response to mechanical stimulation by remodelling of ECM components, mainly by synthesizing and degrading collagen types I, III, V, VI, and XII components (Lekic and McCulloch, 1996, Bumann et al., 1997, Cantarella et al., 2006, He et al., 2004).

The PDL becomes oedematous, hyperaemic, and infiltrated with acute inflammatory cells during the initial phase of OTM when the tooth is displaced within the PDL space (Krishnan and Davidovitch, 2009, Niklas et al., 2013). The viscoelastic characteristics of both the bone and the PDL are affected by the increase in inflammatory fluid and cellular infiltration

in the PDL and surrounding alveolar bone. The tensile strength of collagen bundles gradually decreases due to the release of matrix metalloproteinases (MMPs) and other catabolic agents that disturb the cross-linkages and molecular integrity of the ECM (Henneman et al., 2008, Feller and Lemmer, 2004).

1.1.2.2 The gingival tissues

The gingiva is the soft tissue surrounding the tooth and comprises the oral epithelium, oral sulcular epithelium, junctional epithelium, and underlying connective tissue. Sixty percent of the connective tissue is composed of collagen fibres, with type I and III collagens accounting for 90 % of the collagen network (Schroeder and Listgarten, 1997, Bartold and Narayanan, 2006). Like the PDL, the predominant cell type in the gingiva is the fibroblasts, with their primary function to synthesize and remodel ECM components (Bartold and Narayanan, 2006).

Gingival changes in the overall response to orthodontic forces are crucial, and even so, they have been given little attention. Sustaining the integrity of the gingiva requires highly coordinated tissue remodelling and balanced collagen synthesis and degradation. Following the application of orthodontic forces, two distinct processes occur in the gingiva. Firstly, the gingival connective tissue is injured, as seen by ripped and torn collagen fibres; secondly, the genes for collagen and elastin are activated, while those for tissue collagenases are repressed (Krishnan and Davidovitch, 2006, Redlich et al., 1999, Redlich et al., 1998).

Gingival and periodontal fibroblasts have distinct functions in the remodelling of paradental tissues. Whilst PDL fibroblasts are primarily responsible for the synthesis and breakdown of their own ECM, gingival fibroblasts are involved in bone remodelling processes (Krishnan and Davidovitch, 2009). Like PDL fibroblasts, gingival fibroblasts respond to inflammatory and mechanical stimulation; both cell types upregulate MMP-1, MMP-2, TIMP-

1, and TIMP-2 expression after being stretched (Bolcato-Bellemin et al., 2000, Williams et al., 2016).

1.1.2.3 The alveolar bone

The alveolar bone is an extension of the maxilla and mandible and is composed of mineralized tissue, organic matrix, and water. Whilst the bulk of alveolar bone is trabecular, the PDL space is surrounded by a plate of compact bone termed the lamina dura. PDL fibres attach to the alveolar bone by penetrating the lamina dura while connecting to the cementum on the other end. Numerous cell types, including osteoblasts, osteoclasts, and osteocytes, play key roles in alveolar bone function and homeostasis. Furthermore, macrophages, adipocytes, and endothelial cells can be present in the alveolar bone (Li et al., 2018).

The alveolar processes, comprising the tooth sockets, are composed of dense cortical bone surrounding cancellous bone. Type I and III collagens are the main organic components of bone, similar to the other tissues of the periodontium (Bartold and Narayanan, 2006). Monocytes in the PDL area are stimulated to form osteoclasts in response to prolonged forces, which first appear within the compressed PDL 30 to 40 hours after force application (Apajalahti et al., 2003). Osteoclasts utilize a distinct acidic cathepsin-dependent mechanism for the dissolution of mineralized matrices (Birkedal-Hansen, 1993).

The orthodontic force causes micro damage to the alveolar bone, which results in changes in fluid flow in the lacuna-canalicular network and triggers apoptosis in osteocytes (Verborgt et al., 2000, Henneman et al., 2008). Signals from apoptotic osteocytes can draw osteoclasts to the area of microdamage, where they, together with other bone cells, prompt bone remodelling. Following bone injury, the local release of inflammatory mediators, cytokines, and growth factors such as endothelin, prostaglandin E2, and vascular endothelial

growth factor (VEGF) induces bone remodelling, allowing for OTM (Miyagawa et al., 2009, Sprogar et al., 2008, Feller et al., 2015b, Andrade et al., 2012).

1.1.3 Role of inflammation in OTM

Following orthodontic force application, the early phase of OTM is characterised by instantaneous displacement of the tooth in its alveolar socket with the PDL fibres being stretched and compressed in the tension and compression sites of the PDL, respectively. This triggers an immediate response characterised by an aseptic acute inflammatory response devoid of bacteria (Krishnan and Davidovitch, 2006, Andrade et al., 2012, Rygh et al., 1986, Davidovitch et al., 1988).

This acute inflammation is characterized by blood vessel dilatations in the surrounding periodontal tissues. Leukocytes and plasma cells migrate out of the capillaries and produce cytokines that interact directly or indirectly with the resident paradental cells. These cytokines, along with other systemic and local signal molecules, evoke the production of growth factors, cytokines, and prostaglandins by their target cells. The acute inflammatory reaction lasts for one or two days, then it subsides and is replaced by a chronic aseptic inflammation that continues until the orthodontic appliance is re-activated at the next orthodontic treatment appointment and thus inducing another acute inflammatory reaction. During chronic inflammation, the leukocytes keep their migration into the surrounding periodontal tissues, and the resident cells, including fibroblasts, endothelial cells, osteoblasts, and alveolar bone marrow cells, continue proliferating (Krishnan and Davidovitch, 2006)

Neurovascular mechanisms have important roles in OTM as well. The nerve endings, which are closely associated with blood vessels, are distorted in both the tension and compression sites of the PDL, leading to the release of neurotransmitters such as CGRP and

substance P. These neurotransmitters interact with the capillary endothelial cells, with the later express receptors which bind to circulating leukocytes, inducing them to migrate out of the capillaries into the surrounding periodontal tissues. This, in turn, leads to the release of inflammatory mediators that promote tissue remodelling (Krishnan and Davidovitch, 2006, Wise and King, 2008). Although inflammation is essential for OTM, excessive or uncontrolled inflammation may lead to unwanted effects, mainly orthodontically induced inflammatory root resorption (Li et al., 2018).

Upregulation of IL-1β, IL-1α, TNF-α, IL-6, and IL-8 and other inflammatory mediators has been demonstrated in the gingival crevicular fluid (GCF) of orthodontic patients as early as 1 minute (Dudic et al., 2006), 1 hour (Tuncer et al., 2005, Karacay et al., 2007, Hamamcı et al., 2012), 4 hours (Grant et al., 2013), and 24 hours (Ren et al., 2002, Grieve et al., 1994, Lee et al., 2004, Uematsu et al., 1996, Alikhani et al., 2013) following force application, confirming the presence of the acute aseptic inflammation. However, these inflammatory cytokines have returned to baseline levels at 48 hours, 7 days, 14 days, and 21 days (Sarı and Uçar, 2007, Karacay et al., 2007, Grieve et al., 1994).

1.1.3.1 Cytokines

Cytokines, a broad family of secreted proteins essential for cell-to-cell communications, are low-molecular-weight proteins produced by specific cells. Cytokines are not constitutively released but are produced in response to local stimuli; they have a high affinity for adjacent receptors and an extremely short half-life. They modify and control the actions of their cell of origin (autocrine) and adjacent cells (paracrine). They include interleukins (ILs), tumour necrosis factors (TNFs), growth factors, interferons, and colony-stimulating factors (Meikle, 2006, Meager, 1999, Krishnan and Davidovitch, 2006) and are categorized as pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines include TNF-α, IL-1, IL-2,

IL-6, and IL-8, whereas anti-inflammatory cytokines include IL-4, IL-10, and IL-13 (Stoycheva and Murdjeva, 2005). Cytokines have two features that make their study exceedingly difficult, one is pleiotropy (the ability of a single cytokine to trigger a broad range of responses in a variety of cell types), and the other is redundancy (the overlapping actions by multiple cytokines) (McFarlane et al., 2022, Meikle, 2006).

Cytokines have a major role in starting, exacerbating, maintaining, and alleviating inflammatory reactions (Stoycheva and Murdjeva, 2005, Başaran et al., 2006a, Krishnan and Davidovitch, 2006). They have a significant role in osteoclast differentiation, survival and function (Henneman et al., 2008), and a previous study reported that cytokine activity inhibition by soluble receptors decreased the number of osteoclasts and the amount of tooth movement in rats confirming their primary role in OTM (Jäger et al., 2005). Cytokines play a dual function in remodelling mineralized and non-mineralized connective tissues. Specifically, pro-inflammatory cytokines, such as IL-1 and TNF-α, induce tissue resorption and inhibit apposition, whereas anti-inflammatory cytokines induce tissue apposition and inhibit resorption (Krishnan and Davidovitch, 2009).

The early phase of OTM consists of an acute inflammatory reaction (Saito et al., 1991), manifested by capillary vasodilatation, followed by leukocyte migration and the production of cytokines. IL-1, the most potent among the cytokines, is one of the earliest cytokines to be released and an increase in its levels is evident at 1 minute (Dudic et al., 2006). IL-1 is released locally by stimulated cells in response to mechanical signals and is mainly produced by fibroblasts, macrophages, cementoblasts, osteoblasts, and osteoclasts. IL-1 has two isoforms: IL-1α and IL-1 β, having similar biological functions and play a vital role in the inflammatory process and regulating connective tissue and bone remodelling during OTM (Davidovitch et al., 1988, Alhashimi et al., 2001, Ren and Vissink, 2008, Bletsa et al., 2006). IL-1ß intensifies the inflammatory response by inducing the release of various pro-inflammatory cytokines, such

as IL-6 and TNF-α in periodontal fibroblasts and osteoblasts (Grimm et al., 2020), and it overlaps with TNF-α and IL-6 in their biological functions (d'Apuzzo et al., 2013). In addition, IL-1β blocking by soluble receptors reduces OTM (Jäger et al., 2005).

IL-6 is expressed by immune cells, periodontal fibroblasts, and osteoblasts. It regulates immune responses in inflammatory sites and is essential for bone and connective tissue remodelling (Rosselli-Murai et al., 2013, Tantilertanant et al., 2019, Garlet et al., 2007, Li et al., 2021b). IL-8 plays a crucial role in neutrophil recruitment and activation during inflammation and is primarily secreted by monocytes (Başaran et al., 2006a, Li et al., 2021b, Tuncer et al., 2005). Additionally, the release of IL-8 during OTM has been related to the recruitment and activation of osteoclasts (Asano et al., 2011). TNF-α is expressed by macrophages, monocytes, epithelial cells, and osteoblasts. It has a prevalent catabolic effect and plays a major role in inducing osteoclastogenesis and MMP production (Kobayashi et al., 2000, Li et al., 2021b, Andrade et al., 2012). Several clinical trials have shown raised levels of IL-1α, IL-1β, TNF-α, IL-6, and IL-8 in the GCF of orthodontic patients during the initial phase of OTM (Alikhani et al., 2013, Grieve et al., 1994, Uematsu et al., 1996, Tuncer et al., 2005, Başaran et al., 2006b, Başaran et al., 2006a).

Changes in the cytokine levels between compression and tension areas in the GCF of orthodontic patients have been studied in several studies. In one longitudinal study, GCF samples were collected from the tension and compression sites before tooth extraction and fixed appliance placement and then at 4 hours, 7 days, and 42 days after applying distalizing forces to the maxillary canine teeth. The results demonstrated that tension sites exhibited increases in IL-1β, IL-8, and TNF-α levels across all time points, whilst compression sites showed increases in IL-1β and IL-8 levels after 4 hours of force application (Grant et al., 2013). Another study demonstrated that compression areas showed increased levels of TNF-α, whereas tension areas exhibited higher levels of IL-10 (Garlet et al., 2007). Furthermore,

another study found that IL-8 production in the GCF of orthodontic patients was higher in tension areas than compression areas, with maximal levels in tension areas at day 6 and compression areas after 1 and 24 hours of force application (Tuncer et al., 2005).

1.1.4 Extracellular matrix remodelling of soft tissues

Under physiological circumstances, paradental tissue degradation and synthesis are kept to a minimum to preserve tissue homeostasis. When an external force is applied, this balance is disrupted, and greater remodelling of alveolar bone and PDL leads to tooth movement (Henneman et al., 2008). PDL respond to mechanical force stimulation during OTM and creates a milieu for cellular responses and tissue remodelling. Although the bone remodelling process during OTM has been widely studied, the remodelling of collagenous ECM in PDL in response to mechanical stimuli is largely unknown (Rangiani et al., 2016). Remodelling of gingival and PDL fibres is considered as a wound-healing phenomenon, and the biological cell activity underpinning OTM is characterised by substantial collagen turnover (Ten Cate et al., 1976, Bumann et al., 1997).

The ECM, which is mainly composed of fibrous proteins embedded in a hydrated polysaccharide gel, is mainly composed of macromolecules, with collagen being the main component. These macromolecules are released locally by cells such as fibroblasts, osteoblasts, and chondroblasts. The PDL is one of the most metabolically active tissues within the body, with the half-life of mature collagen turnover is two days, compared to five days for gingiva and six days for the alveolar bone (Krishnan and Davidovitch, 2006, Feng et al., 2016).

Orthodontic forces compress, stretch, or twist collagen fibres and change fluid flow in the PDL space, altering the structure of ECM proteins and exposing molecules that can stimulate fibroblasts through integrins and focal adhesion domains. This leads to the expression

of genes which encodes numerous proteins (collagen and fibronectin) and enzymes (proteases) required for the PDL ECM remodelling (Feller et al., 2015b, Masella and Meister, 2006, Krishnan and Davidovitch, 2006). ECM remodelling of the periodontal tissues involves both degradation of the ECM molecules and formation of new ECM molecules. Extensive collagen fibres formation and degradation were observed in both compression and tension areas of the PDL (Rygh et al., 1986).

On the compression region, orthodontic forces cause bioelastic and bioplastic deformations of the alveolar process, leading to circulation disruptions, ischaemia, disruption of collagen fibers, cell death and hyalinized areas of PDL. Collagen demonstrates degradation accompanied by altered expression of type I collagen, and macrophages remove the altered tissue and collagen in the hyalinized areas. Cellular activity increases, and PDL fibroblasts secrete and form new functionally oriented collagen fibres. Furthermore, osteoclasts and PDL fibroblasts produce a glycosaminoglycan layer on the new resorbed bone surface (Storey, 1973, Bumann et al., 1997, Krishnan and Davidovitch, 2006, Feng et al., 2016).

In the tension region, the PDL fibres are stretched, and an increase in local vascular activity and vasodilation occurs (Rygh et al., 1986). Collagen fibres exist in coiled forms, and when tooth movement exceeds the intrinsic fibres length, new fibres are formed and integrated into the ligament proper (Roberts and Chase, 1981). Electron microscopy revealed a considerable decrease in collagen fibre diameter in the tension areas (Martinez and Johnson, 1987). It has been assumed that the extension of fibres during the remodelling process leads to the elongation of these fibers, allowing for OTM (Bumann et al., 1997).

Collagen synthesis occurs at both the compression and tension sides following orthodontic force application (Bumann et al., 1997). Activated PDL cells express several mediators, such as members of the transforming growth factor- β (TGF-β) superfamily, which

enhance the synthesis of collagen and non-collagenous ECM proteins and inhibit its degradation (Henneman et al., 2008, Krishnan and Davidovitch, 2006). It has been demonstrated that cells can perceive two different mechanical stimulus forms and respond differently relative to ECM synthesis and degradation. One study reported that the application of compressive forces decreased type I collagen and fibronectin while increasing MMP-2, whereas tensional forces enhanced type I collagen and MMP-2 (He et al., 2004). Another study found substantially higher expression of collagen type I on the tension side than on the compression side in rats after orthodontic force application (Nakagawa et al., 1994).

Collagen degradation also occurs at both the compression and tension areas. Several proteases have been implicated in the degradation of collagen and other macromolecules in the ECM. This is confirmed by their elevated levels in compression and tension areas during OTM, emphasising their crucial role in ECM remodelling (Apajalahti et al., 2003, Ingman et al., 2005, Krishnan and Davidovitch, 2009). Several studies have reported the crucial role of MMPs in ECM degradation during OTM (Apajalahti et al., 2003, Holliday et al., 2003a, Domon et al., 1999, Bolcato-Bellemin et al., 2000, Li et al., 2018).

1.1.4.1 Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a group of zinc-dependent endopeptidases, comprising at least 23 different secreted or membrane-bound types in human tissues and belong to the metzincins superfamily of proteases. MMPs share similar core structure and are divided into six main groups based on their substrate specificity and the organization of their structural domains, including collagenases, gelatinases, stromelysins, membrane-type MMPs, matrilysins, and others (Cui et al., 2017, Nagase et al., 2006, Chung et al., 2004, Klein and Bischoff, 2011, Liu and Khalil, 2017). MMPs have an auto-inhibitory prodomain which has to be removed for activation, a C-terminal hemopexin-like domain which is frequently involved

in MMP substrate recognition and positioning, a catalytic domain with catalytic zinc, and a linker peptide (Franco et al., 2017, Mysliwy et al., 2006, Cui et al., 2017) (Figure 1.3). They have a pivotal role in several processes, such as ECM remodelling, angiogenesis, wound healing, embryogenesis, and regulation of inflammation at both physiological and pathological levels (Chung et al., 2004, Laronha and Caldeira, 2020, Nagase et al., 2006, Cui et al., 2017).

Collagen has a triple helical form making it highly resistant to degradation, with MMPs being the only enzymes able to degrade them. The collagenases (MMP-1, MMP-8, MMP-13) initiate tissue remodelling by degrading interstitial collagen I, II, and III and other ECM and non-ECM molecules (Laronha and Caldeira, 2020, Zhu et al., 2018, Visse and Nagase, 2003). Collagenase-1 (MMP-1) is produced by a wide variety of cells, such as fibroblasts, keratinocytes, macrophages, osteoblasts, endothelial cells, and others (Birkedal-Hansen, 1993, Romanelli et al., 1999). Although collagenase-2 (MMP-8) is produced mainly by polymorphonuclear leukocytes (PMNs) (stored in specific granules and released upon stimulation), MMP-8 can be produced by periodontal and gingival fibroblasts and by sulcular epithelial cells and endothelial cells (Ingman et al., 1993, Apajalahti et al., 2003, Ingman et al., 1996, Tervahartiala et al., 2000, Romanelli et al., 1999, Ye, 2015). Collagenase-3 (MMP-13) is produced by gingival sulcular epithelial cells, gingival fibroblasts, and macrophage-like cells (Tervahartiala et al., 2000).

Gelatinases A (MMP-2) and B (MMP-9) have a broad range of substrate specificities and are best known for degrading gelatin which is the denatured form of collagen and type IV collagen (Chung et al., 2004, Fischer et al., 2019, Nagase et al., 2006). MMP-9 is produced by PMNs, epithelial cells, and oral keratinocytes (Ingman et al., 1993). However, MMP-2 is fibroblasts, osteoblasts, keratinocytes, macrophages, chondrocytes, and endothelial cells (Birkedal-Hansen, 1993).

Stromelysins (MMP-3, MMP-10, MMP-11) have a domain structure similar to collagenases; however, they do not degrade interstitial collagens; instead, they degrade other ECM molecules and play a role in activating pro-MMPs (Nagase et al., 2006, Laronha and Caldeira, 2020). Matrilysins (MMP-7, MMP-26) have a wide range of substrate specificity. MMP-7, which is produced mainly by epithelial cells, can digest a wide range of ECM molecules such as gelatin, laminin, fibronectin, and elastin (Nagase et al., 2006, Klein and Bischoff, 2011) and can activate other pro-MMPs including MMP-8 (Tervahartiala et al., 2000). Four of the Membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-24) are transmembrane proteins, while two (MMP-17, MMP-25) are glycosylphosphatidylinositol-anchored. These MMPs are expressed on the cell surface after being activated intracellularly (Nagase et al., 2006, Laronha and Caldeira, 2020).

MMPs are tightly controlled under physiological conditions at various levels, including mRNA expression, pro-MMPs activation to the active form, and endogenous tissue inhibitors of metalloproteinases (TIMPs) counteraction. Hence, uncontrolled MMP activity can cause pathological conditions like inflammation, arthritis, fibrosis, and cancer (Löffek et al., 2011, Visse and Nagase, 2003, Birkedal-Hansen, 1993). MMPs are commonly secreted as inactive pro-MMPs, and to become active, the prodomain must be cleaved by several different proteases, including other MMPs, trypsin, kallikrein, neutrophil elastase, cathepsin B, and others (Cui et al., 2017, Ingman et al., 1993, Chung et al., 2004, Birkedal-Hansen, 1993).

TIMPs are made up of 184-194 amino acids and have been shown to inhibit all MMPs studied. Four TIMPs (TIMP-1, TIMP-2, TIMP-3, TIMP-4) have been identified in humans; these TIMPs bind in a 1:1 ratio to both MMPs and Pro-MMPs (Nagase et al., 2006, Cui et al., 2017). TIMPs preserve the balance between ECM degradation and regeneration by inhibiting MMP activity (Nakasone et al., 2009, Lisboa et al., 2013). All TIMPs are broad-spectrum MMP inhibitors, yet their specificity for MMPs varies (Löffek et al., 2011). They are produced by a

variety of cell types, including fibroblasts, keratinocytes, endothelial cells, and macrophages (Birkedal-Hansen, 1993) and have been detected in the periodontal tissues (Andrian et al., 2007, Nakasone et al., 2009). Changes in MMPs or TIMPs levels alter the ratio of MMP to TIMP; this ratio determines the net activity of MMPs (Cui et al., 2017).

1.1.4.1.1 MMPs and periodontitis

ECM is a network of macromolecules consisting of fibers, proteoglycans, and polysaccharides, with collagen fibers being the most abundant molecule. MMPs play a pivotal role in ECM turnover and remodelling by promoting the degradation of most ECM macromolecules (Ingman et al., 1993, Sorsa et al., 1994, Cui et al., 2017, Laronha and Caldeira, 2020, Restaíno et al., 2007). Periodontitis, a chronic inflammatory disease, is characterized by periodontal supporting tissue destruction that ultimately leads to the loss of teeth. It is well-established that MMPs play a key role in periodontal diseases (Franco et al., 2017, Sorsa et al., 1994, Ingman et al., 1996).

PMNs and other inflammatory cells are activated by cytokines, prostaglandins, and bacterial products, which in turn lead to the release of MMPs and serine proteinases (cathepsin G, elastase, and proteinase 3) which degrade the different ECM components and have a crucial role in the progression of periodontal tissue destruction. Higher levels of these proteases have been detected in saliva, GCF, and inflamed gingival tissues of subjects with periodontal diseases. Additionally, the activity and levels of these proteases have decreased after successful periodontal treatment (Sorsa et al., 1994, Golub et al., 1995, Westerlund et al., 1996).

MMP-8 and MMP-9 have been found to predominate in periodontitis (Ingman et al., 1993, Westerlund et al., 1996), suggesting their crucial role in periodontal tissue destruction. Moreover, MMP-8- and MMP-13 levels have been demonstrated to be greater in the gingiva of individuals with periodontitis compared to healthy tissues (Tervahartiala et al., 2000). When

assessed in a longitudinal study, salivary levels of MMP-8 were reduced after periodontal therapy, implying their potential use in monitoring the status of periodontal diseases (Sexton et al., 2011).

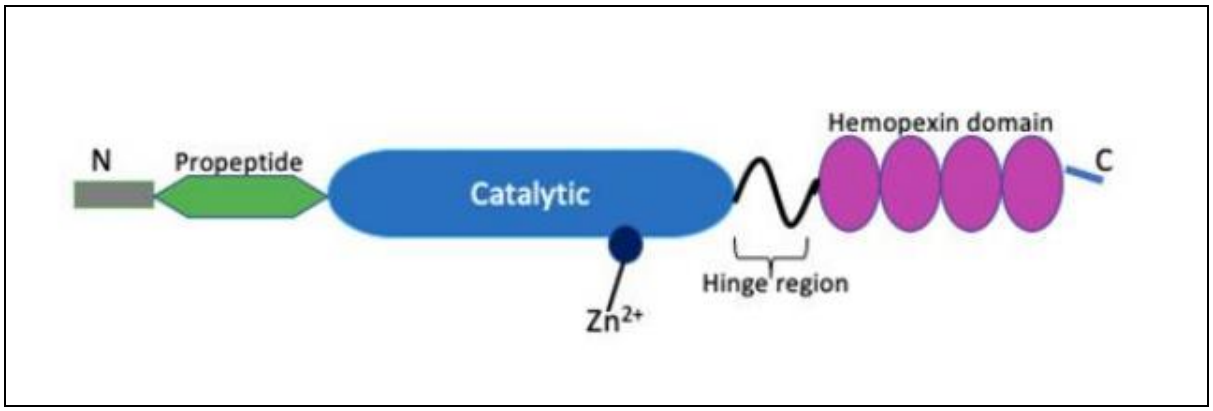

Figure 1.1.3 Matrix metalloproteinases (MMPs) structure.

MMPs have an auto-inhibitory pro-peptide, catalytic domain with catalytic zinc, hinge region (linker peptide), and a C-terminal hemopexin-like domain. Adapted from (Khuda et al., 2021).

1.1.4.1.2 MMPs and orthodontics

MMP levels are very low in normal conditions; however, their expression is elevated in inflamed tissues or those undergoing remodelling in both physiological and pathological conditions (Birkedal-Hansen, 1993, Ye, 2015). Orthodontic forces result in an aseptic inflammatory response characterised by vascular changes and inflammatory cell infiltration (Garlet et al., 2007, Krishnan and Davidovitch, 2006). Hence, several studies have investigated the effect of orthodontic forces on the production of MMPs.

Changes in MMPs and TIMPs levels during OTM have been confirmed by several in vivo studies in humans (Zhang et al., 2020, Apajalahti et al., 2003, Grant et al., 2013). Additionally, several animal studies demonstrated reduced rates of OTM with the use of synthetic inhibitors of MMPs, indicating their significant role in OTM (Holliday et al., 2003a, Bildt et al., 2007). Differential expression of MMPs in the tension and compression sides has

been observed during OTM (Takahashi et al., 2006). Indeed, a particular study showed increased MMP-1 expression levels in the compression and tension sides of the PDL, with the compression side exhibiting higher expression levels, suggesting that MMP-mediated ECM protein degradation may be more crucial at the compression site (Garlet et al., 2007).

Despite the large number of in vivo and in vitro studies that have been performed to investigate the effects of orthodontic forces on the expression of various MMPs, substantial variabilities have been observed in the literature between those studies.

1.1.4.1.2.1 In vivo studies

Levels of MMPs have been thoroughly investigated in the GCF of orthodontic patients (Cantarella et al., 2006, Alikhani et al., 2018, Bildt et al., 2009, Apajalahti et al., 2003, Saloom et al., 2017, Capelli Junior et al., 2011, Ingman et al., 2005, Grant et al., 2013, Zhang et al., 2020). In contrast, few studies have investigated their levels in saliva (Sioustis et al., 2021, Xu et al., 2020), bone tissues (Chang et al., 2008), and PDL tissues (Garlet et al., 2007) in relation to orthodontic forces. Differences in force application methods, force magnitudes, observation times, appointment intervals, appliance types and other factors were observed between the studies mentioned above, which might have impacted the findings.

Elevated MMP-8 levels were observed in the GCF of orthodontic patients within eight hours of orthodontic force application; however, no changes in MMP-1 levels were detected (Apajalahti et al., 2003). Similarly, two other studies found higher levels of MMP-8 in the GCF of orthodontic patients over one month period (Ingman et al., 2005) and after three months of orthodontic force application (Ribagin and Rashkova, 2012). In contrast, no changes in MMP-8 levels were detected in the GCF of orthodontic patients after 6 and 12 months of orthodontic appliance placement (Shirozaki et al., 2020).

In a non-randomized clinical study, MMP-9 levels were measured in the GCF of adolescents and adults undergoing orthodontic treatment. GCF samples were collected prior to orthodontic treatment and at 1, 7, 14, and 28 days after canine retraction. The results showed increases in MMP-9 levels in both adolescents and adults 1, 7, and 14 days following canine retraction and these increases were greater in adults than in adolescents (Alikhani et al., 2018). Several other studies have confirmed increased MMP-9 levels in the GCF during orthodontic treatment (Surlin et al., 2014, Zhang et al., 2020, Bildt et al., 2009). On the other hand, one study reported no changes in MMP-9 levels in the GCF of orthodontic patients over time (Rody et al., 2014).

Numerous studies have investigated changes in MMPs and TIMPs levels in the compression and tension sides during orthodontic treatment. One particular study assessed MMP-3, MMP-9, and MMP-13 levels longitudinally in the GCF collected from the compression and tension sides of orthodontic patients. Their levels were assessed at the following time points: 7 days before force application, baseline, and then after 1 hour, 24 hours, 14 days, 21 days, and 80 days of beginning of OTM. MMPs levels oscillated during the observation period, increasing within 1 hour of force application and then decreasing after 24 hours; thereafter, MMPs levels increased steadily (Capelli Junior et al., 2011). A further controlled longitudinal study investigated changes in MMP-9, TIMP-1, and TIMP-2 GCF levels in patients undergoing orthodontic treatment. Here, GCF samples were collected at baseline (prior to appliance and teeth extractions) and then at 4 hours, 7 days, and 42 days after applying the distalization forces from both the compression and tension sides. MMP-9, TIMP-1, and TIMP-2 levels were elevated in tension areas at all time points following distalization, whereas MMP-9 levels were elevated in compression areas after 7 and 42 days of distalizing force application (Grant et al., 2013).

MMP levels in saliva, in relation to orthodontic treatment, have been reported sparsely. In two previous studies, salivary MMP-8, MMP-9, and MMP-12 levels were increased at 1 hour (Xu et al., 2020) and one week (Sioustis et al., 2021) following orthodontic force application and positively correlated with OTM. However, MMP-1, MMP-3, and MMP-13 levels did not change over time, and MMP-7 levels were decreased in the saliva after eight weeks of force application (Xu et al., 2020).

Overall, MMP-9 and MMP-8 are key mediators of the initial tissue response and remodelling during OTM and can act as biomarkers for monitoring the ECM remodelling of the periodontal tissues during OTM.

1.1.4.1.2.2 In vitro studies

In vitro, MMPs levels have been studied extensively in periodontal fibroblast (Schröder et al., 2021, Chen et al., 2013, Grimm et al., 2020, Jacobs et al., 2014, Lisboa et al., 2009, Long et al., 2002, Redlich et al., 2004, Proff et al., 2014, Wescott et al., 2007), and to a lesser extent in gingival fibroblast (Bolcato-Bellemin et al., 2000, Nan et al., 2019), periodontal mesenchymal stromal cells (Behm et al., 2021a), bone-derived cells (Chang et al., 2008, Tasevski et al., 2005), and macrophages (Schröder et al., 2020b) in relation to simulated orthodontic forces.

Several approaches have been used in vitro to simulate orthodontic forces (Figure 1.4). The substrate deformation-based model involves using a substrate that is mainly an elastic membrane. The cells are cultured on the membrane that is deformed by force, and the cells are stretched at the same time. An example of this approach is the Flexercell tension system used by several studies to simulate tensile orthodontic forces generated on the tension side of the PDL (Long et al., 2002, Wescott et al., 2007, Zhong et al., 2008, Bolcato-Bellemin et al., 2000, Saminathan et al., 2012). Alternatively, the weight approach has been increasingly used to

generate static compressive forces to simulate forces generated on the compression side of the PDL. In general, a weight is placed on the cultured confluent cells, causing gravity to be imposed and producing unidirectional static compressive forces. Different force magnitudes have been used with a range of 0.5-5 gm/cm^2, with 2 gm/cm^2 being the most used magnitude (Kang et al., 2010, Yamaguchi et al., 2006, Schröder et al., 2021, Kanzaki et al., 2002, Liu et al., 2006). Other less common in vitro approaches used include the centrifugation approach, which involves centrifuging the cells in a horizontal microplate rotor to generate compressive forces (Redlich et al., 2004, Zhao et al., 2008, Grimm et al., 2020), and the hydrostatic pressure approach, which involves generating multidirectional centripetal compressive force that can be static or gradually oscillating by increasing the air pressure within an incubator to simulate forces generated according to the hydrostatic pressure hypothesis (Yousefian et al., 1995).

Previous studies demonstrated changes in the expression of various MMPs in human PDL cells between and among the different types of mechanical forces applied. Specifically, these studies demonstrated that compressive forces increased MMP-1 (Huang et al., 2008, El-Awady et al., 2013, Hacopian et al., 2011, Redlich et al., 2004), MMP-3 (Lisboa et al., 2013), MMP-8 (Grimm et al., 2020, Nettelhoff et al., 2016), and MMP-13 (Proff et al., 2014) levels in human periodontal cells. However, other studies showed that compressive forces decreased MMP-2 (Lisboa et al., 2009) and MMP-9 (El-Awady et al., 2013) levels in human PDL cells.

Multiple studies reported that tensile forces increased MMP-1 (Tantilertanant et al., 2019, Kook et al., 2011, Bolcato-Bellemin et al., 2000, Nemoto et al., 2010), MMP-2 (Tantilertanant et al., 2019, Chen et al., 2013, Bolcato-Bellemin et al., 2000), MMP-3 (Tantilertanant et al., 2019), MMP-8 (Jacobs et al., 2014, Jacobs et al., 2018), and MMP-13 (Ziegler et al., 2010) levels in human periodontal cells. Other studies reported no changes in MMP-1 (Behm et al., 2021a, Tsuji et al., 2004), MMP-2 (Behm et al., 2021a, Tsuji et al., 2004, Wescott et al., 2007), MMP-3 (Long et al., 2002), MMP-8 (Tantilertanant et al., 2019, Schröder

et al., 2020a, Wescott et al., 2007), and MMP-9 (Wescott et al., 2007) levels in human periodontal cells. In contrast, MMP-3 (Nemoto et al., 2010) and MMP-8 levels (Ma et al., 2015, Saminathan et al., 2012) were found to be decreased in human periodontal cells subjected to tensile forces.

Most in vitro studies have been conducted on human periodontal cells; however, other cell types have been used to a lesser extent. In one study, compressive forces decreased MMP-1 levels in human gingival fibroblasts subjected to compression using the weight approach (Nan et al., 2019). In contrast, in another study, compressive forces increased MMP-3 but had no effect on MMP-1, MMP-2, MMP-9, or MMP-13 levels in MG-63 osteoblast-like cells (Chang et al., 2008).

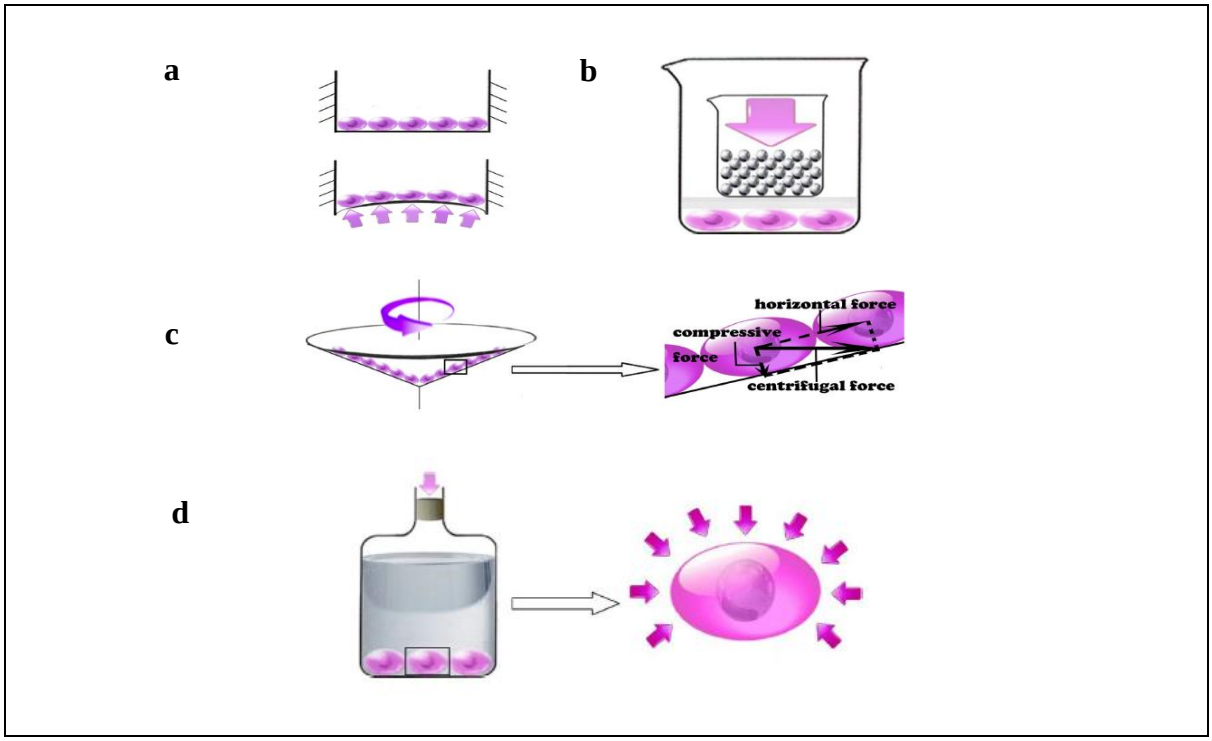

Figure 1.4 Schematic representation of in vitro mechanical loading approaches.

Several mechanical loading models have been used in vitro to simulate orthodontic forces, including substrate deformation-based approach (a), weight approach (b), centrifugation approach (the centrifugal force is divided into a perpendicular compressive force and a horizontal frictional force, the magnitude of which is controlled by the rotation speed) (c), and the hydrostatic pressure approach (d). Adapted from (Yang et al., 2015a).

1.1.5 Alveolar bone remodelling

Bone remodelling is a physiological process that involves both osteoclast-mediated bone resorption and osteoblast-mediated bone formation. The monocyte/macrophage lineage of bone marrow haematopoietic stem cells gives rise to bone-resorbing osteoclasts. Various cytokines, hormones, and growth factors drive osteoclast differentiation, with RANKL-RANK-osteoprotegerin (OPG) signalling pathway being a crucial regulator (Feller et al., 2015b, Yamaguchi, 2009).

RANKL is produced by osteoblasts, stromal and activated T-cells as a membrane-bound and soluble ligand. These cells express OPG as well, which is a soluble decoy receptor for RANKL that prevents RANKL from binding to RANK and hence from differentiating further. RANKL exerts its effects by binding to its receptor (RANK) on osteoclast precursors, prompting their differentiation, maturation, and activation, whilst OPG suppresses RANK-RANKL-mediated osteoclastogenesis indirectly. Therefore, the balance of RANKL and OPG activities will influence the amount and pace of bone resorption (Garlet et al., 2007, Yamamoto et al., 2011, Yamaguchi, 2009). Several inflammatory cytokines such as IL-6, IL-8, IL-11, IL-17, and TNF-α stimulate osteoclastogenic in the early phase of OTM by upregulating the expression of RANK on osteoclasts or promoting the expression of RANKL by osteoblast, PDL fibroblasts, and osteocytes; whereas other cytokines such as IL-10, IL-4, and IL-18 inhibit osteoclastogenesis and bone resorption (Jiang et al., 2015, Andrade et al., 2012, Yamamoto et al., 2011, Yamaguchi, 2009).

Osteoblasts are produced from a multipotent mesenchymal progenitor cell that can differentiate into bone marrow stromal cells and adipocytes (Jiang et al., 2015). Osteoblasts control both bone resorption and formation processes of alveolar bone remodelling by controlling osteoclasts' recruitment and function. Furthermore, osteoblasts produce collagenolytic proteases that allow osteoclasts to reach the mineralized tissue by degrading the

non-mineralized osteoid that covers the surface of the resting bone (Ingman et al., 2012). Osteoclasts, which play a role in bone resorption, are multinucleated giant cells that arise from haematopoietic stem cells (Alhashimi et al., 2001). Osteoclast recruitment and differentiation in the compression areas of PDL are crucial for bone resorption and OTM. It has been demonstrated that pro-inflammatory cytokines, such as IL-1, IL-6 and IL-8, play an essential role in OTM through the regulation of osteoclast differentiation (Jäger et al., 2005).

Alveolar bone resorption, which occurs at the compression region, involves the solubilization of mineralized tissue and the degradation of the organic collagenous matrix (Domon et al., 1999). Before actual bone resorption commences, the non-mineralized osteoid layer is removed by the lining osteoblasts, which produce various proteolytic enzymes, particularly MMPs (Birkedal-Hansen, 1993, Hill, 1998). This facilitates the attachment of mature osteoclasts to the underlying mineralized bone surface by means of specific integrins (Gay and Weber, 2000). These attached osteoclasts change their morphology and develop distinct functional properties: a clear zone that isolates the bone surface from its surroundings and the ruffled border under which resorption occurs. The activated osteoclast resorbs the bone by producing hydrogen ions, which in conjunction with proton pumping, promotes the dissolution of inorganic crystalline apatite. This is followed by the degradation of organic collagenous matrix by proteolytic enzymes, mainly MMPs and cathepsins (Henneman et al., 2008, Sprogar et al., 2008, Hill, 1998).

In the tension region, osteoblasts are responsible for forming the new bone. Stretched PDL and alveolar bone can increase osteogenic gene expression and induce bone formation. Bone formation is the consequence of a complex series of events that includes the proliferation of primitive mesenchymal cells, differentiation into osteoblast precursor cells, maturation of osteoblasts, ECM synthesis, and mineralization. When the new layer of bone thickens, some osteoblasts become entrapped in the bone and transform into osteocytes, and Sharpey's fibres

(principal fibres of PDL) will likewise be entrapped in the newly formed bone (Hill, 1998, Masella and Meister, 2006, Henneman et al., 2008, Krishnan and Davidovitch, 2009).

Nitric oxide (NO) is a key regulator of bone responses to mechanical stress that is generated by eNOS or inducible nitric oxide synthase (iNOS). NO is a significant bone remodelling regulator during OTM; it is involved in adaptive bone formation, osteocyte protection against apoptosis, and osteoclastic activity. Endothelial NOS is involved in bone formation in the tension region, whereas iNOS is involved in bone resorption in the compression region (Tan et al., 2009).

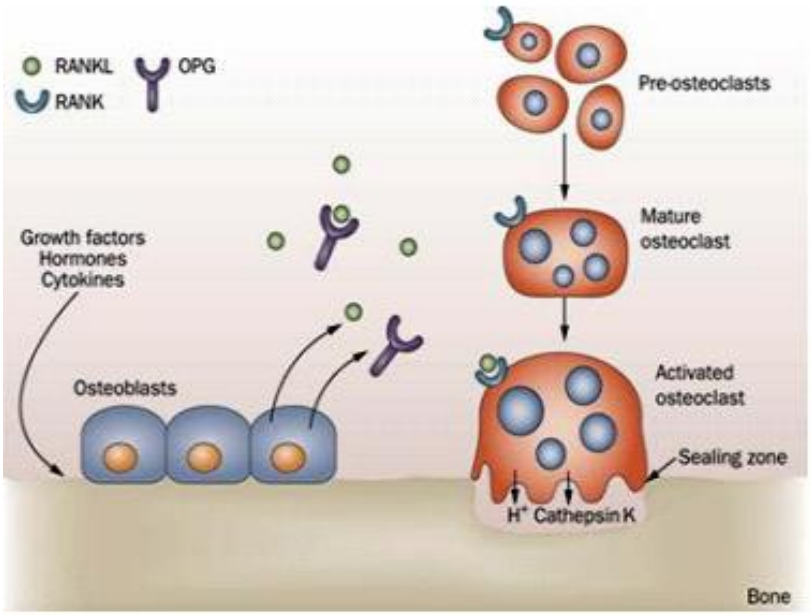

Figure 1.5 RANKL/RANK/OPG pathway.

Pre-osteoclasts are differentiated from hematopoietic stem cells. Osteoblasts derived from mesenchymal stem cells produce receptor activator of nuclear factor kappa B ligand (RANKL). RANKL binds to RANK receptor on pre-osteoclast producing mature osteoclasts which resorb bone. Osteoprotegerin (OPG) produced by osteoblasts inhibits this process by binding to RANKL. Adapted from (Patil and Desai, 2014).

1.1.5.1 Role of MMPs in alveolar bone resorption

Bone resorption by osteoclasts necessitates removal of both the mineral and organic matrix components, and it entails two processes: inorganic bone matrix demineralization by acidification and organic matrix (mainly collagen type I) dissolution by proteases (Domon et al., 1999, Delaissé et al., 1993, Rice et al., 1997). Collagenases, produced by osteoblasts, degrade the organic unmineralized osteoid covering the resting bone surface, allowing osteoclasts access to the m mineralized tissue (Everts et al., 1992). Additionally, it has been shown that interstitial collagenases stimulate bone resorption by activating osteoclasts to resorb bone; inhibition of these collagenases with a particular anti-collagenase inhibitor decreased bone resorption (Holliday et al., 2003b).

Cathepsins and MMPs are both involved in collagenous bone matrix degradation (Domon et al., 1999, Sires et al., 1995). MMP-1 (Domon et al., 1999, Delaissé et al., 1993) and cathepsin K (Bossard et al., 1996, Drake et al., 1996, Domon et al., 1999, Littlewood-Evans et al., 1997) cleave native collagen type I and thus are considered to be crucial in bone resorption. MMP-1, produced by osteoblasts, degrade the unmineralized organic osteoid from bone surfaces exposing the mineralized bone matrix to osteoclasts (Domon et al., 1999).

MMP-9 is abundantly expressed by osteoclast in human and mice developing bone tissues (Rice et al., 1997, Okada et al., 1995) and degrades collagenous bone matrix in concert with MMP-1 and Cathepsin K (Okada et al., 1995). MMPs, in addition to direct bone collagen matrix degradation, can regulate bone resorption via osteoclast activation and differentiation (Franco et al., 2017). MMP-9 has a pivotal role in the recruitment of osteoclasts, as it has been shown that MMP-9 knockout mice demonstrated a delay in osteoclast recruitment (Engsig et al., 2000).

1.2 Duration and rate of OTM

Duration of orthodontic treatment is one of the first things new orthodontic patients ask about. The success of orthodontic treatment is greatly dependent on the accurate estimation of the proposed treatment duration (Shia, 1986). Patients who are provided with accurate information about proposed treatment duration seem to be better consumers of dental services, having more realistic expectations of treatment outcomes and higher levels of satisfaction with their overall care (Klein, 1988, Mavreas and Athanasiou, 2008, Cunningham et al., 1996, Popowich et al., 2006, O'Connor, 2000).

Orthodontic treatment is lengthy and can last from one to three years, with non-extraction cases taking 21-27 months and extraction cases taking 25-35 months (Buschang et al., 2012). Prolonged treatment times are associated with iatrogenic consequences of treatment, including increased risk of root resorption (Kurol et al., 1996, Segal et al., 2004), decalcification (Årtun and Brobakken, 1986), and periodontal problems (Ristic et al., 2007). Furthermore, prolonged treatment times may result in poor profitability in practices (Turbill et al., 2001) and adversely influence patient compliance (Fleming et al., 2015) and satisfaction with the treatment outcomes (Pachêco-Pereira et al., 2015).

Comprehensive orthodontic treatment consists of alignment and levelling, correction of molar relationship and space closure, and finishing phases (Proffit et al., 2019). Alignment of teeth is one of the main objectives of orthodontic treatment. Correct orthodontic diagnosis and treatment planning needs an accurate assessment of dental crowding and the space needed to alleviate it. Multiple methods have been reported in the literature to assess crowding, including the visual approach (Beazley, 1971), the brass wire technique and its modifications (Carey, 1958), the use of a catenometer, a mathematical model to compute arch length (Musich and Ackerman, 1973), and the addition of straight segments of the arch techniques (Lundstrom,

1955). However, these methods often rely on an estimate of the arch perimeter, which might introduce inconsistency (Johal and Battagel, 1997).

In 1975, Little's irregularity index (LII) was proposed as a valid and reliable quantitative method to assess lower anterior alignment. The proposed method entails measuring the linear displacement between the anatomical contact points of the lower anterior teeth, with the total of these five displacements representing the relative degree of anterior irregularity (Little, 1975). This was originally developed to evaluate the irregularity of the lower incisor segment, which is a limiting factor in treatment and stability. The application of this index is simple and quick, and considerable interest has been in its usage in a community setting to estimate arch length discrepancy (Bernabé and Flores-Mir, 2006). It is a reliable measure for the rate of alignment used by numerous studies to assess the efficiency of different treatment approaches in alleviating the irregularity of anterior teeth, which is measured as the difference in irregularity index before and after alignment divided by the duration of alignment (Woodhouse et al., 2015, Scott et al., 2008, Uribe et al., 2017, Songra et al., 2014, Ulhaq et al., 2017, Pandis et al., 2007, Ong et al., 2011, Little and Spary, 2017, Charavet et al., 2019, Gibreal et al., 2019).

Extraction space closure is the most time-consuming phase of orthodontic treatment, accounting for 1/3 to 1/2 of the total treatment duration. Canine retraction rates with traditional treatments range from 0.5 to 1 mm per month, depending on the patient's age and gender, with 5 to 9 months required to complete canine retraction (Abbas et al., 2016). Canine retraction is a widely held experimental model during investigations of the effectiveness of different treatment modalities during orthodontic treatment. Quantification of the distance the canine moves relative to reference points have been conducted by several studies to measure the rate of canine retraction (Aboul-Ela et al., 2011, Al-Shafi et al., 2021, Doshi-Mehta and Bhad-Patil,

2012, Karci and Baka, 2021, Varella et al., 2018, Mistry et al., 2020, Alkebsi et al., 2018, Abbas et al., 2016).

1.2.1 Factors affecting duration of orthodontic treatment

Knowledge of the factors affecting the duration of orthodontic treatment might be beneficial for several reasons, leading to more effective patient counselling, more accurate treatment cost estimation, enhanced cost efficiency, and therefore improved clinical practice (Beckwith et al., 1999, Popowich et al., 2006, Skidmore et al., 2006, Fink and Smith, 1992). There are four major categories of possible determinants of orthodontic treatment time: sociodemographic parameters, patient cooperation, malocclusion features, and treatment method (Skidmore et al., 2006).

The impact of sociodemographic variables such as age, gender, and socioeconomic status on treatment duration is not yet fully acknowledged. Some studies found that age was not associated with treatment duration (Beckwith et al., 1999, Fink and Smith, 1992, Robb et al., 1998), while others demonstrated that the older the patient, the shorter the treatment duration, attributed to older patients' greater compliance (Vig et al., 1990, Popowich et al., 2005). The literature contains controversial information regarding the effect of gender on treatment duration (Skidmore et al., 2006, Aljehani and Baeshen, 2018, Clemmer and Hayes, 1979). In addition, there is disagreement over the impact of socioeconomic status on cooperation and treatment time, with no clear consensus on whether a lower socioeconomic status is related to a shorter or longer treatment time (Egolf et al., 1990, Turbill et al., 2001, Starnbach and Kaplan, 1975).

Patient cooperation and compliance factors have a significant effect on treatment duration. Non-compliance problems, such as missed appointments, failure to use accessory

devices, poor oral hygiene, band or bracket replacement, poor elastic wear, and appliance breakage, all contribute to prolonged treatment duration (Beckwith et al., 1999, Skidmore et al., 2006, Fink and Smith, 1992, Vu et al., 2008, Robb et al., 1998). Patients who maintain good oral hygiene are also more likely to cooperate with other aspects of treatment (Egolf et al., 1990). Because compliance is crucial, it is suggested that patients' motivation be maintained during orthodontic treatment. Texting patients using mobile applications helped to promote patient compliance, resulting in 7.3 weeks less treatment duration, 7% fewer missed appointments, 10% fewer late patients, and 4% less appliance breakage (Li et al., 2016).

Treatment method chosen has been found to affect treatment duration, with premolar extractions tending to prolong treatment duration (Fink and Smith, 1992, Fisher et al., 2010, Skidmore et al., 2006, Leon-Salazar et al., 2014, Janson et al., 2006, Papageorgiou et al., 2017, Germeç and Taner, 2008, O'Brien et al., 1995). This can be attributed to an association between extractions and complex cases, as well as the requirement for an additional space closure step (Papageorgiou et al., 2017). It has been reported that interproximal stripping to avoid extractions in borderline cases may reduce treatment duration by eight months (Germeç and Taner, 2008). Moreover, the treatment duration of patients treated in two or more phases was nearly eight months longer than those treated in one phase (Beckwith et al., 1999), consistent with other studies (Vig et al., 1990, O'Brien et al., 1995). Prescribing headgear wear during orthodontic treatment prolonged treatment duration on average from 3.7 to 6.1 months (Beckwith et al., 1999, Vu et al., 2008). Additionally, patients who had rapid palatal expansion or orthognathic surgery had considerably longer treatment times (7.4 and 3.4 months, respectively) (Vu et al., 2008).

Some studies have focused on treatment duration associated with specific malocclusions. One study found that the duration of treatment was five months longer in Class II division 1 than in Class I malocclusion (Vig et al., 1998). This agrees with other studies

reporting that Class II treatment lasted longer than Class I treatment (Aljehani and Baeshen, 2018, Vu et al., 2008, Skidmore et al., 2006, Popowich et al., 2005). In addition, ANB angle (Popowich et al., 2005, Fink and Smith, 1992), large overjet (Robb et al., 1998, Popowich et al., 2005, Fisher et al., 2010), buccal occlusion (Robb et al., 1998, Turbill et al., 2001), and vertical pattern (Fink and Smith, 1992) can contribute to prolonged Class II treatment. One study reported that Class III malocclusion treatment was significantly associated with an increase in treatment time by 4.1 months compared to class I (Vu et al., 2008).

The duration of treatment is dictated by the rate of OTM, which is controlled by the rate of bone remodelling and osteoclast activity. Numerous variables may affect remodelling activity and, eventually, tooth movement, either alone or in combination, including occlusion forces (Lee, 1995), type of movement (tipping or bodily movement) (Shpack et al., 2008, Lee, 1995), drug consumption (Knop et al., 2011, Bartzela et al., 2009), systemic conditions (Saloom et al., 2017), periodontal disease (Okamoto et al., 2009), age and intrinsic genetic variables (Dudic et al., 2013), and individual differences in PDL remodelling, bone density, and bone metabolism (Pilon et al., 1996).

1.2.2 Acceleration of OTM

Duration of treatment is influenced by a variety of factors, among which is the rate of OTM. When continuous forces are applied to the teeth, The rate of OTM is estimated to be 0.8-1.2 mm per month (Sugimori et al., 2018). The duration of treatment is a big burden for many people undergoing orthodontic treatment and a major concern for both orthodontists and patients; indeed, there is evidence that orthodontic treatment using fixed appliances might negatively influence the quality of life (Johal et al., 2014). The duration of treatment is often beyond patient expectations, and both patients and orthodontists have become recently

fascinated and receptive to methods that might accelerate tooth movement and hence shorten treatment duration (Uribe et al., 2014), especially for adult patients who desire to complete their treatment with the shortest time possible for social and aesthetic considerations (Rosvall et al., 2009). Therefore shortening treatment duration is desirable, with time-saving for both orthodontists and patients, as well as a potential reduction in related expenses, discomfort, and iatrogenic consequences of orthodontic treatment (Fleming et al., 2015).

A wide range of novel conventional and non-conventional approaches for accelerating the rate of OTM has been proposed in recent years. The conventional treatment interventions involve substantial innovations in the design and manufacturing of fixed appliances (Songra et al., 2014, Samuels et al., 1993, Hayashi et al., 2004, Mandall et al., 2006, Pandis et al., 2009, Scott et al., 2008, Fleming et al., 2009, Ulhaq et al., 2017, Penning et al., 2017); however, there is insufficient evidence to demonstrate that bracket materials and design, ligation methods, initial archwires, or archwire sequence may significantly impact how rapidly teeth move (Papageorgiou et al., 2014a, Papageorgiou et al., 2014b, Jian et al., 2013). The non-conventional approaches include surgical and non-surgical adjuncts to orthodontic treatment advocated for accelerating OTM.

1.2.2.1 Surgical approaches

Several surgical approaches with varied degrees of invasiveness have been proposed to accelerate the rate of OTM, including corticotomy, piezocision, micro-osteoperforation (MOP), and others. These techniques can potentially reduce treatment duration but are rather invasive. In general, surgical procedures to accelerate OTM rely on increasing osteoclastic activity by triggering a regional acceleratory phenomenon (RAP) (Wilcko et al., 2001, Fleming et al., 2015). RAP is a tissue reaction to noxious stimuli that promotes healing capability (Frost, 1989). These cellular processes decrease bone density, which may reduce the obstruction to

tooth movement (Teixeira et al., 2010). Based on evidence from low to moderate quality studies, surgically assisted orthodontics appears safe for oral tissues and is characterised by a transient phase of accelerated tooth movement, which can significantly shorten treatment duration (Hoogeveen et al., 2014). In addition, a Cochrane review reported on low quality evidence regarding the effectiveness of surgical interventions in accelerating OTM (Fleming et al., 2015).

Corticotomy is a surgical technique that involves intentional surgical injury to cortical bone. It was introduced in 1959 to speed up OTM (Köle, 1959). It involves the elevation of a full-thickness mucoperiosteal flap to expose buccal and lingual alveolar bone, followed by vertical incisions made between the roots of the teeth through the cortical bone and horizontal cuts connecting the vertical cuts 2-3 mm above the apices. Trauma to the bone triggers RAP, leading to an increase in bone turnover and a decrease in bone mineral content and thus accelerates OTM (Baloul et al., 2011, Wang et al., 2009). Corticotomy has been shown to dramatically increase the rate of canine tooth movement in the first two months following the surgery. However, a significant decrease in tooth movement rate is observed after the third month of observation (Aboul-Ela et al., 2011). Similarly, this is consistent with the results of two other studies, which reported faster rates of OTM in the corticotomy groups (Al-Naoum et al., 2014, Abbas et al., 2016). Nevertheless, this technique is invasive and accompanied by increased cost, discomfort, and morbidity for the patient. It negatively impacts oral health-related quality of life, with only a partial recovery after seven days (Cassetta et al., 2012). Two recent systematic reviews reported that corticotomy accelerates OTM and results in shorter treatment times; however, more robust evidence-based research is needed to support these findings (Gil et al., 2018, Viwattanatipa and Charnchairerk, 2018).

Minimally invasive surgical approaches have been introduced into orthodontic treatment as an alternative to invasive conventional corticotomy, such as piezocision, MOPs,

and laser-assisted flapless corticotomy. These minimally invasive surgical approaches rely on RAP for accelerating OTM. A recent systematic review has indicated that minimally invasive surgical approaches have some influence on accelerating tooth movement and do not increase pain, periodontal parameters, or root resorption following their application (Fu et al., 2019).

MOP is considered a minimally invasive surgical method for accelerating OTM because there is no need to elevate a full-thickness flap or perform additional soft tissue incisions before the osteoperforation. It involves creating small shallow perforations directly through the gingival tissues into the bone by the tooth to which the orthodontic force is applied. MOP solves most of the issues associated with conventional corticotomy procedures, and unlike other less invasive surgical approaches, they can be performed by orthodontists utilising commonly used orthodontic instruments (Shahabee et al., 2020). MOP with clinical replication in humans were first introduced in 2013. Those authors reported that MOP are an effective and safe procedure that increased the canine retraction rate by 2.3 folds compared to the control group and decreased orthodontic treatment duration by 62% (Alikhani et al., 2013). On the other hand, a spilt-mouth RCT evaluated the effect of MOP on the rate of canine retraction and found no significant effect of MOP on the rate of OTM compared with the control side in a 3-month period. (Alkebsi et al., 2018). A systematic review concluded that there is insufficient evidence to indicate if a single application of MOP can speed up tooth movement and that there is low-quality evidence that flapless corticotomy treatments may hasten tooth movement (Fu et al., 2019). Another recent systematic review concluded that MOPs increase the rate of tooth movement, although, in at least one study, greater root resorption was observed (Shahabee et al., 2020).

Piezocision, a minimally invasive surgical procedure for accelerating OTM, was introduced in 2009 (Dibart et al., 2009). It involves making incisions in the buccal gingiva parallel to the long axis of the teeth without flap elevation, followed by incisions in the buccal

cortical plates with a piezo-surgery knife. Piezocision is regarded as one of the best and safest surgical procedures due to its superior periodontal tissue response and aesthetic outcomes while being the least invasive surgical approach with no side effects on periodontal tissue (Nimeri et al., 2013, Kilinc and Baka, 2022). Piezocision has recently shown promising results in speeding the rate of OTM and shortening treatment duration. Several clinical trials have been undertaken to assess the effect of piezocision on the rate of OTM. A split-mouth clinical trial comparing corticotomy or piezocision procedures to conventional maxillary canine retraction following first premolar extraction revealed that both treatments might enhance the rate of OTM (Abbas et al., 2016). Likewise, two other RCTs demonstrated a significant decrease in overall treatment duration in piezocision groups compared to conventional treatment groups (Charavet et al., 2016, Charavet et al., 2019). On the other hand, one RCT reported no evidence that piezocision was more efficient in alleviating mandibular anterior crowding (Uribe et al., 2017). Two systematic reviews have claimed that there is weak evidence to demonstrate that this approach is efficient in accelerating OTM (Hoffmann et al., 2017, Viwattanatipa and Charnchairerk, 2018). However, these findings should be interpreted cautiously since the number of included studies was small and might not be representative. A recent RCT compared the effectiveness of piezocision and that of MOPs approaches in accelerating the alignment of the mandibular anterior teeth and found that piezocision enhanced the levelling of mandibular front teeth during 16-week period, mainly in the first 12 weeks, whereas MOPs had no impact (Kilinc and Baka, 2022).

1.2.2.2 Non-surgical approaches

Non-surgical approaches advocated to accelerate OTM include resonance vibration, pulsed electromagnetic fields, photobiomodulation, and pharmacological approaches. It has been proposed that these interventions could serve as a bio-stimulus to stimulate bone cell

(osteoblast and osteoclast) activity, so the enhanced rate of bone remodelling can accelerate the rate of OTM, thereby shortening the time of orthodontic treatment (Tortamano et al., 2009, El-Angbawi et al., 2015). A Cochrane review conducted in 2015 concluded that there is insufficient evidence to determine if non-surgical adjunctive procedures to accelerate OTM are beneficial (El-Angbawi et al., 2015).

Vibrational devices have been proposed as a method of increasing the rate of OTM by enhancing periodontal and bone remodelling. This approach involves applying low-level vibration to the teeth while subjected to orthodontic forces. Vibrational forces have been found to help maintain bone mass in postmenopausal women (Rubin et al., 2004) and people with limited mobility and prolonged bed rest (Holguin et al., 2009). Additionally, data from animal models indicate that vibration induced higher rates of tooth movement, osteoclastic activity, and bone remodelling within the periodontium (Darendeliler et al., 2007, Nishimura et al., 2008). These data have aided in the development of commercial vibrational devices for clinical usage. AcceleDent is one example of a commercially available vibrational device. It is a rechargeable device comprising an activator and a removable mouthpiece, which provides a vibrational frequency of 30 Hz and a force of 0.2 N. Patients are asked to bite on the vibrating plastic wafer for 20 minutes per day (Woodhouse et al., 2015). A 3-arm parallel-group RCT (An AcceleDent group, an AcceleDent sham group, and a no-device group) was conducted to assess the effect of supplemental vibrational forces on the rate of OTM and duration of orthodontic treatment during the alignment and space closure phases of fixed-appliance orthodontic treatment. No evidence was found to indicate that supplemental vibrational forces might increase the initial tooth movement or space closure rate. Likewise, no evidence was found that they can reduce the time needed to achieve final alignment or overall treatment duration (Woodhouse et al., 2015, DiBiase et al., 2018). A systematic review published in 2018 assessing the effectiveness of vibrational forces that included five RCTs indicated that the

results from all but one included trial demonstrated no advantage from using vibrational devices during orthodontic treatment (Aljabaa et al., 2018). The consensus in the literature at present is that supplemental vibrational forces do not cause a clinically significant increase in OTM rate in initial alignment or space closure phases (Miles et al., 2012, Miles and Fisher, 2016, Woodhouse et al., 2015, Katchooi et al., 2018, DiBiase et al., 2018).

Photobiomodulation approach involves using low-level lasers or light-emitting diodes to modify cellular function by exposing cells or tissues to low levels of red and near-infrared light (600–1000 nm) (Miles, 2017). This triggers a photochemical reaction in which the light energy is absorbed by photoreceptors and transformed into adenosine triphosphate by mitochondria, enhancing DNA, RNA, and protein production and increasing cellular proliferation and activity. Several clinical trials have been conducted to assess the effect of low level laser therapy (LLLT) (Doshi-Mehta and Bhad-Patil, 2012, Dakshina et al., 2019, Varella et al., 2018, Mistry et al., 2020) and light-emitting diode lights (Al-Shafi et al., 2021, Ekizer et al., 2016) on the rate of OTM. A split-mouth RCT demonstrated that LLLT caused an increase of 30% in the rate of OTM and had no adverse effects on the vitality or the periodontium of the teeth (Doshi-Mehta and Bhad-Patil, 2012). In addition, another split-mouth RCT showed that OTM with LLLT was approximately two times faster than conventional treatment (Varella et al., 2018). On the other hand, two other clinical trials found no effect of LLLT on canine retraction rate (Limpanichkul et al., 2006b, Mistry et al., 2020). Likewise, a recent randomized split-mouth trial demonstrated that light-emitting diode light did not affect canine retraction rate (Al-Shafi et al., 2021). There is low to moderate evidence that photobiomodulation is effective and can increase OTM rates (Gkantidis et al., 2014, Sonesson et al., 2016, Miles, 2017).

Pulsed electromagnetic fields approach is a non-invasive, non-thermal treatment that uses pulsating electromagnetic fields in tissue to induce healing by enhancing blood circulation

(Strauch et al., 2009). It has been suggested that pulsed electromagnetic fields impact the activity of intracellular cyclic adenosine monophosphate and cyclic guanosine monophosphate. This may accelerate bone remodelling and, thus, OTM (Darendeliler et al., 1995). An integrated circuit in a removable appliance and a watch battery generates a 1 Hz electric current to the teeth (Bhad Patil and Karemore, 2022). The application of pulsed electromagnetic fields increased the rate of tooth movement in animals (Stark and Sinclair, 1987, Darendeliler et al., 1995). In humans, a non-randomised prospective trial investigating the effects of pulsed electromagnetic fields on OTM revealed a 0.3mm/month increase in canine retraction rate (Showkatbakhsh et al., 2010). Moreover, a recent split-mouth RCT found that pulsed electromagnetic fields caused an increase of 31% in the rate of OTM (Bhad Patil and Karemore, 2022).

Pharmacological approaches to accelerate OTM have also been proposed. This approach aims to alter the biological response to orthodontic forces. In the literature, much of the data comes from animal studies rather than human studies, and even though insight into their effects is provided, the results cannot be used to predict human effects (Miles, 2017). In animal studies, corticosteroid hormones, vitamin D3, thyroxin, and parathyroid hormone have all been demonstrated to enhance OTM (Bartzela et al., 2009). The findings of human trials assessing the effect of prostaglandin on OTM have revealed a potential acceleration (Yamasaki et al., 1984, Spielmann et al., 1989, Patil et al., 2005). One study assessed the rate of OTM with and without a submucosal injection of prostaglandin E1. The results revealed that the rate of canine retraction was about 1.6-fold higher on the experimental injection side compared to the control side (Yamasaki et al., 1984). However, the use of these medications is currently limited due to the requirement for regular administration and the severe pain associated with the injection. On the other hand, several medications, such as nonsteroidal anti-inflammatory drugs, dietary calcium, and bisphosphonates, decreased the rate of OTM (Bartzela et al., 2009).

A recent meta-analysis of 27 animal studies demonstrated that the administration of diazepam, Vitamin C and pantoprazole increased the rate of OTM, whereas simvastatin, atorvastatin, strontium ranelate, calcium compounds, losartan, propranolol, famotidine, cetirizine, and metformin decreased the rate of OTM (Makrygiannakis et al., 2018). Another systematic review concluded that local injection of prostaglandin E1 was found to increase OTM rate, while systemic intake of Nabumetone reduced OTM. On the other hand, tenoxicam administration, drinking water with fluoride or local injection of calcitriol (vitamin D metabolite) had no effect on OTM rate (Kaklamanos et al., 2019).

Recent studies have proposed using platelet-rich plasma and platelet-rich fibrin as alternatives for the local injection of cytokines or medications to reproduce the effects induced in bone during surgery. Platelet-rich plasma is described as an autologous concentrate of platelets in a minute amount of plasma. It has a large number of platelets, coagulation factors, and growth factors (Paoloni et al., 2011). Platelet-rich fibrin is a fibrin structure derived from natural blood that comprises platelets and leukocytes. It does not have anticoagulants, thrombin, or calcium chloride (Koçyiğit et al., 2012, Dohan Ehrenfest et al., 2009). Platelets have numerous secretory granules, which contain various proteins, growth factors and chemokines essential for haemostasis and soft and hard tissue wound healing (Anitua et al., 2004, Miles, 2017). A split-mouth RCT investigated the effect of local injection of platelet-rich plasma on the rate of OTM. Local injection of platelet-rich plasma was done before canine retraction and at 3 and 6 weeks after applying the retracting forces. The results showed a faster canine retraction rate on the intervention side in the first and second months by 15% and 5%, respectively, compared to the control side, indicating a short-term effect and the need for repeated injections to maintain a steady rate acceleration of OTM (El-Timamy et al., 2020). Another recent split-mouth RCT compared the effects of local injection of platelet-rich fibrin and piezocision on canine retraction rate. The results showed that both interventions

accelerated OTM compared to the control, but with no difference between the two groups regarding speed, duration of tooth movement, or periodontal parameters (Karci and Baka, 2021).

1.3 Obesity

Obesity, which is characterised by excessive fat accumulation, is a metabolic disease resulting from a sedentary lifestyle, lack of physical activity, increased calorie intake, hereditary factors, and hormonal disturbance. Obesity is becoming increasingly prevalent in many countries worldwide, with overweight and obesity being associated with more deaths globally than underweight. In addition, obesity has been regarded as one of the most critical public health and medical issues of our time (Giuca et al., 2012, Marcantonio et al., 2021). It is widely considered a major risk factor for various diseases, including cardiovascular disease, rheumatoid arthritis, type 2 diabetes mellitus, and some types of cancer, both in obese adults and children (Chang et al., 2011, Lobstein et al., 2004, Must and Strauss, 1999).

Overweight and obesity have been an issue in economically developed countries in the past few decades. The growing number of overweight adults is concerning, but children and adolescents are also significantly affected (Ogden et al., 2010, Daniels et al., 2009). It is estimated that 287 million school-age children are overweight globally (von Bremen et al., 2013). According to data from the United Kingdom, 22% of boys and 28% of girls aged 2-15 years were overweight or obese in 2002 (Seddon, 2005), with an increasing trend .

Several methods for measuring weight have been described, including waist circumference, body mass index (BMI), and age- and sex-specific BMI percentiles. In adults, persons with a body mass index (BMI) ≥ 25 kg/m^2 are considered overweight, while those with a BMI ≥ 30 kg/m^2 deemed obese (Alberti et al., 2006). On the other hand, age- and sex-specific

BMI percentiles provide a rapid, non-invasive, and easily accessible way to measure children's weight. Obese children are those who are above the 95th percentile; overweight children are those who are between the 85th and 95th percentiles; normal-weight children are those who are between the fifth and 85th percentiles; and underweight children are those who are below the fifth percentile (Mack et al., 2013, Cole, 1979).

Obesity is characterized by chronic subclinical inflammation mediated by the release of systemic pro-inflammatory factors (Deng et al., 2016) and has been demonstrated to increase susceptibility to infection through immune response modulation (Falagas and Kompoti, 2006). Adipose tissue may influence the severity and resolution of inflammatory processes in various tissues (Pierpont et al., 2014, Issa and Griffin, 2012). Adipocytes, the predominant cells in the adipose connective tissue, produce a variety of inflammatory mediators, resulting in a systemic inflammatory state that may impair wound healing (Ouchi et al., 2011). In addition, adipocytes synthesize metabolically active proteins and adipokines that influence metabolic function and inflammation. Adipocyte-derived adipokines, such as pro-inflammatory leptin (Fantuzzi, 2005) and anti-inflammatory adiponectin (Iwayama et al., 2012), are soluble proteins that bind to particular receptors on target cells and they play an important role in inflammatory illnesses such as periodontal disease (Ouchi et al., 2011).

Periodontal diseases are associated with a variety of systemic diseases, including obesity. Chronic periodontitis was found to be significantly associated with obesity in a meta-analysis of 28 studies (Chaffee and Weston, 2010). There is evidence of an increased risk of chronic periodontitis (Suvan et al., 2011) as well as differences in inflammatory and metabolic markers in obese patients with periodontal diseases compared to normal-weight patients (Papageorgiou et al., 2015a). Obese persons have greater blood levels of leptin and lower adiponectin levels than normal-weight persons (Reid, 2008), which may increase the likelihood of periodontal inflammation and destruction (Kraus et al., 2012). The findings of one particular

study into the relationship between periodontal disease and overweight and obese people indicate that being overweight, obesity and increased waist circumference may all be risk factors for the development or worsening of periodontal measures like probing depth, alveolar bone loss, attachment loss, and plaque index (Keller et al., 2015).

Obesity can affect bone metabolism through a variety of mechanisms. Obesity may promote adipocyte differentiation and fat accumulation while decreasing osteoblast differentiation and bone formation. It may directly affect bone formation or indirectly affect bone resorption through upregulated pro-inflammatory cytokine production induced by excessive leptin secretion and decreased adiponectin production in obesity (Cao, 2011). Obesity may promote bone resorption by upregulating pro-inflammatory cytokines such as IL-6 and TNF-α. Both have been demonstrated to stimulate osteoclast activity by modulating the RANKL/RANK/OPG pathway (Khosla, 2001).

1.3.1 Obesity and orthodontics

Obesity is increasing worldwide, and obese individuals are more likely to present for orthodontic treatment in greater numbers. Obesity may substantially impact orthodontic treatment due to its effect on bone metabolism, craniofacial growth, pubertal growth and possibly tooth movement during orthodontic treatment of obese people. In addition, obesity in adolescents is associated with apparent psychological and psychological issues, which might influence patient attitude and compliance with orthodontic treatment. The facial aesthetics of obese people differ from those of normal-weight persons as well, with obese people having larger mandibles and shorter upper facial heights. These factors should be taken into consideration when planning treatment, and obese patients should be treated individually, with special consideration given to their psychosocial status (Neeley and Gonzales, 2007).

There is scarce data on the association between BMI and oral health/cooperation during orthodontic treatment in the literature. A group of authors looked at the differences in the incidence of white spot lesions and gingivitis, cooperation level, and treatment duration among normal-weight, overweight, and obese orthodontic patients undergoing fixed appliance treatment. The results showed that a higher BMI appears to be linked with more oral health issues, including higher white spot lesions and gingivitis incidence (von Bremen et al., 2016). In addition, overweight patients showed poor cooperation and had a more extended treatment duration with more appointments during orthodontic treatment than normal-weight counterparts (von Bremen et al., 2013, von Bremen et al., 2016).

Dental development and skeletal maturation are commonly used in growing children to predict the timing of orthodontic treatment and treatment methods. One retrospective study compared skeletal maturation in obese and normal-weight patients using carpal analysis and cervical vertebral maturation methods. According to the carpal analysis, obese patients exhibited a more significant mean discrepancy between skeletal and chronological ages and a significantly greater cervical vertebral maturation score (Giuca et al., 2012). Another study assessed the relationship between BMI percentile and skeletal and dental maturity using the cervical vertebral method and the Demirjian assessment method, respectively, in adolescent orthodontic patients. The results showed that the cervical vertebral stage and dental age were more advanced in individuals with increased BMI percentiles. Therefore, it is recommended that orthodontists should consider obtaining objective weight data for treatment planning purposes (Mack et al., 2013).

The effect of obesity on OTM was investigated in a recent prospective clinical cohort study, with tooth alignment rate and duration assessed in obese and normal-weight adolescents undergoing fixed-appliance orthodontic treatment. Data were collected at baseline, 1-hour and 1-week after fixed appliance placement, and at the completion of mandibular arch alignment.

The results showed that the rate of OTM was significantly higher in obese patients compared to normal-weight, and obese patients needed less time to achieve tooth alignment than normal-weight, but this was not significant. GCF leptin levels differed significantly between obese and normal-weight patients and were associated with observed rates of OTM (Saloom et al., 2017).

Recently, the effect of obesity on periodontal tissue remodelling induced by orthodontic forces has been investigated in a recent animal study. The results showed that obesity modulates periodontal tissue remodelling during orthodontic movement, resulting in lower bone volume fraction and bone mineral density, more inflammation, and a tendency for faster tooth movement (Marcantonio et al., 2021).

1.3.2 Leptin

Leptin is a 16 kDa non-glycosylated polypeptide hormone produced mainly by adipocytes but also in small amounts by the placenta, stomach, salivary glands, and osteoblasts (Gröschl et al., 2001, Reseland et al., 2001, Ouchi et al., 2011, Zhang et al., 1994, Masuzaki et al., 1997, Bado et al., 1998). Leptin regulates energy expenditure and body weight and modulates appetite and satiety (Sahu, 2003), a function controlled mainly by the hypothalamus (Friedman and Halaas, 1998). Circulating levels of leptin are elevated in obesity (Paul et al., 2011, Considine et al., 1996).

Leptin is a proinflammatory cytokine that plays a key role in the inflammatory responses by modulating the activity of immunocytes such as T-cells, monocytes, and natural killer cells. Leptin directly activates these immune cells, causing an increase in the production of other inflammatory mediators (Zhu et al., 2017). Leptin and its receptor share structural features with IL-6 cytokines and type I cytokine receptors, respectively (Fantuzzi and Faggioni, 2000). The cytokines of the IL-6 family have a wide range of local and systemic actions and

are notably well-studied in terms of inflammation, immunity, and wound healing (Taga and Kishimoto, 1997). Therefore, leptin has been thought to have similar functions, and various investigations have provided evidence to support this notion (Matarese et al., 2005).

Recent studies have reported that leptin is produced in periodontal cells and contributes to periodontal infection and healing (Li et al., 2015). It has been demonstrated that leptin is present within healthy and marginally inflamed gingiva (Johnson and Serio, 2001) and that leptin levels in GCF decrease significantly as periodontal disease progresses, adding to our knowledge of leptin's protective function in periodontal health (Karthikeyan and Pradeep, 2007). On the other hand, another study evaluated leptin levels in four groups (chronic periodontitis, chronic periodontitis with obese, obese, and healthy) and found higher blood levels of leptin in the chronic periodontitis and/or obese groups than in the healthy group (Zimmermann et al., 2013).

Some studies have investigated leptin levels in the saliva and GCF of obese and normal-weight orthodontic patients. Leptin levels in the GCF of normal-weight patients are reduced during OTM in a time-dependent manner (Dilsiz et al., 2010, Sar et al., 2019). In a prospective clinical trial, GCF levels of leptin and RANKL were significantly different between obese and normal-weight patients and were significantly associated with observed rates of OTM (Saloom et al., 2017). Another study reported that leptin levels in the saliva of obese patients were three times greater than those of normal-weight patients during orthodontic treatment, demonstrating a strong correlation between leptin and tooth movement in these patients (Jayachandran et al., 2017).

One in vitro study investigated the effects of leptin on the expression profile of human periodontal fibroblasts during simulated orthodontic mechanical strain. The cells were exposed to mechanical forces using the weight method with or without different leptin concentrations.

The results showed that high leptin concentrations resulted in increased expression of pro-inflammatory factors and RANKL in compressed periodontal fibroblasts. Therefore, increased osteoclastogenesis can be assumed to accelerate bone resorption and, consequently, the rate of OTM in the orthodontic treatment of obese patients (Schröder et al., 2021).

1.3.3 Adiponectin

Adiponectin, a secretory protein produced by adipocytes, performs different activities in the targeting of different types of cells. Specifically, it has been found to possibly affect insulin resistance, inflammation, and cardiovascular systems (Funahashi et al., 1999). Adiponectin has to bind to its receptors (AdipoR1 and AdipoR2) to exert its effects. Both receptors are present in in the fibroblasts of gingiva and PDL and exert anti-inflammatory effects (Iwayama et al., 2012). This adipokine regulates metabolic (Choi et al., 2007) and immune processes by suppressing pro-inflammatory cytokines and enhancing anti-inflammatory cytokines production (Polyzos et al., 2010, Iwayama et al., 2012, Deschner et al., 2014). Adiponectin levels are lower than normal in inflammatory disorders such as obesity, insulin resistance, and diabetes (Ouchi et al., 2003).

Adiponectin contributes to periodontal infection and inflammation (Nokhbehsaim et al., 2014), with its serum levels decreasing in periodontitis and increasing again following periodontal therapy (Deschner et al., 2014). Other studies have also found a lower number of adiponectin receptors in individuals with severe periodontitis compared to their healthy counterparts (Yamaguchi et al., 2010, Saito et al., 2008). A recent meta-analysis found that patients with periodontitis had higher blood levels of leptin and lower serum levels of adiponectin compared to controls in the BMI ≤ 30 group. Also, leptin and adiponectin serum

levels do not change significantly following periodontal therapy in systemically healthy patients with periodontitis (Zhu et al., 2017).

There are extremely scarce data in the literature on the effects of Adiponectin on OTM. One particular study investigated the effect of local administration of adiponectin on experimental OTM in rats. This study found that local injection of adiponectin reduced OTM in rats in a dose-dependent manner but had no effect on bone density, periodontal cell count, or collagen content (Haugen et al., 2017). However, It has been found that adiponectin increases osteoblast proliferation while inhibiting osteoclastogenesis in adiponectin knockout mice (Williams et al., 2009). This might explain the slower tooth movement effect induced by adiponectin.

1.4 Human salivary proteome and peptidome

1.4.1 Whole mouth saliva

Whole mouth saliva (WMS), the fluid that bathes the mouth and oral cavity, is composed of both salivary and non-salivary components. It contains salivary gland secretions (parotid, submandibular, and sublingual glands, as well as minor salivary glands) as well as non-salivary components such as GCF, nasal and bronchial secretions, serum and blood derivatives from wounds, desquamated epithelial linings, food debris, and microorganisms residing in the oral cavity (Kaufman and Lamster, 2002). Salivary glands comprise two types of epithelial cells, acinar and ductal. Saliva is produced in acinar cells and stored in granules, which are released when secretory stimulation occurs. The salivary ducts are lined with ductal cells, which guide secreted saliva into the mouth (Humphrey and Williamson, 2001, Loo et al., 2010).

WMS comprises water (99% of saliva), peptides and proteins (including enzymes), hormones, carbohydrates, lipids, and inorganic substances such as sodium, chloride, potassium,

magnesium, calcium, phosphate, and bicarbonate. WMS have crucial roles in the oral cavity. It lubricates, hydrates, and bathes the mouth cavity, so facilitating speech and mastication; contains digestive enzymes and mediates the perception of taste; protects both soft and hard oral tissues from chemical, mechanical, and thermal irritants; works as an ion reservoir to promote tooth remineralization; and prevents dental demineralization; has antibacterial properties and protects the teeth and oral cavity from microorganisms (Humphrey and Williamson, 2001, Loo et al., 2010).

Healthy adults secrete 600-1000 ml per day of saliva, with a resting salivary flow rate of 0.2-0.4 ml per minute (Nanci, 2018), but large inter-personal variation is observed (Carpenter, 2013). Numerous factors affect salivary flow rate and composition, including systemic medical conditions (hypertension, depression, and allergies), individual hydration, posture, smoking, the circadian rhythm, and medication (Navazesh, 1993, de Almeida Pdel et al., 2008).

1.4.2 Human saliva as diagnostic body fluid

Saliva has become a useful tool for diagnosing and monitoring disease progression. The specific contribution of GCF to WMS supplies it with circulation-derived biomarkers. This gingival contribution makes it possible to use WMS diagnostically to monitor disease biomarkers generally seen in the serum. This is particularly interesting because WMS collection is not invasive, economical, safe, easy, and stress-free, and the analysis methods are simple and accessible for standard laboratories (Kaufman and Lamster, 2002). In addition, WMS collection is readily available from most individuals and can be easily repeated, stored, and transported as well as safe to handle compared to other biological materials (Schulz et al., 2013, Loo et al., 2010).

WMS contains proteins and other components that are expressed locally and can be used as disease biomarkers. Evidence for WMS diagnostic capabilities includes the identification of MMP-8, MMP-9 and OPG as biomarkers for periodontal diseases (Ramseier et al., 2009), variations in WMS inflammatory cytokine patterns in asthma exacerbations (Blicharz et al., 2009), variation in WMS biomarkers in Sjogren's syndrome (Hu et al., 2007), the association between WMS transcriptome markers and pancreatic cancer (Zhang et al., 2010), variation in WMS amylase in cardiovascular diseases (Adam et al., 1999), WMS HIV-1 detection (Malamud, 1997), and WMS C-reactive protein, myeloperoxidase, and myoglobin biomarkers for acute myocardial infarction detection (Floriano et al., 2009). Recently, It has been reported that WMS may be superior to serum in disease differentiation because discriminatory biomarkers, particularly oral cancer biomarkers, are present only in WMS (Dawes and Wong, 2019).

1.4.3 Human salivary proteome

The proteome is the complete set of proteins of a specified biological system and proteomics is the study of the proteome. This concept was introduced in 1995 and is an abbreviation for the entire "PROTEin" complement expressed by "genOME" or cells or tissues (Wasinger et al., 1995, Wilkins et al., 1996). The protein composition of WMS differs according to the kind of gland, with different glands releasing various kinds of proteins. Salivary glands produce most of the salivary proteome, with proline-rich proteins (PRP), mucins, cystatins, histatins, amylases, and statherin being the major salivary protein families (Carpenter, 2013).

WMS contains numerous proteins and peptides, each of which performs multiple important biological functions. In general, these proteins play key functions in immune defence, the endocrine system, and the maintenance of mucosal tissue and dental health (Dodds

et al., 2015, Fábián et al., 2012). These proteins may also provide information about both local and systemic diseases (Jasim et al., 2016). The protein content in WMS is around one-quarter that of blood which makes it easier to choose and investigate low abundant proteins (Pfaffe et al., 2011, Schulz et al., 2013).

A comprehensive study of the WMS proteome is essential to fully appreciate its diagnostic potential. Protein analysis tools have advanced significantly in recent decades, combined with bioinformatics, creating a new revolution in WMS proteomics (Dawes and Wong, 2019). Recent proteomic platforms evaluated the human WMS proteome, identifying around 3000 differentially expressed proteins and peptides, many of which were derived from desquamated epithelial cells, GCF, and the oral microbiome (Castagnola et al., 2017, Dawes and Wong, 2019).

Proteomics platforms, according to the sample used, are divided into bottom-up and top-down platforms. Top-down proteomics analyses native proteins or peptides, avoiding sample changes as much as possible. Bottom-up proteomics is based on pre-digesting the sample (often with trypsin), followed by a high-throughput analysis of peptide fragments. Protein presence in the sample is inferred by detecting one or more of its specific fragments (Messana et al., 2013).

In the last two decades, high-throughput proteomic technologies, such as mass spectrometry, have been developed. Mass spectrometry is an analytical method that separates ions based on their mass/charge ratio. It enables the study of proteins when used in conjunction with liquid chromatography and macromolecular ionisation. The separation, ionisation, and detection of macromolecules like peptides produce mass fingerprints that can be compared to peptides in curated databases and then assigned to proteins or determined by de novo

sequencing using bioinformatics. These technologies enable the identification of unknown targets (Forsgard et al., 2010, Mallick and Kuster, 2010).

1.4.4 Human salivary peptidome and proteases

The salivary peptidome is the entire set of peptides at a given time and conditions in the WMS of an individual. The salivary peptidome comprises around 2000 peptides, only 400-600 of which are directly derived from salivary glands, implying a considerable peptide input from other sources. Proteolysis events are the primary source of peptides, and significant efforts have been undertaken to identify the resultant fragments, cleavage sites, and implicated proteases (Amado et al., 2010, Vitorino et al., 2009). The majority of WMS peptides belong to proline-rich proteins (PRPs), histatin, and statherin families (Trindade et al., 2015a).

Most peptide fragments seen in saliva are the consequence of in-situ proteolysis. Proteolysis is the irreversible hydrolysis of peptide and isopeptide bonds, which affect every protein at some point throughout its life cycle by hydrolytic proteases (Doucet et al., 2008). These proteases hydrolyse the bonds of the peptides by a nucleophilic attack. However, the catalytic mechanism of action is different among proteases, and they are categorised on this basis. Aspartic, glutamic, and metalloproteases use a coordinated water molecule to disrupt substrate peptide bonds, while cysteine, serine, and threonine proteases exploit these amino acids in their active sites as nucleophiles (Magalhães et al., 2018). Proteases are the largest family of proteins, with 553 genes encoding proteases or protease homologues in the human genome (Puente et al., 2003, Mulkern et al., 2020, Chung et al., 2004).

Proteases are intriguing candidates for saliva-derived biological markers since they are engaged in several fundamental physiological processes, and their activity is tightly regulated by several mechanisms (Garreto et al., 2021). Proteolysis modulates critical mechanisms such

as embryonic development, immune response, blood coagulation, and overall metabolism (Butler and Overall, 2009, Garreto et al., 2021). Furthermore, studies have linked protease activity to autoimmune disorders (Butler and Overall, 2009, Garreto et al., 2021). MMPs and serine proteases are believed to be the main proteases involved in periodontal diseases and other diseases such as rheumatoid arthritis, skin diseases, and cancer (Birkedal-Hansen, 1993, Ingman et al., 1996, Westerlund et al., 1996).

Mass spectrometry and bioinformatics technologies have improved the profiling of endogenous peptides in body fluids as well as the identification of potentially associated proteases. Proteasix, an open-source peptide-centric tool, can predict in silico the proteases involved in naturally occurring peptide synthesis identified by mass spectrometry (Klein et al., 2013). Proteasix incorporates data from protease databases such as MEROPS, which contains around 8000 cleavage sites for over 2400 proteases (Barrett, 2004).

1.5 Aims

A major determinant of orthodontic treatment duration is the rate of OTM, which is primarily influenced by PDL and alveolar bone remodelling. Recently, orthodontists and patients have become interested in shortening treatment duration; and accelerating the rate of OTM has become the central focus of much research. A wide range of conventional and non-conventional approaches have been proposed to accelerate OTM, with evidence relating to the efficiency of most of these approaches being weak and insufficient. Evaluating the current evidence on rate of OTM and understanding the biological mechanisms that potentially influence this process are critical for the development of new approaches aimed at accelerating OTM. Therefore, the aims of this thesis were:

- To systematically review the evidence on duration of treatment and rate of OTM during the alignment phase of orthodontic treatment with fixed appliances;
- To systematically review the evidence on duration of treatment and rate of OTM during canine retraction phase of orthodontic treatment with fixed appliances;
- To characterise the salivary peptidome and protease profile during the alignment stage of fixed appliance orthodontic treatment using a peptidomic approach aided by mass spectrometry and bioinformatics;
- To assess the effect of adipokines on inflammation and ECM remodelling biomarkers in compressed human periodontal and gingival fibroblasts in the presence or absence of inflammation; and
- To assess the effect of appointment interval (2 weeks vs. 8 weeks) on the duration and rate of orthodontic tooth alignment in a RCT.

Chapter 2 Duration of tooth alignment with fixed appliances: a systematic review and meta-analysis

2.1 Introduction

Orthodontic treatment can improve the function and aesthetic of the orofacial region in children and adults by aligning the teeth, arch-coordination, and establishing normal occlusion. However, comprehensive orthodontic treatment with fixed appliances is time-consuming, with an average treatment duration of 20-30 months (Abbing et al., 2020, Tsichlaki et al., 2016). Patients are often reluctant to wear fixed appliances for long periods, and prolonged treatment might have detrimental effects on oral health. Perhaps it is for these reasons that both patients and orthodontists are interested in methods that may help to shorten orthodontic treatment duration (Uribe et al., 2014).

The rate of OTM is controlled through the biological response, and numerous host and treatment-related factors can affect how teeth move (Huang et al., 2014, Dudic et al., 2013). Orthodontists have focussed on treatment mechanics to influence OTM for many years, but robust clinical data are lacking. Indeed, there is limited data on something as fundamental as optimal force levels (Theodorou et al., 2019). Furthermore, despite substantial diversity in fixed appliance design and archwire material selection, there is little evidence that they can influence OTM to any clinically significant level (Papageorgiou et al., 2014a, Riley and Bearn, 2009, Wang et al., 2018). This all presents a paradox because orthodontic clinical research has recently become increasingly preoccupied with evaluating clinical interventions advocated to accelerate OTM and shorten treatment duration.

Alignment of dentition is a primary objective during orthodontic treatment with fixed appliances (McLaughlin and Bennett, 2015) and is a frequently used metric in clinical trials

assessing treatment interventions and the rapidity of OTM (Fleming et al., 2009, Miles, 2005, Miles et al., 2006, Pandis et al., 2007, Scott et al., 2008, Ulhaq et al., 2017, Woodhouse et al., 2015, Abdelrahman et al., 2015, Charavet et al., 2019). Although multiple methods are used to measure tooth alignment, Little's irregularity index (LII) is a commonly used method (Little, 1975). It measures the linear distances between the anatomical contact points of the mandibular anterior teeth from canine to canine, and the sum of these five measurements represents the irregularity index. This index is a simple, quick, and reliable indicator of the alignment progress (Antoszewska-Smith et al., 2017, Bernabé and Flores-Mir, 2006, Goonewardene et al., 2008). Intuitively, the more irregularity in the dental arch, the longer arch alignment with a fixed appliance is predicted to take. However, the reported alignment durations vary greatly between studies (Fleming et al., 2009, Miles, 2005, Miles et al., 2006, Pandis et al., 2007, Pandis et al., 2009, Scott et al., 2008). Interestingly, it has recently been shown that piezocision-assisted surgical intervention can shorten alignment duration by 46 days, although the evidence is weak (Afzal et al., 2020). Given the significance of arch alignment during orthodontic treatment and the broad utilisation of alignment measurements in orthodontic clinical research, it is essential to understand the normal parameters associated with this clinical outcome.

Accurate prediction of orthodontic treatment duration may affect success in orthodontic practice (Shia, 1986). In addition, timely completion of treatment helps more precisely expect financial costs and improves patient satisfaction (Skidmore et al., 2006). Therefore, it is beneficial for both the patient and the orthodontist to present reliable information about the duration of treatment. Moreover, knowledge of the average alignment rate and duration that can be expected during fixed appliance would add to orthodontic knowledge and improve treatment delivery and clinical efficiency, which would lead to better planning of future RCTs and developing systems that provide more efficient and safer treatment for all orthodontics patients.

The primary objective of this systematic review was to determine treatment duration needed to achieve whole-arch alignment of the mandibular dentition using fixed orthodontic appliances. The secondary objectives were to determine the time required to achieve incisor alignment in the mandibular arch and alignment rates during each treatment phase.

2.2 Materials and methods

2.2.1 Protocol and registration

This review's protocol was made a *priori* and registered in the prospective register of systematic reviews (PROSPERO) (CRD42019143204). This review was conducted and reported according to Cochrane Handbook (Higgins and Green, 2011) and Preferred Reporting Items for Systematic Reviews and Meta-Analyses statement (PRISMA) (Liberati et al., 2009), respectively.

2.2.2 Eligibility criteria

According to the Participants-Intervention-Comparison-Outcome-Study design (PICOS) schema, the following were included: (P) human participants of any age, sex, ethnicity, or malocclusion; (I) using fixed orthodontic appliances with or without treatment adjuncts; (C) trials of any comparison (O) assessing duration and/or rate of mandibular teeth alignment; (S) RCTs. No limitations regarding language, publication year or status were applied. Excluded studies included those involving animals, non-clinical and non-randomised studies, case reports or series, cross-sectional studies, those involving patients who had undergone any previous orthodontic treatment, growth modification or multidisciplinary treatment, and those including patients with systematic diseases, craniofacial abnormalities or without comprehensive orthodontic treatment or eligible outcomes.

The primary outcome of this review was treatment duration to achieve whole-arch (complete) alignment of the mandibular dentition measured as days needed to align the lower teeth or time to passively insert a stainless-steel rectangular working archwire. Time to achieve alignment of the mandibular incisor teeth (incisor-alignment) and tooth alignment rate (irregularity-change per unit time) in each treatment phase were evaluated as secondary outcomes. We focused on alignment of the mandibular dentition because irregularity is most measured in the mandible in interventional trials rather than the maxilla. In addition, mandibular irregularity is an important factor taken into consideration when deciding on extraction or non-extraction treatment during treatment planning.

2.2.3 Information sources and search

Eight electronic databases (MEDLINE, Embase, Cochrane Database of Systematic Reviews, Cochrane Central Register of Controlled Trials, Scopus, Web of Science, Latin American and Caribbean Health Sciences Literature and Database of Abstracts of Reviews of Effects) were searched systematically without restrictions for publication date, language or type from inception up to January 7, 2021, while Directory of Open Access Journals (DOAJ), Digital Dissertations, metaRegister of Controlled Trials, Google Scholar as well as reference/citation lists of eligible articles or existing systematic reviews were manually searched for any additional trials. A detailed search strategy was developed for each database. Individual search strategies were based on the search strategy developed for MEDLINE but modified appropriately for every database (Appendix 2.1).

2.2.4 Study selection, data collection, and items

Two reviewers independently screened titles, abstracts, and full texts of identified studies to check for eligibility. Any differences between reviewers were resolved by discussion with a

third reviewer. Data extraction was conducted independently by two reviewers, with similar discrepancy resolution using pre-determined and piloted extraction forms covering: (1) study characteristics (design, clinical setting, country); (2) patient characteristics (age/sex); (3) malocclusion, treatment characteristics; (4) appliance type; (5) intervention and/or adjunct interventions; (6) follow-up; (7) outcome details.

2.2.5 Risk of bias of individual studies

The risk of bias of included studies was assessed according to Cochrane guidelines with the Risk of Bias 2.0 tool for randomised trials (Sterne et al., 2019) independently by two reviewers with the same discrepancy resolution approach.

2.2.6 Data synthesis and summary measures

An effort was made to maximise data output from included trials; where data were missing, raw data from trials on the primary outcome were requested from the authors, and we calculated the needed data. As orthodontic treatment outcome is inevitably affected by patient and treatment-related characteristics, a random-effects model was used to calculate the average distribution of true effects based on clinical and statistical reasoning (Papageorgiou, 2014) and a restricted maximum likelihood variance-estimator was used according to recent guidance (Langan et al., 2019). Data synthesis was performed on two different levels. Initially, indirect analysis of pooled averages was undertaken to calculate average alignment duration or alignment change during orthodontic treatment with its corresponding 95% confidence intervals (CIs), following pooling multiple trial arms before meta-analysis. After that, direct analysis was undertaken to compare different trial arms within each trial and pooling mean differences (MDs) and their 95% CIs across studies.

Between-study heterogeneity was assessed by inspecting forest plots and calculating τ^2 (absolute heterogeneity) and I^2 (relative heterogeneity). I^2 describes the proportion of total variability in the result explained by heterogeneity and not by chance. We considered I^2 >75% arbitrarily to denote considerable heterogeneity while as well considering the direction of heterogeneity (localization on the forest plot) and uncertainty intervals around heterogeneity estimates (Higgins et al., 2003). Ninety-five per cent predictive intervals were calculated for meta-analyses of ≥3 studies to include existing heterogeneity and provide a range of possible effects for a future clinical setting, essential for the correct interpretation of random-effects meta-analyses (IntHout et al., 2016).

All analyses were run in Stata version 14.0 (StataCorp LP, Texas, USA), and the dataset was openly provided (Wazwaz et al., 2020). All P values were two-sided with α=5%, except the test of between-studies or between-subgroups heterogeneity where α-value was 10% (Ioannidis, 2008).

2.2.7 Additional analyses, risk of bias across studies and quality of evidence

Possible sources of heterogeneity were sought through subgroup analyses and random-effects meta-regression in meta-analyses of at least five studies according to patient age, sex, baseline irregularity, extraction incorporation, and bracket slot size. Reporting biases (including the possibility of publication bias) were assessed for meta-analysis with ≥10 trials with contour-enhanced funnel plots and Egger's test. Individual-Patient-Data (IPD) from studies assessing the primary outcome (whole-arch alignment duration) were obtained from trials corresponding authors, pooled appropriately and meta-analysed after making them compatible across trials.

The overall quality of meta-evidence (and thus, the strength of clinical recommendations) was rated using Grades of Recommendations, Assessment, Development and Evaluation (GRADE) (Guyatt et al., 2011) and revised summary of findings tables were

made using the newly proposed improved format (Carrasco-Labra et al., 2016). The minimal clinically significant, large, and very large effects were defined as half, one and two standard deviations of the post-treatment response (for continuous outcomes) (Norman, Sloan et al. 2003). Forest plots were augmented with contours representing the magnitude of observed effects to assess heterogeneity, clinical relevance, and imprecision.

2.2.8 Sensitivity analyses

The robustness of results was checked for meta-analyses of ≥3 studies with sensitivity analyses based on (1) inclusion or exclusion of studies with low risk-of-bias; (2) inclusion or exclusion of large studies (arbitrarily set as studies with >30 patients).

2.3 Results

2.3.1 Study selection

The electronic literature search yielded 3016 results (Figure 2.1). Following duplicate removal, 1131 titles and abstracts were screened, and the full-text of 127 publications was checked against eligibility criteria according to established inclusion criteria (Appendix Table 2.1). Eventually, 35 publications reporting 35 trials were included.

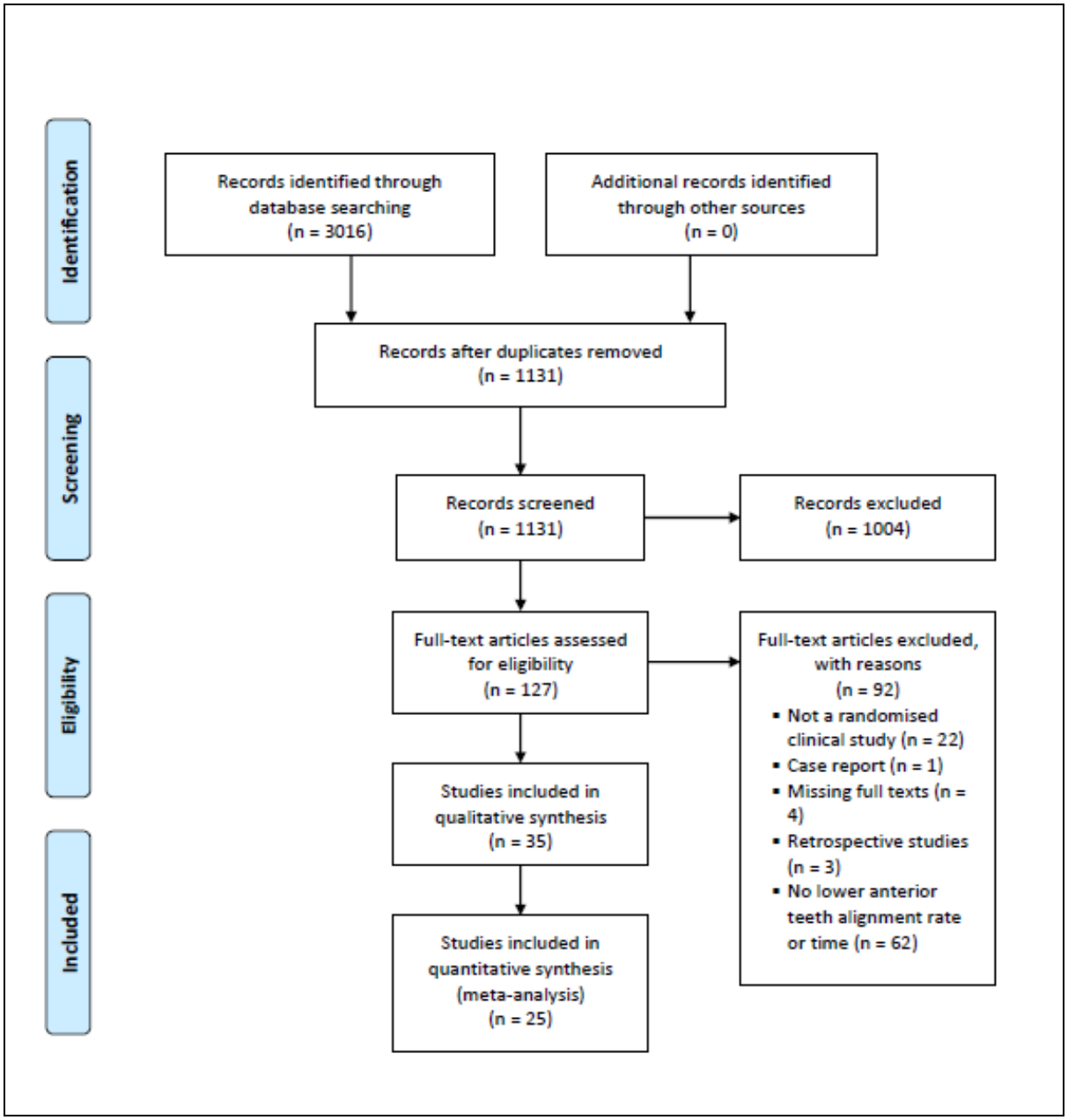

Figure 2.1 Preferred Reporting Items for Systematic Reviews and Meta-Analyses diagram for the identification and selection of studies eligible in this review.

2.3.2 Study characteristics

The characteristics of included trials are shown in Table 2.1. Included trials were conducted in university-clinics (n=18; 51%), private-practice (n=6; 17%), hospitals (n=9; 26%) or both private-practice and hospital environments (n=2; 6%) and originated from 15 different countries, including Australia, Brazil, Belgium, China, Egypt, Greece, India, Iran, Iraq, Jordan,

Syria, Turkey, United Arab Emirates, United Kingdom, and the United States of America. Of the 35 trials included, 30 (86%) were single- and 5 (14%) were multi-centre. Thirty-one studies included 2258 patients with a mean age of 17.8 years (reported in 33 studies). Out of 32 studies reporting on patient gender, 862/2202 patients were male (39%) and 1340 (61%) were female.

Sixteen trials (46%) involved non-extraction treatment, 6 (17%) both extraction and non-extraction, 7 (20%) extraction-only, and 6 (17%) did not report on whether extraction was performed or not. Most of the trials (21; 60%) did not report malocclusion type, 8 trials included any type of malocclusion (23%), 5 trials included class I (14%), and one trial included patients with either class I or class II division1 malocclusion (3%). The average baseline mandibular arch irregularity in the included studies ranged from 2.1 to 11.8 mm.

Twenty studies reported on changes in alignment rate or irregularity index, 7 reported on alignment duration, 8 reported on alignment rate and duration. Changes in mandibular arch incisor irregularity were measured using Little's irregularity index in most trials (n=33), either from stone models (n=24), intra-orally (n=6) or scanned models (n=3) with one trial not reporting method of outcome assessment. One trial estimated crowding by comparing the space required to align the teeth and mesiodistal widths of the teeth.

Rate of mandibular alignment was calculated as the difference in irregularity index taken at two different time points divided by the number of days or weeks between measurements. Time points in included studies were 0, 4, 8, 12, 16, 20, 24 weeks, with changes in irregularity index calculated for the subsequent time intervals 0-4, 0-8, 0-12, 0-16, 4-8, 8-12, 12-16 weeks. Four studies reported on whole-arch alignment duration measured as time to align the mandibular teeth and passively insert a working stainless-steel archwire; whereas 9 studies reported duration of incisor-alignment measured as time taken to align the mandibular anterior teeth irrespective of the posterior.

Alignment duration and/or rate were compared between different interventions, including initial archwire types (n=13), archwire sequences (n=2), types of brackets (n=9), ligation methods (n=1) and placement of laceback ligatures (n=1). Other studies used adjuncts to orthodontic treatment, including surgery (n=5), laser (n=1), vibration (n=2) or photobiomodulation (n=1).

2.3.3 Risk of bias within studies

The risk of bias assessment for the 35 included studies is shown in Figure 2.2. The detailed risk of bias assessment for the included studies can be found in Appendix Table 2.2. A high risk of bias was seen in 5 studies (14%) for at least one domain. Three studies were at high risk of bias due to the randomisation process, with two lacking allocation concealment and one demonstrating deviation from the intended interventions and missing outcome data. Two studies were at high risk of bias due to lack of blinding and missing outcome data. Ten studies (29%) presented concerns with the randomisation process, mainly a lack of information regarding allocation concealment (n=6) and randomisation sequence generation (n=4). The remaining 20 studies (57%) presented a low risk of bias except for the absence of a priori protocols, which would rule out selective reporting.

Table 2.1 Characteristics of included studies.

Study	Design; setting; country*	Patients (M/F); age†	Maloc-clusion	Ex / Non-Ex	LII	Appliance	Intervention / Supplemental	FU in wks (interval)	Outcome
(Abdelrahman et al., 2015)	RCT; Uni; JOR	A (SE NiTi): 25 (10/15); 19.4 B (HA NiTi): 25 (10/15); 17.4 C: (Conv NiTi): 24 (8/16); 19.3	Any	Both	6.0	Labial CLB (Roth)	Different archwire types / None	0-(2)-16	AlignRate; AlignDur
(Aydin et al., 2018)	RCT; Uni; TUR	EXP (CuNiTi): 30 (10/20); 15.9 CNT (NiTi): 36 (10/26); 14.7	Cl. I	Non-Ex	10.4	Labial CLB (Roth)	Different archwire types / None	0-(6)-12	AlignRate
(Bansal et al., 2019)	RCT; Uni; IND	EXP: 15 (7/8); 15.9 CNT: 15 (7/8); 15.3	NR	Non-Ex	5.3	Labial CLB (MBT)	Micro-osteoperforation using mini-implants	0-(3)-15	AlignDur
(Celikoglu et al., 2014)	RCT; Uni; TUR	EXP: 22 (17/5); 15.5 CNT: 24 (18/6); 14.7	Cl. I / Non-Ex	Non-Ex	6.7	EXP: SLB CNT: Labial CLB	Different bracket types / None	0-(8)-16	AlignRate
(Charavet et al., 2019)	RCT; Hosp; BEL	EXP:12 (5/7); 29.0 CNT:12 (4/8); 27.0	NR	NR	3.1	Customized Labial SLB	Piezocision	0-(2)-End	AlignDur
(Cobb 3rd et al., 1998)	RCT; Uni; USA	A (Implanted NiTi): (NR);15.2 B (NiTi): (NR); 17.3 C (Multi-strand SS): (NR); 16.3	NR	NR	7.5	Labial CLB	Different archwire types / None	0-(4)-End	AlignRate
(de Araújo Gurgel et al., 2020)	RCT; Uni; BRA	A: 24 (12/12); 18.6 B: 25 (8/17); 20.0	Any	NR	4.9	Labial CLB (Roth)	Different archwire sequences / None	0-(4)-24	AlignRate
(El Shehawy et al., 2020)	RCT; Uni; EGY	EXP/CNT: 30 (12/18); 19.23	NR	Non-Ex	6.9	Labial CLB (Roth)	Photobiomodulation	0-(4)-12	AlignRate
(Fleming et al., 2009)	RCT; Hosp; GBR	EXP: 32 (14/18); 15.9 CNT: 33 (8/25); 16.6	Any	Non-Ex	6.4	EXP: SLB (MBT) CNT: Labial CLB	Different bracket types / None	0-(-)-8	AlignRate
(Gibreal et al., 2019)	RCT; Uni; SYR	EXP: 17 (8/9); 20.29 CNT: 17 (7/10); 20.35	NR	Lower 4s	11.5	Labial CLB (MBT)	Piezocision	0-(4)-End	AlignRate; AlignDur
(Huang et al., 2010)	RCT; Hosp; CHN	EXP (HA NiTi): 40 (20/20); (12.0-14.0) CNT (NiTi): 40 (20/20); (12.0-14.0)	Cl. I	Ex	NR	Labial CLB (MBT)	Different archwire types / None	0-(NR)-End	AlignDur
(Irvine et al., 2004)	RCT; Hosp; GBR	EXP: 30 (12/18); 13.6 CNT: 32 (14/18); 13.8	Cl. I-II/1 with crowding	4PMs	3.1	EXP: Labial CLB (Andrews) CNT: Labial CLB (Andrews)	Use of laceback ligatures / None	0-(6)-End	AlignRate (Change in LLS irregularity)
(Jahanbin et al., 2019)	RCT; Pract; IRN	EXP: 15 (0/15); 16.3 CNT: 15 (0/15); 16.1	NR	Non-Ex	5.1	EXP: SLB CNT: Labial CLB (MBT)	Different bracket types / None	0-(4)-16	AlignRate
(Little and Spary, 2017)	RCT; Hosp; GBR	A: EXP (Ex): 30 (16/14); 14.2 B: EXP(Non-Ex): 30 (14/16); 14.0 C: CNT (Ex): 30 (14/16); 13.7	NR	Both	8.9	Labial CLB (MBT)	Ligation method (Conv vs. figure-of-eight) / None	0-(6)-12	AlignRate

		D: CNT (Non-Ex): 30 (15/15); 14.0							
(Miles, 2005)	RCT; Pract; AUS	EXP/CNT: 58 (26/32); 17.1	NR	Both	5.8	EXP: Labial SLB (MBT) CNT: Labial CLB (MBT)	Different bracket types / None	0-(10)-20	AlignRate
(Miles et al., 2006)	RCT; Pract; AUS	EXP/CNT: 58 (18/40);16.3	NR	NR	2.1	EXP: Labial SLB CNT: Labial CLB (MBT)	Different bracket types / None	0-(10)-20	AlignRate
(Miles et al., 2012)	RCT; Pract; AUS	EXP: 33 (12/21); 13.0 CNT: 33 (14/19); 13.1	NR	Non-Ex	5.6	Labial CLB (MBT)	Vibration	0, 5, 8, 10	AlignRate
(Mahmoudzadeh et al., 2018)	RCT; Uni; IRN	EXP (HA NiTi): 29 (10/19); 17.9 CNT (A-NiTi): 30 (11/19); 17.92	NR	Non-Ex	6.1	Labial CLB (MBT)	Different archwire types / None	0-(-)-4	AlignRate
(Mandall et al., 2006)	RCT; Hosp & Pract (multicenter); GBR	A: 51 (31/20); 13.8 B: 50 (13/37); 14.4 C: 53 (18/35); 14.4	Any	Both	6.1	Labial CLB	Different archwire sequences / None	NR	AlignDur
(Nabbat and Yassir, 2020)	RCT; Uni / Pract; IRQ	A (HA NiTi): 15 (5/10); 20.5 B (SE NiTi): 16 (4/12); 17.8	NR	Non-Ex	4.79	Labial CLB (MBT)	Different archwire types / None	0-(4)-8	AlignRate
(Nahas et al., 2017)	RCT; Uni; ARE	EXP: 18 (NR); 21.8 CNT: 16 (NR); 21.1	NR	Non-Ex	5.6	Labial SLB (MBT)	Photobiomodulation	0-(2)-End of alignment	AlignDur
(Nordstrom et al., 2018)	RCT; Uni; USA	EXP (Gummetal): 14 (5/9); 15.43 CNT (NiTi): 14 (6/8); 16.50	Any	Non-Ex	7.2	Labial CLB	Different archwire types / None	0-(5)-10	AlignRate
(Ong et al., 2011)	RCT; Pract; AUS	A (3M Unitek): 44 (14/30); 14.4 B (GAC): 44 (19/25); 15.5 C (Ormco): 44(19/25); 16.1	Any	Both	6.7	Labial CLB (MBT)	Different archwire types / None	BL, T2 before 2nd archwire, T3 before working archwire	AlignDur; AlignRate
(Pandis et al., 2007)	RCT; Uni; GRC	EXP:27 (4/23); 13.48 CNT:27 (7/20); 13.92	Any	Non-Ex	5.4	EXP: Labial SLB CNT: Labial CLB (Roth)	Different bracket types/ None	0-(4)-End of alignment	AlignDur; AlignRate
(Pandis et al., 2009)	RCT; Pract; GRC	EXP (CuNiTi): 30 (9/21); 13.4 CNT (NiTi): 30 (5/25); 12.8	Any	Non-Ex	5.5	Labial SLB	Different types of archwires / None	0-(4)-24	AlignDur; AlignRate
(Sandhu et al., 2012)	RCT; Uni; IND	G1 (SE NiTi): 24 (12/12); 15.0 G2 (Multistrand SS): 25 (13/12); 15.1 G3 (SE NiTi): 24 (13/11); 15.2 G4 (Multistrand SS): 23 (11/12); 15.4	NR	Both	NR	G1-2: SW labial CLB G3-4: Standard Begg	Different brackets & wires / None	0-(-)-6	AlignRate

(Scott et al., 2008)	RCT; Hosp (multicenter); GBR	EXP: 33 (13/20); 16.19 CNT: 29 (20/9); 16.38	NR	Lower 4s	11.8	EXP: Labial SLB CNT: Labial CLB (Roth)	Different bracket types / None	BL, 1st archwire change, end of alignment	AlignRate; AlignDur
(Sebastian, 2012)	RCT; Uni; IND	EXP (Coaxial NiTi): 12 (0/12); 13.6 CNT (NiTi): 12 (0/12); 13.8	Cl. I	Non-Ex	8.8	Labial CLB (MBT)	Different archwire types / None	0-(4)-12	AlignRate
(Sebastian et al., 2019)	RCT; Uni; IND	EXP (Coaxial NiTi): 20 (0/20); 15.30 CNT (NiTi): 20 (0/20); 14.85	Cl. I	Ex	9.1	Labial CLB (MBT)	Different archwire types / None	0-(4)-12	AlignRate
(Serafim et al., 2015)	RCT; Uni; BRA	EXP/CNT: 22 (NR); 16.68	NR	NR	4.5	Labial CLB (Roth)	Different archwire sequences / None	0-(4)-20	AlignRate
(Sirri et al., 2020)	RCT; Uni; SYR	EXP: 30 (9/21); 21.1 CNT: 30 (10/20); 21.4	NR	Non-Ex	4.4	Labial CLB (MBT)	Corticision	0-(2)-End	AlignDur
(Songra et al., 2014)	RCT; Hosp; GBR	A: 42 (17/25); 14.3, 14.2 B: 38 (12/26); 14.1, 13.8 C: 20 (8/12); 14.3, 13.2	NR	Ex	8.1	A: Labial SLB passive (Roth) B: Labial SLB active (Roth) C: Labial CLB(Roth)	Different bracket types/ None	0-(12)-End	AlignDur
(Ulhaq et al., 2017)	RCT; Hosp (multicenter); GBR & ITA	A (BioCosmetic): 75 (33/42); 17.54 B (Titanol): 75 (35/40); 16.32 C (TP Aesthetic): 75 (28/47); 15.95 D (Tooth Tone): 75 (26/49); 17.21	NR	NR	NR	Labial CLB	Different archwire types / None	0-(-)-8	AlignRate
(Uribe et al., 2017)	RCT; Uni; USA	EXP: 16 (6/10); 30 CNT: 13 (6/7); 29.4	NR	Non-Ex	7.4	Labial SLB	Piezotome-corticision	0-(4)-End of alignment	AlignRate; AlignDur
(Woodhouse et al., 2015)	RCT; Hosp (multicenter); GBR	A (Accel): 29 (15/14); 13.9 B (Accel sham): 25 (13/12); 14.1 C (CNT): 27 (12/15); 14.4	NR	Lower 4s	8.4	Labial CLB (MBT)	Vibration	BL, 2nd arch wire change and end of alignment	AlignRate; AlignDur

* countries given with their alpha-3 codes.

† patient age is given either as mean (one value in without parenthesis) or if mean is not reported as range (two values in parenthesis).

AlignDur, alignment duration; AlignRate, alignment rate; BL, baseline; Cl., (Angle's) Class; CLB, conventionally ligated brackets; Conv, conventional; CNT, control; Ex, extraction; EXP, experimental; FU, follow-up; Hosp, hospital; HA, heat-activated; LII, Little's Irregularity Index for the mandibular arch at baseline; M/F, male / female; MBT, MacLaughlin-Bennet-Trevisi prescription; mo, month; NiTi, nickel titanium; NR, not reported; Pract, private practice / clinic; RCT, randomized clinical trial; SE, superelastic; SLB, self-ligating bracket; SS, stainless steel; Tx, treatment; Uni, university clinic; wk, week.

Figure 2.2 Risk of bias assessments for included trials.

2.3.4 Data synthesis

2.3.4.1 Indirect analyses of pooled averages across trials

The pooled duration to attain whole-arch alignment of mandibular dentition was estimated at 263.0 days (4 trials; translated to 8.8 months; 95% CI=6.2-11.3 months; Figure 2.3a); and 100.7 days (9 trials; translated to 3.4 months; 95% CI=2.8-3.9 months; Figure 2.3b) to achieve alignment of the mandibular incisor teeth. Average tooth alignment through changes in irregularity index from baseline was 2.88 mm at 4 weeks (10 trials; 95% CI, 2.12-3.63), 4.37 mm at 8 weeks (12 trials; 95% CI, 3.48-5.26), and 5.55 mm at 12 weeks (8 trials; 95% CI, 4.39-6.71) (Figure 2.4). Extreme heterogeneity across studies was seen for all indirect poolings (I^2>75%); thus, 95% CIs might be more informative than the pooled averages. Subgroup and meta-regression analyses were conducted to investigate sources of heterogeneity (Table 2.2). Treatment with premolar extractions was associated with more tooth alignment than non-extraction for weeks 0-4 (5.3 vs. 2.3 mm) and weeks 0-8 (7.5 vs. 3.8 mm). Baseline irregularity was associated with more alleviation of irregularity between weeks 0-4 and weeks 0-8 (+0.4 and +0.5 mm alleviation for each initial mm of crowding for both).

2.3.4.2 Direct comparisons within and across-trials

Direct comparisons between different archwires, fixed appliances or treatment adjuncts were conducted in individual single studies (Tables 2.3 and 2.4) and meta-analyses (Table 2.5) with only few significant results. Single studies showed that subjective incisor alignment duration in the mandibular dentition was shorter using photobiomodulation (vs none; MD=-19.5 days; P=0.03) and self-ligating brackets (vs conventional ligation; MD=-23.5 days; P=0.03). Tooth alignment was more with premolar extraction (vs non-extraction; 0-12 weeks; MD=2.0 mm; P<0.001) and with coaxial superelastic Nickel-Titanium (NiTi) archwires (vs single superelastic NiTi archwires; 0-8 weeks; MD=1.3 mm; P=0.04).

Meta-analyses found no difference in whole-arch alignment duration between self-ligating and conventional brackets (n=2 trials; P=0.26) and no difference in duration of incisor-alignment duration between thermal and conventional NiTi archwires (n=2 trials; P=0.94) (Table 2.3; Figure 2.5a). Meta-analysis of 4 studies found a considerable reduction in incisor-alignment duration with surgically-assisted orthodontics (MD=44.9 days less; 95% CI=20.0 to 68.9 days; P<0.001; Figure 2.5b) although with substantial heterogeneity (I^2=93%) and this did not affect our conclusion about the effectiveness of treatment. The quality of evidence, according to GRADE (Table 2.6), was high both for the lack of benefit for thermal NiTi archwires and self-ligating brackets and for the surgically-assisted orthodontics.

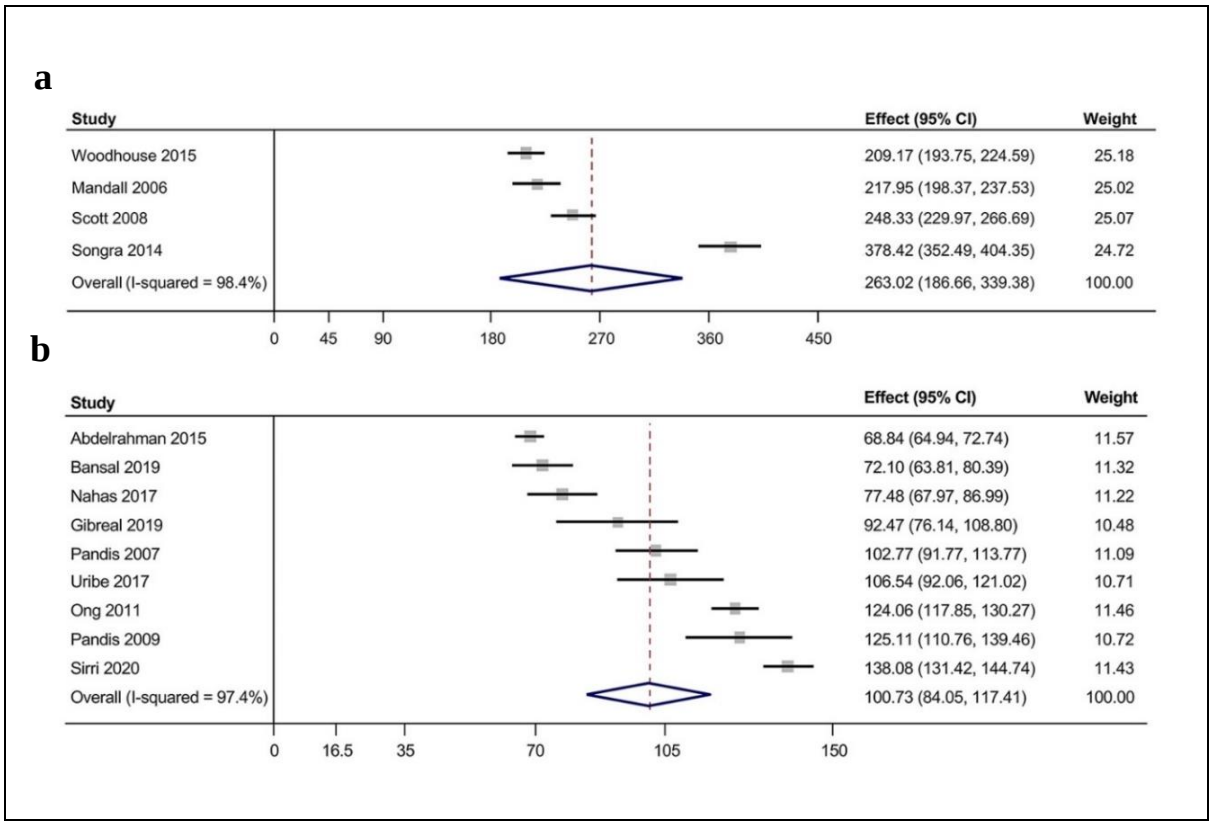

Figure 2.3 Forest plots depicting duration to achieve whole-arch alignment of the mandibular dentition (a) and mandibular arch incisor alignment (b) in days.

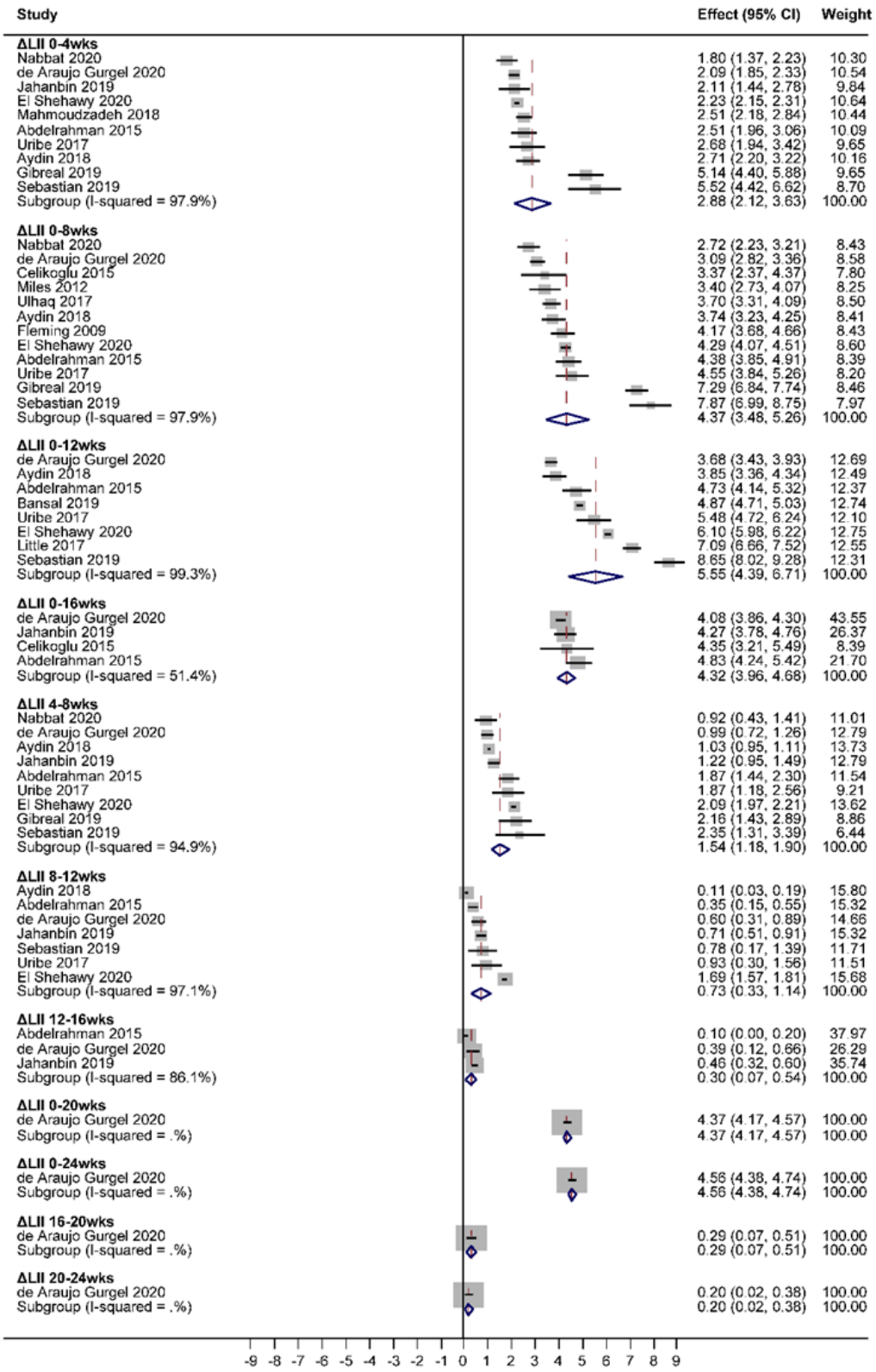

Figure 2.4 Forest plot of the pooled average for incisor irregularity changes.

Table 2.2 Meta-regression / subgroup analyses for possible factor influencing the pooled average incisor-alignment duration and irregularity changes across identified randomized trials.

		Initial alignment			Irregularity change 0-4 weeks			Irregularity change 0-8			Irregularity change 0-12			Irregularity change 4-8		
Factor	**Category**	**n**	**Estimate (95% CI)**	**P§**	**n**	**Estimate (95% CI)**	**P§**	**n**	**Estimate (95% CI)**	**P§**	**n**	**Estimate (95% CI)**	**P§**	**n**	**Estimate (95% CI)**	**P§**
Age	Per year	9	b: -0.24 (-4.65, 4.18)	0.90	9	b: -0.04 (-0.31, 0.11)	0.75	11	b: 0.03 (-0.25, 0.31)	0.81	7	b: -0.07 (-0.45, 0.30)	0.64	8	b: 0.04 (-0.08, 0.15)	0.46
Male %	Per 10%	8	b: -12.70 (-36.36, 10.95)	0.24	8*	b: 1.02 (-0.56, 2.61)	0.17	11*	b: 0.05 (-0.71, 0.81)	0.89	7*	b: 1.18 (-0.69, 3.05)	0.17	7*	b=0.65 (-0.28, 1.58)	0.13
Baseline LII	Per mm	9	b: -1.90 (-12.94, 9.14)	0.70	**10**	**b: 0.38 (0.11, 0.65)**	**0.01**	**11**	**b: 0.51 (0.12, 0.89)**	**0.02**	8	b: 0.34 (-0.40, 1.07)	0.30	8	b: 0.01 (-0.30, 0.32)	0.96
Extraction	Both	2	Average 96.40 (42.28, 150.51)	0.64	**1**	**Average 2.51 (1.96, 3.06)**	**0.001**	**2**	**Average 4.02 (3.35, 4.68)**	**0.001**	2	Average 5.92 (3.61, 8.23)	0.50	1	Average 1.87 (1.44, 2.30)	0.86
	Ex	1	Average 92.47 (76.15, 108.79)		**2**	**Average 5.26 (4.64, 5.88)**		**2**	**Average 7.45 (6.94, 7.96)**		1	Average 8.65 (8.02, 9.28)		2	Average 2.22 (1.63, 2.82)	
	Non-Ex	6	Average 103.57 (82.59, 124.55)		**6**	**Average 2.31 (2.05, 2.57)**		**7**	**Average 3.77 (3.28, 4.25)**		4	Average 5.08 (4.13, 6.03)		5	Average 1.42 (0.95, 1.89)	
Slot	18”	1	Average 124.06 (117.85, 130.27)	0.19	1	Average 2.71 (2.20, 3.22)	0.65	2	Average 3.62 (3.21, 4.02)	0.68	1	Average 3.85 (3.36, 4.34)	0.54	**1**	**Average 1.03 (0.95, 1.11)**	**0.01**
	22”	8	Average 97.71 (79.97, 115.45)		9	Average 2.90 (2.05, 3.75)		10	Average 4.53 (3.48, 5.58)		6	Average 5.57 (4.22, 6.93)		**8**	**Average 1.62 (1.24, 2.01)**	

* one study with only female patients excluded.

§ from meta-regression or subgroup analysis

b, meta-regression coefficient; CI, confidence interval; Ex, extraction.

Table 2.3 Direct estimates (MD) from single trials on duration

Nr	Outcome	Timepoint	Trial	Control	Experimental	MD (95% CI)	P
1	Initial alignment duration	-	Abdelrahman 2015	Nitinol wire	Superelastic NiTi wire	2.10 (-7.97, 12.17)	0.68
2	**Initial alignment duration**	**-**	**Nahas 2017**	**No photomodulation**	**Photomodulation**	**-19.50 (-37.45, -1.55)**	**0.03**
3	Initial alignment duration	-	Ong 2011	Wire sequence A	Wire sequence B	12.00 (-3.04, 27.04)	0.12
4	Initial alignment duration	-	Ong 2011	Wire sequence A	Wire sequence C	0 (-15.22, 15.22)	1.00
5	**Initial alignment duration**	**-**	**Pandis 2007**	**Conventional brackets**	**Self-ligating brackets**	**-23.48 (-44.74, -2.22)**	**0.03**
6	Complete alignment duration	-	Mandall 2006	Wire sequence A	Wire sequence B	36.00 (-11.63, 83.63)	0.14
7	Complete alignment duration	-	Mandall 2006	Wire sequence A	Wire sequence C	12.00 (-36.84, 60.84)	0.63
8	Complete alignment duration	-	Woodhouse 2015	No vibration	Vibration	9.50 (-28.79, 47.79)	0.63
9	Complete alignment duration	-	Woodhouse 2015	No vibration	Sham vibration	16.80 (-21.97, 55.57)	0.40

CI, confidence interval; Ex, extraction; MD, mean difference.

Table 2.4 Direct estimates (MD) from single trials on irregularity change

Nr	Outcome	Timepoint	Trial	Control	Experimental	MD (95% CI)	P
1	Irregularity change	0-4 weeks	Sebastian 2019	Wire: superelastic NiTi	Wire: coaxial superelastic NiTi	1.28 (-0.32, 2.88)	0.12
2	Irregularity change	0-4 weeks	Abdelrahman 2015	Wire: Nitinol	Wire: superelastic NiTi	-0.25 (-1.71, 1.21)	0.74
3	**Irregularity change**	**0-4 weeks**	**Jahanbin 2019**	**Conventional brackets**	**Self-ligating brackets**	**1.10 (0.35, 1.87)**	**0.004**
4	Irregularity change	0-4 weeks	Mahmoudzadeh 2018	Wire: superelastic NiTi	Wire: thermal NiTi	-0.34 (-1.02, 0.34)	0.33
5	**Irregularity change**	**0-4 weeks**	**El Shehawy 2020**	**No laser**	**Laser**	**0.21 (0.12, 0.30)**	**<0.001**
6	Irregularity change	0-8 weeks	Miles 2012	No vibration	Vibration	0.80 (-0.51, 2.11)	0.23
7	**Irregularity change**	**0-8 weeks**	**Sebastian 2019**	**Wire: superelastic NiTi**	**Wire: coaxial superelastic NiTi**	**1.31 (0.03, 2.11)**	**0.04**
8	Irregularity change	0-8 weeks	Abdelrahman 2015	Wire: Nitinol	Wire: superelastic NiTi	0.25 (-1.19, 1.69)	0.73
9	Irregularity change	0-8 weeks	Ulhaq 2017	Wire: Nitinol	Wire: cosmetic (Biocosmetic)	0.36 (-0.84, 1.56)	0.56
10	Irregularity change	0-8 weeks	Ulhaq 2017	Wire: Nitinol	Wire: cosmetic (TP)	-0.36 (-1.30, 0.58)	0.45
11	Irregularity change	0-8 weeks	Ulhaq 2017	Wire: Nitinol	Wire: cosmetic (Toothtone)	0.13 (-0.92, 1.18)	0.81
12	Irregularity change	0-8 weeks	El Shehawy 2020	No laser	Laser	0.16 (-0.14, 0.46)	0.30
13	**Irregularity change**	**0-12 weeks**	**Little 2017**	**Non-Ex**	**Ex**	**2.04 (1.13, 2.95)**	**<0.001**
14	Irregularity change	0-12 weeks	Little 2017	Ligature: conventional	Ligature: figure-8	-0.45 (-1.32, 0.42)	0.31
15	Irregularity change	0-12 weeks	Sebastian 2019	Wire: superelastic NiTi	Wire: coaxial superelastic NiTi	0.87 (-0.41, 2.15)	0.18
16	Irregularity change	0-12 weeks	Abdelrahman 2015	Wire: Nitinol	Wire: superelastic NiTi	-0.10 (-1.64, 1.44)	0.90
17	Irregularity change	0-12 weeks	Abdelrahman 2015	Wire: superelastic NiTi	Wire: thermal NiTi	-0.30 (-1.65, 1.05)	0.66
18	Irregularity change	0-12 weeks	El Shehawy 2020	No laser	Laser	0.03 (-0.21, 0.27)	0.80
19	Irregularity change	0-16 weeks	Abdelrahman 2015	Wire: Nitinol	Wire: thermal NiTi	-0.30 (-1.76, 1.16)	0.69
20	Irregularity change	0-16 weeks	Abdelrahman 2015	Wire: Nitinol	Wire: superelastic NiTi	0.10 (-1.46, 1.66)	0.90
21	Irregularity change	0-16 weeks	Abdelrahman 2015	Wire: superelastic NiTi	Wire: thermal NiTi	-0.40 (-1.76, 0.96)	0.56
22	**Irregularity change**	**4-8 weeks**	**Jahanbin 2019**	**Conventional brackets**	**Self-ligating brackets**	**-0.70 (-1.21, -0.19)**	**0.007**
23	Irregularity change	4-8 weeks	Sebastian 2019	Wire: superelastic NiTi	Wire: coaxial superelastic NiTi	0.03 (-1.46, 1.52)	0.97
24	Irregularity change	4-8 weeks	Abdelrahman 2015	Wire: Nitinol	Wire: superelastic NiTi	0.50 (-0.69, 1.69)	0.41
25	Irregularity change	4-8 weeks	El Shehawy 2020	No laser	Laser	-0.09 (-0.32, 0.14)	0.45
26	Irregularity change	8-12 weeks	Uribe 2017	No SAO	SAO	-1.11 (-2.34, 0.12)	0.08
27	**Irregularity change**	**8-12 weeks**	**Jahanbin 2019**	**Conventional brackets**	**Self-ligating brackets**	**-0.70 (-1.00, -0.40)**	**<0.001**
28	Irregularity change	8-12 weeks	Sebastian 2019	Wire: superelastic NiTi	Wire: coaxial superelastic NiTi	-0.44 (-1.30, 0.42)	0.31
29	Irregularity change	8-12 weeks	Abdelrahman 2015	Wire: Nitinol	Wire: superelastic NiTi	-0.35 (-0.83, 0.13)	0.15
30	Irregularity change	8-12 weeks	Abdelrahman 2015	Wire: superelastic NiTi	Wire: thermal NiTi	0.10 (-0.41, 0.61)	0.70
31	Irregularity change	8-12 weeks	El Shehawy 2020	No laser	Laser	0.11 (-0.13, 0.35)	0.37
32	Irregularity change	12-16 weeks	Abdelrahman 2015	Wire: Nitinol	Wire: thermal NiTi	0.10 (-0.14, 0.34)	0.41
33	Irregularity change	12-16 weeks	Abdelrahman 2015	Wire: Nitinol	Wire: superelastic NiTi	0.20 (-0.04, 0.44)	0.10
34	Irregularity change	12-16 weeks	Abdelrahman 2015	Wire: superelastic NiTi	Wire: thermal NiTi	-0.10 (-0.31, 0.11)	0.34
35	Irregularity change	12-16 weeks	Jahanbin 2019	Conventional brackets	Self-ligating brackets	-0.18 (-0.43, 0.07)	0.16

CI, confidence interval; Ex, extraction; MD, mean difference; NiTi, Nickel-Titanium; SAO, surgically assisted orthodontics.

Table 2.5 Direct meta-analytical comparisons with Mean Differences (MDs) on alignment duration and irregularity change

Outcome	Time-point	Comparison (studies)	MD (95% CI)	P	I^2 (95% CI)	tau^2 (95% CI)
Incisor-alignment duration	-	Thermal NiTi versus NiTi wire (n=2)	-0.39 (-9.91, 9.13)	0.94	0% (NC)	0 (NC)
Incisor-alignment-duration	-	SAO versus no SAO (n=4)	-44.31 (-68.90, -20.04) *	<0.001	94% (58%, 99%)	534.17 (48.12, >1000.00)
Whole-arch alignment-duration	-	Self-ligating versus conventional brackets (n=2)	83.79 (-63.27, 230.86)	0.26	95% (NC)	>1000.00 (NC)
Irregularity change	0-4 weeks	Thermal NiTi versus NiTi wire (n=2)	-0.22 (-1.04, 0.61)	0.61	0% (0%, 99%)	0 (0, 45.07)
Irregularity change	0-8 weeks	Thermal NiTi versus NiTi wire (n=2)	-0.05 (-0.87, 0.77)	0.91	0% (0%, 98%)	0 (0, 17.79)
Irregularity change	0-12 weeks	Thermal NiTi versus NiTi wire (n=2)	-0.22 (-1.06, 0.62)	0.61	0% (0%, 98%)	0 (0, 20.45)
Irregularity change	4-8 weeks	Thermal NiTi versus NiTi wire (n=2)	0.04 (-0.49, 0.57)	0.88	36% (0%, 100%)	0.09 (0, 29.76)
Irregularity change	8-12 weeks	Thermal NiTi versus NiTi wire (n=2)	-0.15 (-0.27, -0.02)	0.02	0% (0%, 98%)	0 (0, 1.66)
Irregularity change	0-4 weeks	Thermal NiTi versus superelastic NiTi wire (n=2)	0.01 (-0.71, 0.73)	0.98	0% (0%, 99%)	0 (0, 36.95)
Irregularity change	0-8 weeks	Thermal NiTi versus superelastic NiTi wire (n=2)	-0.19 (-0.98, 0.59)	0.63	0% (0%, 98%)	0 (0, 18.18)
Irregularity change	4-8 weeks	Thermal NiTi versus superelastic NiTi wire (n=2)	-0.10 (-0.78, 0.58)	0.78	0% (0%, 99%)	0 (0, 15.79)
Irregularity change	0-8 weeks	Self-ligating versus conventional brackets (n=2)	0.49 (-0.31, 1.30)	0.23	0% (0%, 99%)	0 (0, 29.22)
Irregularity change	0-16 weeks	Self-ligating versus conventional brackets (n=2)	0.25 (-0.61, 1.11)	0.57	0% (0%, 99%)	0 (0, 54.17)
Irregularity change	0-4 weeks	SAO versus no SAO (n=2)	2.30 (-2.36, 6.95)	0.33	97% (78%, 100%)	10.92 (1.31, >1000.0)
Irregularity change	0-8 weeks	SAO versus no SAO (n=2)	1.48 (-1.75, 4.70)	0.37	94% (60%, 100%)	5.09 (0.48, 679.70)
Irregularity change	0-12 weeks	SAO versus no SAO (n=2)	-0.10 (-2.27, 2.07)	0.93	88% (21%, 100%)	2.20 (0.08, 312.13)
Irregularity change	4-8 weeks	SAO versus no SAO (n=2)	-0.95 (-2.36, 0.47)	0.19	67% (0%, 100%)	0.72 (0, 133.57)

CI, confidence interval; MD, mean difference; NiTi, Nickel-Titanium; SAO, surgically-assisted orthodontics.

* 95% predictive interval -157.13 to 68.51 days.

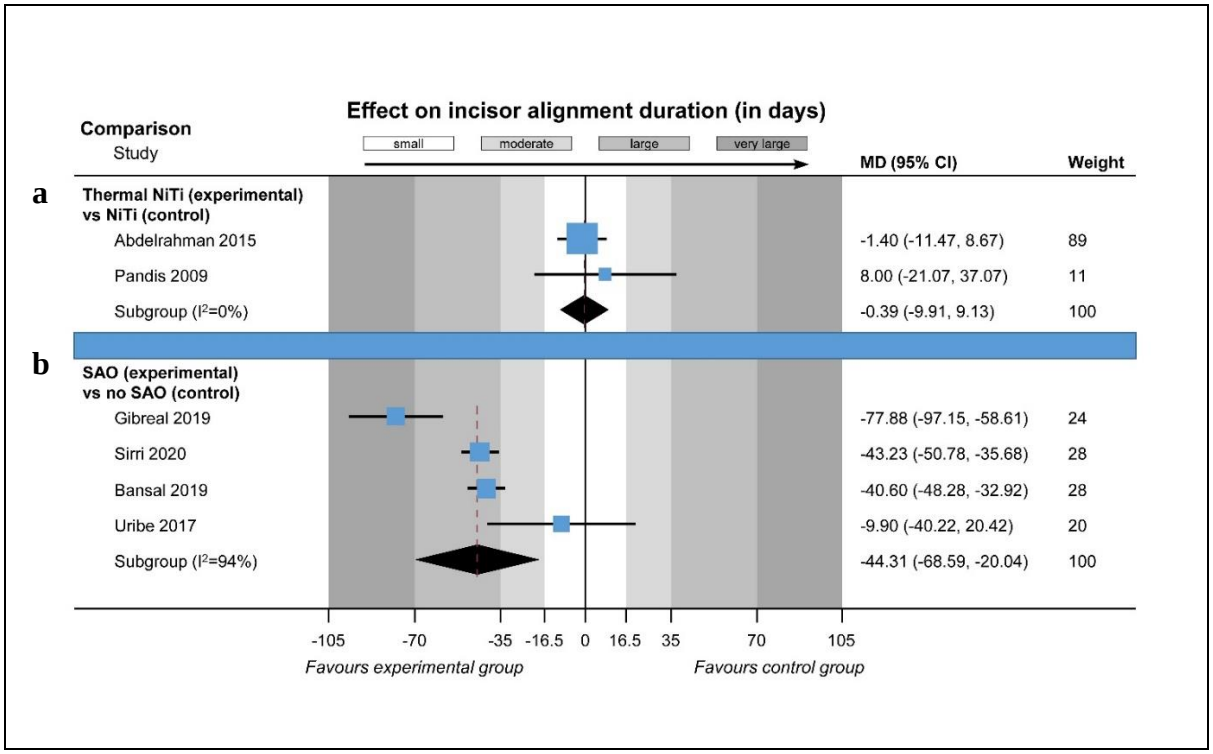

Figure 2.5 Contour-enhanced Forest plot of effect on incisor alignment duration.

It shows the effects of thermal NiTi archwires vs conventional NiTi for change in the duration of incisor alignment in days (a), and the effect of surgically-assisted orthodontics vs non-surgically assisted orthodontics for change in incisor alignment duration in days (b). Colour contours indicate increasing effect magnitude from the middle to the ends of the forest plot: small effects (white), moderate effects (light grey), large effects (mid-grey), and very large effects (dark grey). CI, confidence interval; MD, mean difference; NiTi, Nickel-Titanium wire; SAO, surgically-assisted orthodontics.

Table 2.6 Summary of findings table according to the GRADE approach.

	Anticipated absolute effects (95% CI)				
Outcome Studies (patients)	**Control group[a]**	**Experimental group**	**Difference in experimental group**	**Quality of the evidence (GRADE)[b]**	**What happens with experimental treatment**
	NiTi	**Thermal NiTi**			
Incisor-alignment duration 2 trials (90 patients)	94.2 days	-	0.4 day less (9.9 less to 9.1 more)	□□□□ high	Little to no difference in initial alignment duration
	No surgical insult	**Surgically-assisted orthodontics**			
Incisor-alignment duration 4 trials (153 patients)	124.2 days	-	44.3 days less (20.0 to 68.9 less)	□□□□ high[c,d] due to bias	Shorter initial alignment duration
	Conventional brackets	**Self-ligating brackets**			
Whole-arch alignment duration 2 trials (158 patients)	245.4 days	-	83.8 days more (63.3 less to 230.9 more)	□□□□ high	Little to no difference in complete alignment duration

Intervention: orthodontic treatment with fixed appliances with/without extractions and with/without adjuncts / Population: adolescent and adult patients with crowding / Setting: university clinics, hospitals, and private practice (Greece, India, Jordan, Syria, United Kingdom, United States of America).

[a] Response in the control group is based on random-effects meta-analysis duration among the control groups.

[b] Starts from "high"

[c] Downgrading by one level should be done for bias (3 trials in low risk of bias and 1 in high risk of bias), but omission of the trial in high risk of bias led to even larger effect estimates (MD=-52.3 days; 95% CI=-74.1 to -30.48 days; P<0.001); therefore, no downgrading was done.

[d] Considerable inconsistency observed (I^2=93%), but this does not affect our decision about surgical assisted orthodontics, as all trials were on the same side of the forest plot. However, caution is warranted by the quantification of the actual reduction in alignment duration.

CI, confidence interval; GRADE, Grading of Recommendations Assessment, Development and Evaluation.

2.3.4.3 Individual-patient-data

Apart from aggregate data available in certain articles, raw IPD was obtained for 3 out of 4 studies reporting on the primary outcome of duration to achieve whole-arch alignment in the mandibular dentition (Scott et al., 2008, Songra et al., 2014, Woodhouse et al., 2015). Modification of acquired datasets (237 adolescent/adult patients in total) was undertaken to create three datasets with overlapping characteristics (n=143 adolescent patients) compatible for re-analysis (Tables 2.7 and 2.8). Amongst patients with great irregularity (>7.0 mm), patient age was significantly associated with increased duration, 12.7 days on average per additional patient year (n=3 trials; 95% CI=7.7-17.7 days; P<0.001; Figure 2.6; Table 2.9). Whilst, among patients with small to moderate irregularity (<7.0 mm), alignment duration was increased by 17.5 days per additional mm of baseline-irregularity (n=2 trials; 95% CI=9.8-25.2 mm; P<0.001).

2.3.5 Additional analyses

Only two indirect meta-analyses included ≥10 studies and could be assessed for reporting biases. The contour-enhanced funnel plots exhibit some hints of asymmetry (Figure 2.7), which was not confirmed by Egger's test for weeks 0-4 (P=0.10) or weeks 0-8 (P=0.50). Sensitivity analyses according to the sole inclusion of studies with low risk of bias or studies with adequate sample size (>30 patients) found that results were consistent with original analyses (Table 2.10). Significant differences were found by both sensitivity analyses for alignment changes at weeks 4-8 and weeks 8-12; however, these were clinically irrelevant.

Table 2.7 Characteristic of the datasets available for re-analysis.

Variable	Data	Scott 2008†	Songra 2014¥	Woodhouse 2015
Patients	n	62	98	77
Age	Mean (SD) [range]	16.1 (4.4) [10.5, 37.6]	14.0 (1.3) [11.0, 17.08]	14.0 (1.7) [12.0, 19.0]
Age>18	n (%)	14 (23%)	0 (0%)	3 (4%)
Male	n (%)	32 (52%)	35 (36%)	38 (49%)
Centre1	n (%)	22 (35%)	-	33 (43%)
Centre2	n (%)	40 (65%)	-	15 (19%)
Centre2	n (%)	-	-	29 (38%)
LII	Median (IQR) [Range]	11.5 (9.2, 13.6) [6.7, 21.4]]	7.4 (5.6, 10.9) [1.7, 19.8]	7.9 (5.5, 10.4) [0.4, 23.3]]
Differences	By intervention*	P=0.27	P<0.001	P=0.56
Differences	By centre*	P=0.02	-	P=0.02

Table 2.8 Characteristic of the refined datasets available for re-analysis with age & LII overlap (with age<18 years and LII>7).

Variable	Data	Scott 2008†	Songra 2014¥	Woodhouse 2015
Patients	n	47	54	42
Age	Mean (SD) [range]	14.2 (1.6) [10.5, 17.9]	14.1 (1.4) [11.6, 17.1]	13.9 (1.5) [12.0, 17.0]
Age>18	n (%)	0 (0%)	0 (0%)	0 (0%)
Male	n (%)	27 (57%)	20 (37%)	20 (48%)
Centre1	n (%)	13 (28%)	-	24 (57%)
Centre2	n (%)	34 (72%)	-	6 (14%)
Centre2	n (%)	-	-	12 (29%)
LII	Median (IQR) [Range]	11.6 (9.1, 13.5) [7.1, 21.4]	10.1 (8.1, 12.6) [7.1, 19.8]	10.0 (8.2, 11.7) [7.0, 23.3]
Differences	By intervention*	0.21	0.03	0.76
Differences	By centre*	0.12	-	0.02

Note. Comment on the compatibility of the 3 available trial datasets: an overlap among the available 3 trial samples is refined with age<18 y and LII>7 mm to analyze them in a parallel manner.
IQR, interquartile range; LII, Little's irregularity index; SD, standard deviation; y, year.
* P value from 1-way analysis of variance on the transformed alignment duration; † Re-analyzing the data of Scott 2008 in order to assess the effect of baseline patient age on alignment duration according to patient age category (underage <18 years vs overage >18 years), indicates that age has a different effect on underage patients (coefficient=11.45; 95% Confidence Interval [CI]=4.04 to 18.85) and overage patients (coefficient=-0.25; 95% CI=-0.45 to -0.05)—a difference that is statistically significant (P for subgroups=0.02); ¥ Re-analyzing the data of Songra 2014 in order to assess the effect of baseline patient age on alignment duration according to Little's Irregularity Index (LII) category (LII <7 mm vs. LII >7 mm), indicates that age has a different effect on patients with LII<7 mm (coefficient=-11.52; 95% Confidence Interval [CI]=-35.59 to 12.54) and patients with LII>7 mm (coefficient=20.88; 95% CI=-7.18 to 48.94)—a difference that is statistically significant (P for subgroups=0.08).

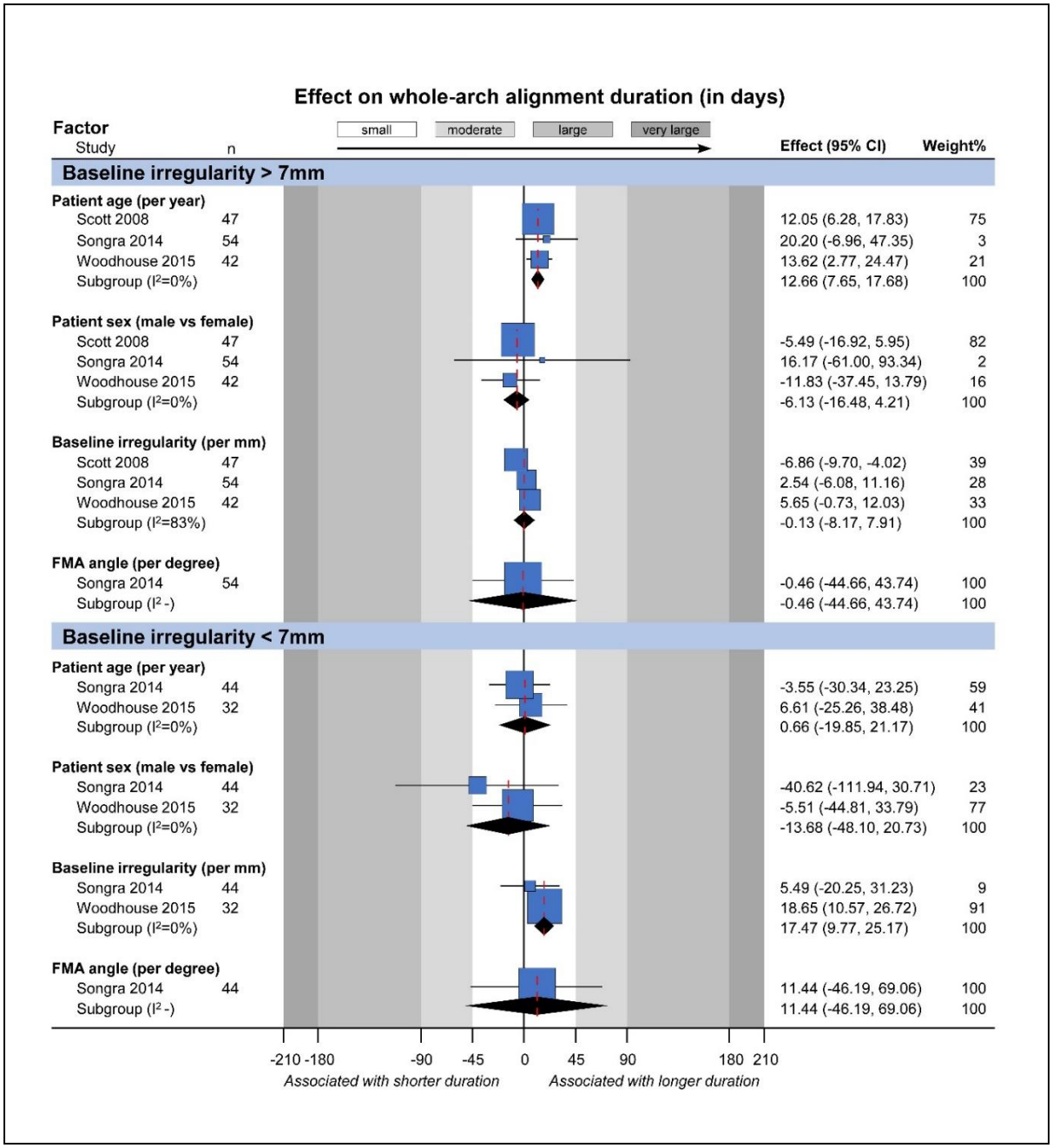

Figure 2.6 Contour-enhanced Forest plot of effect on whole-arch alignment duration.

It shows the effect of patient baseline age, sex, Little's irregularity index, and Frankfort mandibular plane angle on whole-arch mandibular alignment duration in days. Colour contours indicate increasing effect magnitude from the middle to the ends of the forest plot: small effects (white), moderate effects (light grey), large effects (mid-grey), and very large effects (dark grey). CI, confidence interval; FMA, Frankfort mandibular plane.

Table 2.9 Random-effects meta-analysis of the effect of patient baseline age, sex, Little's irregularity index, and FMA angle on duration of alignment.

		Crude analysis				Adjusted-for-confounders analysis		
Variable	**Studies**	**Coefficient (95% CI)**	**P**	**I^2 (95% CI)**		**Coefficient (95% CI)**	**P**	**I^2 (95% CI)**
Patients aged<18 years & LII>7 mm								
Age	3	14.53 (7.25, 21.81)	<0.001	0% (0%, 89%)		12.67 (7.65, 17.68)	<0.001	0% (0%, 91%)
Sex	3	-8.58 (-24.09, 6.94)	0.28	0% (0%, 100%)		-6.13 (-16.48, 4.21)	0.25	0% (0%, 100%)
LII	3	-0.64 (-8.57, 7.28)	0.87	82% (31%, 99%)		-0.13 (-8.17, 7.91)	0.98	83% (36%, 99%)
FMA	1	-8.08 (-46.64, 30.48)	0.68	-		-0.46 (-45.78, 44.85)	0.98	-
Patients aged<18 & LII<7 mm								
Age	2	-11.28 (-29.97, 7.41)	0.24	0% (0%, 100%)		0.66 (-19.85, 21.17)	0.95	0% (0%, 100%)
Sex	2	-35.51 (-96.85, 25.83)	0.26	56% (0%, 100%)		-13.68 (-48.10, 20.74)	0.44	0% (0%, 100%)
LII	2	19.01 (11.33, 26.69)	<0.001	0% (0%, 100%)		17.47 (9.77, 25.17)	<0.001	0% (0%, 100%)
FMA	1	14.54 (-47.14, 76.23)	0.64			11.44 (-48.03, 70.90)	0.70	

CI, confidence interval; LII, Little's irregularity index.

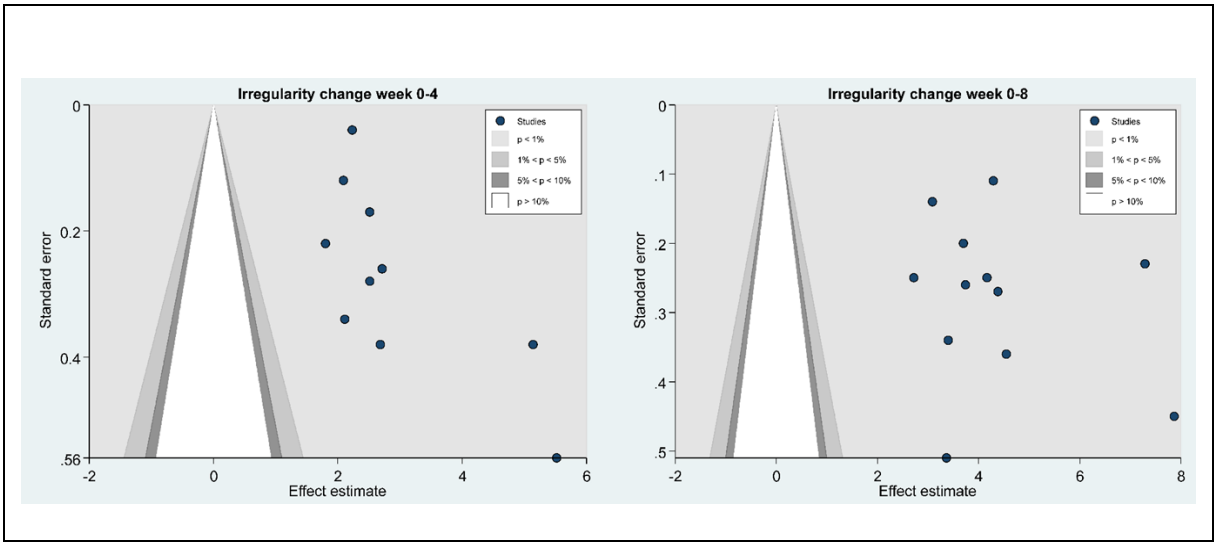

Figure 2.7 Contour-enhanced funnel plot of the indirect meta-analysis of incisor irregularity change between 0-4 and 0-8 weeks.

Table 2.10 Pooled average (indirect meta-analysis) for alignment duration and anterior irregularity changes across identified randomized trials.

Outcome	Time-point	Original analysis	Low risk of bias	Large trials (>30 patients)
		Trials Average (95% CI)	**Trials Average (95% CI) P***	**Trials Average (95% CI) P***
Initial alignment duration	-	n=9 100.73 (84.05, 117.41)	n=6 104.98 (82.50, 127.45) P=0.06	n=7 104.09 (84.45, 123.74) P=0.66
Irregularity change	0-4 weeks	n=10 2.88 (2.12, 3.63)	n=6 3.21 (1.96, 4.46) P=0.93	n=6 2.76 (1.85, 3.68) P=0.72
Irregularity change	0-8 weeks	n=12 4.37 (3.48, 5.26)	n=6 5.05 (3.46, 6.63) P=0.48	n=8 4.06 (3.08, 5.04) P=0.49
Irregularity change	0-12 weeks	n=8 5.55 (4.39, 6.71)	n=5 6.06 (4.38, 7.74) P=0.54	n=5 5.59 (3.69, 7.50) P=0.41
Irregularity change	4-8 weeks	n=9 1.54 (1.18, 1.90)	**n=5 1.68 (1.14, 2.22) P=0.01**	**n=5 1.34 (0.88, 1.80) P=0.002**
Irregularity change	8-12 weeks	n=7 0.73 (0.33, 1.14)	**n=4 0.97 (0.44, 1.49) P=0.009**	**n=3 0.33 (0.05, 0.60) P=0.005**

CI, confidence interval
* P value for subgroup differences according to methodological characteristic.

2.4 Discussion

2.4.1 Summary of evidence

The current systematic review summarises evidence from RCTs on treatment duration to achieve alignment of the mandibular dentition during the initial phase of orthodontic treatment using fixed orthodontic appliances. Out of the initially identified 3016 publications from the literature search, 35 studies were ultimately included, with a total of 2258 patients. The duration of orthodontic treatment was assessed in terms of time to whole-arch or incisor-alignment of the mandibular dentition.

High heterogeneity among studies was observed for the primary outcome of duration to achieve whole-arch alignment, with an average pooled duration of 8.8 months. No significant differences were found between self-ligating and conventional-ligated brackets. However, a combined re-analysis of IPD obtained for three RCTs (Scott et al., 2008, Songra et al., 2014, Woodhouse et al., 2015) showed adolescent patient age significantly associated with the duration of time to achieve whole-arch alignment for (>7 mm) irregularity. After adjusting for confounding effects of baseline irregularity, gender, and mandibular plane angle, each additional patient year had an increased alignment duration of 12.7 days. There have previously been contradictory results for such a relationship (Abbing et al., 2020, Beckwith et al., 1999, Fink and Smith, 1992, Vig et al., 1990, Skidmore et al., 2006), but this could be due to the fact that this type of data is not identifiable from several small separate studies with different patient cohorts, whereas IPD enabled pooling among similar patient cohorts and increased statistical power. Likewise, among adolescent patients with <7mm irregularity, a relationship between baseline-irregularity and duration for whole-arch alignment was found, with 17.5 days added for each additional mm of irregularity, agreeing with previous data (Pandis et al., 2007).

Substantial variation was found in the incisor-alignment duration of the mandibular incisor teeth, with a pooled average of 100.7 days: and incisor irregularity changes during

treatment periods with pooled averages of 2.9 mm (month 1), 1.5 mm (months 1-2), 0.7 mm (months 2-3), 0.3 mm (months 3-4), 0.3 mm (months 4-5), and 0.2 mm (months 5-6). Interestingly, no clinically relevant differences were seen according to different NiTi archwires or the usage of surgical-assisted orthodontics. Nevertheless, there was extreme heterogeneity, which is to be expected due to the combination of different clinical settings, patient demographics, extractions, malocclusions, fixed appliances and treatment adjuncts (Abbing et al., 2020, Vig et al., 1990, Schubert et al., 2020, Vieira et al., 2018, Skidmore et al., 2006, Dudic et al., 2013). Furthermore, different time intervals between appointments were utilized across studies, including 2-weeks (Abdelrahman et al., 2015, Nahas et al., 2017), 3-weeks (Bansal et al., 2019), 4-weeks (Gibreal et al., 2019, Pandis et al., 2007, Uribe et al., 2017) and 10-weeks (Ong et al., 2011). This is potentially a key factor in clinical trials evaluating time periods to achieve teeth alignment, though it is difficult to control in real-world orthodontic care. Although the time between appointments is likely to affect alignment duration, there is no robust current evidence to support an ideal appointment interval (Keim, 2011).
Additionally, limited data is available on the effects of the rapidity of archwire progression during alignment. Overall, the effect of clinical setting variations and environment on treatment duration remains unknown (Mavreas and Athanasiou, 2008). Finally, the fact that initial alignment is a seemingly subjective variable also adds to heterogeneity.

Part of this heterogeneity can also be explained by the application of different treatment methods. Meta-analysis of four trials indicated that surgically-assisted orthodontics was associated with significantly shorter incisor-alignment duration compared to conventional orthodontics, which has previously been indicated (Fleming et al., 2015) and apparently applies to conventional (Afzal et al., 2020) or customised fixed appliances (Charavet et al., 2019) and OTM rate (Hoogeveen et al., 2014). However, no difference was observed for different NiTi archwires, which agrees with other studies demonstrating that there is insufficient evidence to

determine the effectiveness of different archwires in terms of alignment rate or duration (Wang et al., 2018, Riley and Bearn, 2009). Photobiomodulation was associated in a single trial with shorter incisor-alignment duration (Nahas et al., 2017), but contradicting results exist in the literature; some trials supporting (Doshi-Mehta and Bhad-Patil, 2012, AlShahrani et al., 2019, Cruz et al., 2004) and others rejecting (Limpanichkul et al., 2006a, Skidmore et al., 2006) the effectiveness of this intervention. Moreover, many of these studies only investigate the rate of OTM in the canine-retraction phase of treatment. Finally, one study found that self-ligating brackets significantly reduced incisor-alignment duration (Pandis et al., 2007), but this contradicts the findings of multiple other studies that have not reported this advantage (Miles, 2005, Miles et al., 2006, Scott et al., 2008, Songra et al., 2014).

Subgroup and meta-regression analyses showed that treatment-related characteristics were associated with tooth alignment. Premolar-extraction groups demonstrated greater alignment (irregularity-change) both in month 1 and month 2 compared to non-extraction groups (5.3 vs 2.3 mm; 7.5 vs 3.8 mm, respectively). This is consistent with other studies reporting significantly faster rates of alignment in extraction than non-extraction cases (Little and Spary, 2017, Ong et al., 2010, Scott et al., 2008). However, the overall treatment duration with extractions is significantly longer by around six months (Papageorgiou et al., 2017), with many other studies confirming this longer treatment duration with extractions (Alger, 1988, Fink and Smith, 1992, Skidmore et al., 2006). In addition, bracket slot size was associated with alignment alleviation at week 4-8, favouring minimally the 0.022-inch slot over 0.018-inch (1.6 vs 1.0 mm). This is similar to previous results demonstrating that the alignment rate is significantly faster for 0.022-inch bracket slot size in the mandibular arch (Cobb 3rd et al., 1998), but disagrees with another study and systematic review showing no difference (Yassir et al., 2019, Vieira et al., 2018). However, other studies reported longer treatment duration for

a 0.022-inch bracket slot size than a 0.018-inch slot (Detterline et al., 2010, Amditis and Smith, 2000).

2.4.2 Implications for clinical practice and research

This is the first comprehensive systematic review of the duration of teeth alignment during orthodontic treatment. It builds upon previous data on overall treatment duration using fixed appliances (Tsichlaki et al., 2016), the impact of various types of malocclusion (Mavreas and Athanasiou, 2008), treatment of adolescents and adults (Abbing et al., 2020), bracket or archwire design (Papageorgiou et al., 2014a, Riley and Bearn, 2009). The quality of data on overall treatment durations has improved in recent years, but there is now more data on alignment because this is a more often used and convenient metric in studies assessing the effectiveness of treatment interventions using fixed appliances. Researchers should work towards establishing studies that investigate the effect of different interventions on total treatment duration.

According to current data, most orthodontic treatment with fixed appliances will take just under two years to finish and will require approximately 18 visits (Tsichlaki et al., 2016). The findings of the current review indicate that teeth alignment might occupy almost one-third of the whole treatment duration. This indicates that shortening alignment time might contribute to shorter overall treatment durations, and practitioners should manage the alignment phase of treatment carefully. Interestingly, variations in appliance design had minimal influence on alignment rates, which was consistent with the findings of previous reviews (Papageorgiou et al., 2014a, Riley and Bearn, 2009). However, surgically-assisted orthodontics reduced incisor alignment time, and this adjunct treatment appears to be associated with higher rates of tooth movement - at least in the short term - for patients who are willing to undergo such a procedure.

2.4.3 Strengths and limitations

The strengths of this review include a priori registration (Sideri et al., 2018), extensive unrestricted comprehensive literature searching, the inclusion of RCTs (Papageorgiou et al., 2019, Papageorgiou et al., 2015b), contemporary statistics (Langan et al., 2019), assessment quality of meta-evidence according to GRADE (Guyatt et al., 2011) and transparent open dataset provision (Wazwaz et al., 2020).

However, this systematic review also has some limitations. The limitations are dependent upon methodological issues with the conduct of included studies, as well as high levels of heterogeneity between studies, which may introduce bias. Additionally, several studies had moderate to small sample sizes, which might influence the magnitude and precision of estimated effects (Cappelleri et al., 1996). Finally, due to the limited number of included trials and their incomplete reporting, all pre-planned subgroup and meta-regression analyses could not be carried out to identify factors associated with the outcome of interest.

2.5 Conclusions

The present systematic review identified 35 RCTs describing 2258 patients with a mean age of 17.8 years. The pooled duration for whole-arch alignment of the mandibular dentition was 263.0 days, whilst mandibular incisor-alignment took 100.7 days. Extreme heterogeneity was seen among trials for all indirect poolings. IPD analysis from three RCTs revealed that patient age was significantly associated with increased duration for the alignment of >7.0 mm irregularity, whereas for irregularity <7.0 mm, the alignment duration was increased for every mm of baseline irregularity. Future research studies investigating orthodontic tooth alignment rates would benefit from adequate sample sizes and more consistent outcome assessment methods. The data in this systematic analysis provide a basis for future RCTs assessing the rate of orthodontic teeth alignment using fixed appliances.

Chapter 3 Duration of canine retraction with fixed appliances: A systematic review and meta-analysis

3.1 Introduction

Comprehensive orthodontic treatment with fixed appliances can be divided into several phases, including alignment and levelling, space management (either creation or closure), correction of inter-arch relationships, and finishing or detailing of the occlusion. Comprehensive orthodontic treatment with fixed appliances is lengthy, with a previous systematic review reporting an average treatment duration of 24.9 months (Papageorgiou et al., 2017). In addition a recent systematic review reported that an average of 8.8 months (263.0 days) might be required to complete the first phase of teeth alignment (Wazwaz et al., 2022b). When orthodontic treatment involves the extraction of teeth, space closure can represent one of the most challenging phases of treatment (Ribeiro and Jacob, 2016) and can be associated with prolonged treatment duration (Papageorgiou et al., 2017, Mavreas and Athanasiou, 2008, Fisher et al., 2010, O'Brien et al., 1995, Skidmore et al., 2006, Germeç and Taner, 2008). In some cases, orthodontists will perform canine retraction mechanics as a separate stage of treatment, which can help to preserve anchorage during the establishment of inter-arch relationships. Furthermore, canine retraction is a common experimental model during investigations of different variables during OTM and determining average canine retraction duration and rate are useful metrics for evaluating treatment progress and planning future research in this domain.

Although a recent systematic review investigated the rate of canine retraction in patients undergoing orthodontic treatment with fixed appliances, it did not assess complete duration of

canine retraction, which is the most clinically relevant outcome for both patients and orthodontists. Additionally, studies included in the review used non-conventional measures (surgical and non-surgical adjuncts) to accelerate OTM; however, those that used conventional interventions were not included (MacDonald et al., 2020). Hence, the primary objective of this systematic review was to critically appraise clinical evidence from randomized clinical trials (RCTs) evaluating treatment duration required to fully retract maxillary canines following maxillary first premolar extractions in adolescent and adult patients undergoing orthodontic treatment with fixed appliances. The secondary objective was to assess the rate of canine retraction measured as the amount of canine tooth movement per unit time and identify associated factors.

3.2 Materials and methods

3.2.1 Protocol and registration

The protocol for this review was made a *priori* and registered in PROSPERO (CRD42020198596). This review was conducted and reported according to the Cochrane Handbook (version 6.3) (Higgins et al., 2021) and Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) 2020 statement (Page et al., 2021), respectively.

3.2.2 Eligibility criteria

According to the Participants-Intervention-Comparison-Outcome-Study design (PICOS) schema, included were: (P) human participants of any age, sex, ethnicity, or malocclusion in need of maxillary first premolar extraction followed by individual canine retraction as a part of an orthodontic treatment plan with full-arch fixed appliances; (I) retraction of maxillary canines

using full-arch fixed appliances with or without any treatment adjuncts; (C) RCTs of any comparison involving various surgically-assisted or non-surgical treatment procedures, appliances, or adjuncts; (O) assessing duration and/or rate of maxillary canine retraction; (S) parallel-group or split-mouth (within patient randomized) RCTs. No limitations regarding language, publication year or status were applied. Excluded were studies involving animals, case reports or series, non-clinical and non-randomized studies, cross-sectional studies, studies using segmented arch mechanics or en-masse retraction, studies comprising patients with any systematic disease, craniofacial abnormalities, studies without comprehensive orthodontic treatment or eligible outcomes, and studies involving patients who had previously undergone orthodontic treatment, growth modification, or multidisciplinary treatment.

The primary outcome of this review was the duration of maxillary canine retraction in months from the start to completion of retraction. The amount of canine retraction relative to the observation time (canine retraction rate) was assessed as a secondary outcome.

3.2.3 Information sources, search strategy and study selection

Eight electronic databases (MEDLINE, Embase, Cochrane Database of Systematic Reviews, Cochrane Central Register of Controlled Trials, Cochrane Database of Abstracts of Reviews of Effects, Scopus, Web of Science, and Latin American and Caribbean Health Sciences Literature) were searched systematically without restrictions for publication date, language or type from inception up to July 09, 2021, whereas the Directory of Open Access Journals (DOAJ), Digital Dissertations, metaRegister of Controlled Trials, Google Scholar as well as reference/citation lists of eligible articles or existing systematic reviews were manually searched additional papers. A detailed search strategy was created for each database. Individual

search strategies were based on the search strategy developed for MEDLINE but modified appropriately for every database (Appendix 3.1).

3.2.4 Study selection, data items and collection

Two reviewers independently screened titles, abstracts, and full texts of identified studies to check for eligibility, with all discrepancies between reviewers resolved by discussion with a third reviewer. Data extraction was conducted independently by two reviewers, with similar discrepancy resolution using pre-determined and piloted extraction forms covering: (1) study characteristics (design, clinical setting, country); (2) patient characteristics (age/sex); (3) malocclusion and treatment characteristics; (4) appliance type; (5) intervention and/or adjunct interventions; (6) follow-up period; (7) outcome details.

3.2.5 Risk of bias in individual studies

The risk of bias of included studies was assessed according to Cochrane guidelines with the Risk of Bias 2.0 tool (Sterne et al., 2019) independently by two reviewers with the same discrepancy resolution approach.

3.2.6 Data synthesis and summary measures

An effort was made to maximize data output from included trials, and where data were missing, raw data from studies on the primary outcome were calculated by us. Since orthodontic treatment outcome is inevitably affected by patient and treatment-related characteristics, a random-effects model was used to calculate the average distribution of true effects based on clinical and statistical reasoning (Papageorgiou, 2014) and a restricted maximum likelihood

random-effects model was used according to recent guidance (Langan et al., 2019) and with the Hartung-Knapp correction (Hartung and Knapp, 2001). Mean differences (MD) for continuous variables and their corresponding 95% CIs were calculated as effect sizes. Initially, indirect analyses of pooled averages were conducted to calculate average canine retraction duration or rate during orthodontic treatment with 95% CIs. Then direct meta-analysis was performed comparing trial-arms with different retraction methods or treatment adjuncts within each trial and pooling mean differences (MDs) across trials.

Between-study heterogeneity was assessed by inspecting forest plots by calculating τ^2 (absolute heterogeneity) and I^2 (relative heterogeneity) statistics, respectively. We considered I^2 over 75% to arbitrarily denote considerable heterogeneity while as well considering the localization of heterogeneity on the forest plot and the certainty around estimated heterogeneity estimates. Ninety-five per cent prediction intervals were calculated for meta-analyses of ≥3 trials to incorporate and visualize existing heterogeneity and provide a range of probable effects in a future clinical setting.

All analyses were run in R (version 4.0.4)) and the dataset was openly provided (Wazwaz et al., 2022a). All P values were 2-sided (α=5%, except the test of between-studies or between-subgroups heterogeneity where α-value was 10% (Ioannidis, 2008)).

3.2.7 Additional analyses, risk of bias across studies and quality of evidence

Possible sources of heterogeneity were planned a *priori* to be sought through subgroup analyses and random-effects meta-regression in meta-analyses of at least five studies according to patient age, gender, anchorage type, force magnitude, bracket type, bracket slot size and methods of canine retraction. Reporting biases (including the possibility of publication bias)

were assessed for meta-analysis with ≥7 trials with contour-enhanced funnel plots and Egger's test.

The certainty and overall quality of clinical recommendations were rated using the Grades of Recommendations, Assessment, Development, Evaluation (GRADE) framework (Guyatt et al., 2011) and revised summary of findings tables (Carrasco-Labra et al., 2016) were constructed for direct meta-analyses at three months of retraction which was arbitrarily deemed to be clinically relevant because no canine could have been fully retracted yet. Forest plots were augmented with contours representing the magnitude of observed effects to assess heterogeneity, imprecision, and clinical relevance.

3.2.8 Sensitivity analyses

Robustness of results was checked for meta-analyses of ≥3 trials with sensitivity analyses based on (1) RCT design (parallel or split-mouth); (2) precision of trials primarily according to sample size (most-precise half / least-precise half); (3) and risk of bias (low or some concerns; or high).

3.3 Results

3.3.1 Study selection and characteristics

The electronic literature search yielded 2253 results. Following the removal of duplicates, 915 titles and abstracts were screened, and the full text of 167 publications was checked against eligibility criteria (Appendix Table 3.1), whilst six additional studies were found through hand-searching. Finally, 50 publications reporting 50 studies were included, as depicted in the PRISMA flow diagram (Figure 3.1).

The characteristics of included trials are shown in Table 3.1. Among the 50 included RCTs, 44 were split-mouth and six were of parallel-group design. Included studies were undertaken in university clinics (n=37; 74%), private practices (n=2; 4%), hospitals (n=10; 20%), or both private practice and university (n=1; 2%) and originated from 17 different countries, including Australia, Brazil, China, Dominican Republic, Egypt, India, Iran, Jordan, Japan, Malaysia, Pakistan, Switzerland, Saudi Arabia, Syria, Thailand, Turkey and the United States of America. Of the 50 trials included, 48 (96%) were single-centre and 2 (4%) were multi-centre. The 50 studies included 811 patients with a mean age of 19.86 years (reported in 38 trials). Out of the 40 trials reporting on patient gender, 561/844 patients were female (66%), and 283 (34%) were male.

Nineteen studies (38%) did not report on malocclusion type, 3 included class I (6%), 14 included class II division1 malocclusion (28%), 1 included class II malocclusion (2%), 6 included patients with either class I or class II division1 malocclusion (12%), 3 included patients with either class I or class II malocclusion (6%), 1 included patients with either class II division1 malocclusion or crowding (2%), 2 included patients with either severe crowding or protrusion that require first premolars extractions (4%), and 1 included patients with either class II or bimaxillary protrusion (2%).

Forty-six studies reported on the amount of canine tooth movement measured at certain time intervals, and 4 reported the amount of canine tooth movement and duration. Canine retraction duration is assessed as the time between the beginning of force application for canine retraction and the completion of full canine retraction, while the rate of canine retraction was determined as the amount of canine tooth movement divided by the time interval. The amount of canine tooth movement was calculated at a specific time interval by measuring the distance between numerous reference points. Canine tooth movement was assessed, either from stone models (n=13), scanned models (n=19), intra-orally (n=8), intra-oral scans (n=2), on digital

photocopies of models (n=5), both on scanned models and intra-orally (n=2), or both on stone-models and intra-orally (n=1). Time points used in this study were 0, 1, 2, 3, 4, and 5 months, and the amount of tooth movement was measured for the following time intervals 0-1, 0-2, 0-3, 0-4, 0-5, 1-2, 2-3, 3-4, and 4-5 months, if possible.

Canine retraction duration and/or amount of canine tooth movement was compared between different interventions, including different retraction methods (n= 1), different bracket types (n=3), different ligation methods (n=1), and different orthodontic forces (n=1). Most studies used adjuncts to orthodontic treatment, including surgically-assisted orthodontics (n=18), photobiomodulation (n=14), vibration (n=4), low-intensity pulsed ultrasound (n=1), both surgically-assisted orthodontics and photobiomodulation (n=3), both surgically-assisted orthodontics and local injection of platelet-rich fibrin (n=1), and local injection of platelet-rich fibrin or platelet-rich plasma (n=3).

Eighteen studies reported using temporary anchorage devices (TADs) to enhance anchorage: 15 used a transpalatal arch (TPA, including Nance button), 1 used both TADs and TPA, 1 used both TPA and headgear, 4 used ligations of second premolars and first molars together, 2 included the second molar in the anchor unit, 2 used vertical stopped loops, and 7 studies did not report on methods of anchorage reinforcement. In 46 studies, NiTi closed coil springs were used to retract the canines, delivering 150 grams (g) of force in 36 studies, 100 g in one study, 200 g in 3 studies, 120 g in one study, 180 g in one study, 61 g in one study, both 100 and 150 g in one study, and 50, 100, and 150 g in one study, with one study not reporting on force magnitude. At the same time, elastic chains were used in 4 studies delivering 150 g of force. Maxillary first premolars were extracted 0-6 weeks before fixed appliance placement in 22 studies, 6 months before retraction in 3 studies, 3 months before retraction in 2 studies, 0-4 weeks before retraction in 12 studies, with 11 studies not reporting on the timing of extraction.

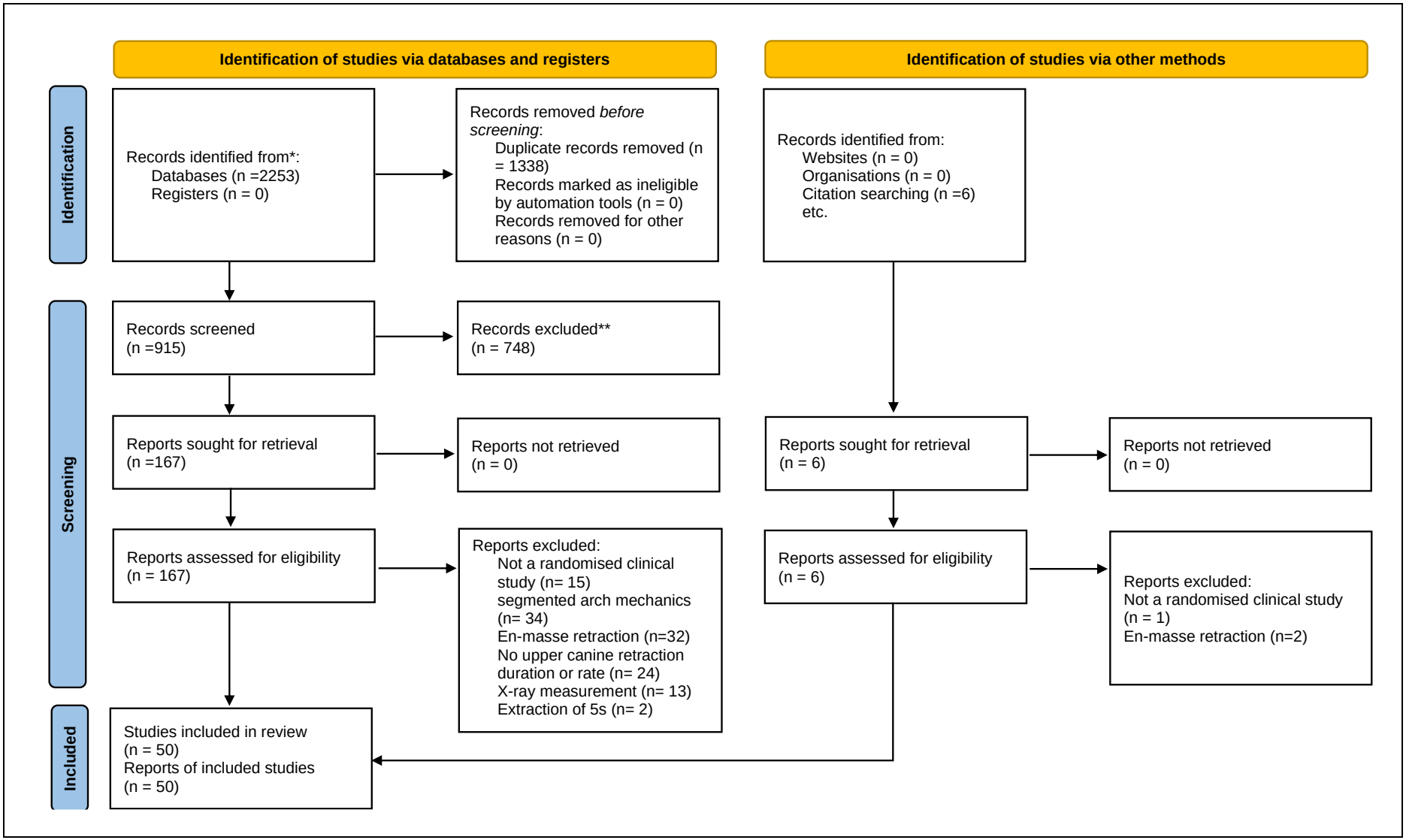

Figure 3.1 Preferred Reporting Items for Systematic reviews and Meta-Analyses diagram for the identification and selection of studies eligible in this review. Reproduced from (Page et al., 2021).

Table 3.1 Characteristics of included studies.

Study	Design; setting; country*	Patients (M/F); age†	Malocclusion / Tx	Appliance	Intervention / Supplemental	FU in wks (interval)	Outcome
(Abbas et al., 2016)	RCT (2 PA SMD); Uni; EGY	A: 10 (NR); 15-25 B: 10 (NR); 15-25 (SMD)	Cl. II/I / Ex of upper 4s	Labial CLB (Roth)	A: Corticotomy B: Piezocision	0-(2)-12	RetractRate
(Ahmad et al., 2020)	RCT (2 PA SMD); Uni; EGY	16 (0/16); 15-20	NR / Ex of upper 4s	Labial CLB (NR)	Mucoperiosteal Flap / LLLT	0-2-6-14-16-End	RetractRate; RetractDur
(Abdelhameed and Refai, 2018)	RCT (3 PA SMD); Uni; EGY	A: 10 (NR); 15-25 B: 10 (NR); 15-25 C: 10 (NR); 15-25	Cl. II or Bimax Prot / Ex of upper 4s	Labial CLB (NR)	A: MOPs B: LLLT C: MOPs + LLLT	0-(2)-12	RetractRate
(Aboalnaga et al., 2019)	RCT (SMD); Uni; EGY	18 (F); 20.50	NR / Ex of upper 4s	Labial CLB (Roth)	MOPs	0-(4)-16	RetractRate
(Aboul-Ela et al., 2011)	RCT (SMD); Uni; EGY	13 (5/8); 19	Cl. II/I / Ex of upper 4s	Labial CLB (NR)	Corticotomy	0-(4)-16	RetractRate
(Alfawal et al., 2018)	RCT (2 PA SMD); Uni; SYR	A: 18 (7/11); 18.7 B: 18 (5/13); 17.47	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	A: Piezocision B: LAFC	0-(4)-16	RetractRate; RetractDur
(Alikhani et al., 2013)	RCT (2 PA SMD); Uni; USA	A: 10 (5/5); 26.8 B: 10 (3/7); 24.7	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	A: MOPs B: CNT	0-(-)-4	RetractRate
(Alkebsi et al., 2018)	RCT (SMD); Uni; JOR	32 (8/24); 19.26	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	MOPs	0-(4)-12	RetractRate
(Alqadasi et al., 2019)	RCT (SMD); Hosp; CHN	8 (NR); 15-40	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	MOPs	0-2-4-8-12	RetractRate
(Alqadasi et al., 2021)	RCT (2 PA SMD); Hosp; CHN	A: 10 (4/6); 20.89 B: 11 (5/6); 20.89	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	A: MOPs B: Piezocision	0-2-4-8-12	RetractRate
(Al-Naoum et al., 2014)	RCT (SMD); Uni; SYR	30 (15/15); 20.04	Cl. II / Ex of upper 4s	Labial CLB (MBT)	Corticotomy	0-1-2-4-8-12	RetractRate
(Al-Shafi et al., 2021)	RCT (SMD); Hosp; CHE	20 (10/10); 15.8	NR / Ex of upper 4s	Labial SLB	Light-emitting diode lights	0-(4)-12	RetractRate
(Araghbidikashani et al., 2017)	RCT (SMD); Uni; IRN	15 (6/9); 14.3	NR / Ex of upper 4s	NR	Different retraction methods / None	0-(4)-16	RetractRate
(Babanouri et al., 2020)	RCT (2 PA SMD); Uni; IRN	28 (NR); 16.3-35.2	Cl. I-II/I / Ex of upper 4s	Labial CLB (MBT)	A: Buccal MOPs B: Buccal and palatal MOPs	0-(4)-12	RetractRate
(Cruz et al., 2004)	RCT (SMD); Pract; BRA	11 (NR); 12-18	Crowding or Bimax Prot / Ex of upper 4s	Labial CLB (Roth)	LLLT	0-(4)-8	RetractRate
(Dakshina et al., 2019)	RCT (SMD); Hosp; IND	24 (NR); > 18	NR/ Ex of upper 4s	NR	LLLT	0-(4)-12	RetractRate
(Deguchi et al., 2007)	RCT (SMD); Uni; JPN	30 (6/24); 21.30	Cl. I-II / Ex of upper 4s	Labial CLB (NR)	Use of Clear Snap	0-(4)-End	RetractRate; RetractDur

(Doshi-Mehta and Bhad-Patil, 2012)	RCT (SMD); Hosp; IND	20 (8/12); 12-23	NR / Ex of upper 4s	Labial CLB (MBT)	LLLT	0-(NR)-12-End	RetractRate
(El-Timamy et al., 2020)	RCT (SMD); Uni; EGY	16 (0/16); 18	Crowding or Bimax Prot / Ex of upper 4s	Labial CLB (Roth)	Local injection of Platelet-rich plasma	0-(4)-16	RetractRate
(Ekizer et al., 2016)	RCT (SMD); Uni; TUR	20 (7/13); 16.77	NR / Ex of upper 4s	Labial CLB (Roth)	Light-emitting diode lights	0-(4)-12	RetractRate
(Farid et al., 2019)	RCT (SMD); Uni; EGY	16 (0/16); 21.5	Cl. I-II / Ex of upper 4s	Labial CLB (Roth)	Combined corticotomy and LLLT	0-(4)-16	RetractRate
(Feizbakhsh et al., 2018)	RCT (SMD); Uni; IRN	20 (12/8); 28	CI. I / Ex of 4s	Labial CLB (Roth)	MOPs	0-(0)-4	RetractRate
(Haliloglu-Ozkan et al., 2018)	RCT; Uni; TUR	EXP: 17 (10/7); 15.27 CNT: 15 (9/6); 16.13	NR / Ex of 4s	Labial CLB (MBT)	MOPs	0-(4)-8	RetractRate
(Ozkan and Arici, 2021)	RCT (2 PA SMD); Uni; TUR	A: 12 (6/6); 17.27 B: 12 (6/6); 18.13	Cl. I-II/I / Ex of upper 4s	Labial CLB (MBT)	A: MOPs B: CNT	0-(0)-4	RetractRate
(Hassan et al., 2016)	RCT (SMD); Uni; SYR	15 (4/11); 20.99	NR / Ex of upper 4s	One side: Labial CLB Other side: Labial SLB	Different bracket types / None	0-(0)-12	RetractRate
(Heravi et al., 2014)	RCT (SMD); Pract; IRN	20 (3/17); 22.1	NR / Ex of upper 4s ± Ex of lower 4s	Labial CLB (Roth)	LLLT	0-(4)-8	RetractRate
(Jaber et al., 2021)	RCT (SMD); Uni; SYR	18 (7/11); 16.9	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	LAFC	1-2-4-8-12	RetractRate
(Jivrajani and Bhad Patil, 2020)	RCT (SMD); Hosp; IND	10 (3/7); 14-24	NR / Ex of upper 4s	Labial CLB (MBT)	LLLT	0-12-End	RetractRate; RetractDur
(Kansal et al., 2014)	RCT (SMD); Uni; IND	10 (NR); NR	NR / Ex of upper 4s	Labial CLB (MBT)	LLLT	0-5-9	RetractRate
(Karci and Baka, 2021)	RCT (2 PA SMD); Uni; TUR	A: 12 (5/7); 16.84 B: 12 (5/7); 16.45	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	A: Piezocision B: Local injection of platelet-rich fibrin	1-(2)-12	RetractRate
(Kundi et al., 2020)	RCT; Uni; SAU	EXP/CNT: 30 (14/16); 27.9	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	MOPs	0-(0)-4	RetractRate
(Liao et al., 2017)	RCT (SMD); Uni; TUR & AUS	13 (NR); 13.6	NR / Ex of upper 4s	Labial CLB (NR)	Vibration	0-(4)-12	RetractRate
(Limpanichkul et al., 2006b)	RCT (SMD); Uni; THA	12 (4/8); 20.11	NR / Ex of upper 4s	Labial CLB (Roth) (SLB on upper 3s)	LLLT	0-(4)-12	RetractRate
(Mahmoudzadeh et al., 2020)	RCT (SMD); Uni & Pract; IRN	12 (3/9); 18.91	NR / Ex of upper 4s	Labial CLB (MBT)	LAFC	0-4	RetractRate
(Mezomo et al., 2011)	RCT (SMD); Uni; BRA	15 (5/10); 18	Cl. I-II / Ex of upper 4s	One side: Labial CLB Other side: Labial SLB	Different bracket types / None	0-(4)-12	RetractRate
(Mistry et al., 2020)	RCT (SMD); Hosp; AUS	22 (7/15); 17.3	NR / Ex of upper 4s	Labial SLB (Hanson)	LLLT	0-(4)-12	RetractRate

(Reyes Pacheco et al., 2020)	RCT (SMD); Uni; DOM	17 (5/12); 33	Cl. I-II/1 / Ex of upper 4s	Labial CLB (MBT)	Using leukocyte-platelet–rich fibrin membranes	0-(4)-20	RetractRate
(Qamruddin et al., 2017)	RCT (SMD); Uni; PAK	22 (11/11); 19.8	Cl. II/I / Ex of upper 4s	Labial SLB (MBT)	LLLT	0-(3)-9	RetractRate
(Qamruddin et al., 2021)	RCT (SMD); Uni; PAK	22 (11/11); 19.18	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	LIPUS	0-(3)-12	RetractRate
(Sharma et al., 2020)	RCT (SMD); Uni; IND	17 (NR); 18.87	Cl. I-II/I / Ex of upper 4s	Labial CLB (MBT)	Corticotomy	0-(3)-End	RetractRate
(Siriphan et al., 2019)	RCT; Uni; THA	EXP1: 20 (3/17); 21.6 EXP2: 20 (5/15); 22.1 CNT: 20 (5/15); 20.9	NR / Ex of upper 4s	Labial CLB (Roth)	Vibration	0-12	RetractRate
(Taha et al., 2020)	RCT; Uni; USA	EXP: 10 (3/7); 15.9 CNT: 11 (4/7); 15.09	NR / Ex of upper 4s	Labial CLB (MBT)	Vibration	0-(4)-12	RetractRate
(Telatar and Gungor, 2021)	RCT (2 PA); Uni; TUR	EXP: 11 (5/6); 15.8 CNT: 8 (5/3); 15.9	Crowding or Cl. II/I / Ex of 4s	Labial CLB (MBT)	Vibration	0-(4)-24	RetractRate
(Thomas et al., 2021)	RCT (SMD); Hosp; IND	33 (9/24); 22.1	Cl. I-II/I / Ex of upper 4s	Labial CLB (MBT)	MOPs	0-(2)-12	RetractRate
(Varella et al., 2018)	RCT (SMD); Hosp; IND	10 (4/6); 17.7	CI. I / Ex of 4s	Labial CLB (MBT)	LLLT	0-(4)-8	RetractRate
(Wahab et al., 2013)	RCT; Uni; MYS	EXP/CNT: 20 (NR); 14-30	Cl. I-II/I / Ex of upper 4s	One side: Labial CLB Other side: Labial SLB	Different bracket types / None	0-(4)-12	RetractRate
(Wahab et al., 2015)	RCT (SMD); Uni; MYS	19 (6/13); 21.3	Cl. II/I / Ex of upper 4s	Labial SLB (MBT)	Different orthodontic forces / None	0-(1)-5	RetractRate
(Yassaei et al., 2016)	RCT (SMD); Uni; IRN	11 (0/11); 19.0	CI. I / Ex of 4s	Labial CLB (NR)	LLLT	0-(4)-16	RetractRate
(Zeitounlouian et al., 2021)	RCT (SMD); Uni; SYR	21 (6/15); 20.85	Cl. II/I / Ex of upper 4s	Labial CLB (MBT)	Local injection of platelet-rich fibrin	0-(4)-20	RetractRate
(Zheng and Yang, 2021)	RCT (SMD); Hosp; CHN	12 (4/8); 18-28	NR / Ex of upper 4s	Labial CLB (MBT)	LLLT	0-(1)-4	RetractRate

* countries given with their alpha-3 codes.

† patient age is given either as mean (one value in without parenthesis) or if mean isn't reported as range (two values in parenthesis).

Bimax Prot, bimaxillary protrusion; Cl., (Angle's) Class; CLB, conventionally ligated bracket; CNT, control; Ex, extraction; EXP, experimental; FU, follow-up; Hosp, hospital; LAFC, laser-assisted flapless corticotomy; LIPUS, Low-intensity pulsed ultrasound; LLLT, low-level laser therapy; M/F, male / female; MBT, MacLaughlin-Bennet-Trevisi prescription; MOPs, micro-osteoperforations; NR, not reported; PA, parallel arms; Pract, private practice / clinic; RCT, randomized clinical trial; RetractDur, retraction duration; RetractRate, retraction rate; SLB, self-ligating bracket; SMD, split mouth design; Uni, university clinic; wk, week.

3.3.2 Risk of bias within studies

The risk of bias assessment for the 50 included studies is shown in Figures 3.2 and 3.3. The detailed risk of bias assessment for the included studies can be found in Appendix Table 3.2. A high risk of bias was observed in 14 studies (28%), with each study having at least one domain judged to have a high risk of bias or multiple domains judged to have some concerns. Nine studies were at high risk of bias due to issues with the randomization process, deviations from the intended interventions and missing outcome data. Four studies were at high risk of bias due to issues with the randomization process (lack of information concerning allocation concealment) and deviations from the intended interventions. One study was at high risk of bias due to a lack of random sequence generation and allocation concealment. Twenty studies (40%) showed concerns with the randomization process, mainly lack of information concerning allocation concealment (n=15), randomization sequence generation (n=1), and both allocation concealment and randomization sequence generation (n=4). The remaining 16 studies (32%) presented a low risk of bias except for the absence of a priori protocols, which would rule out selective reporting.

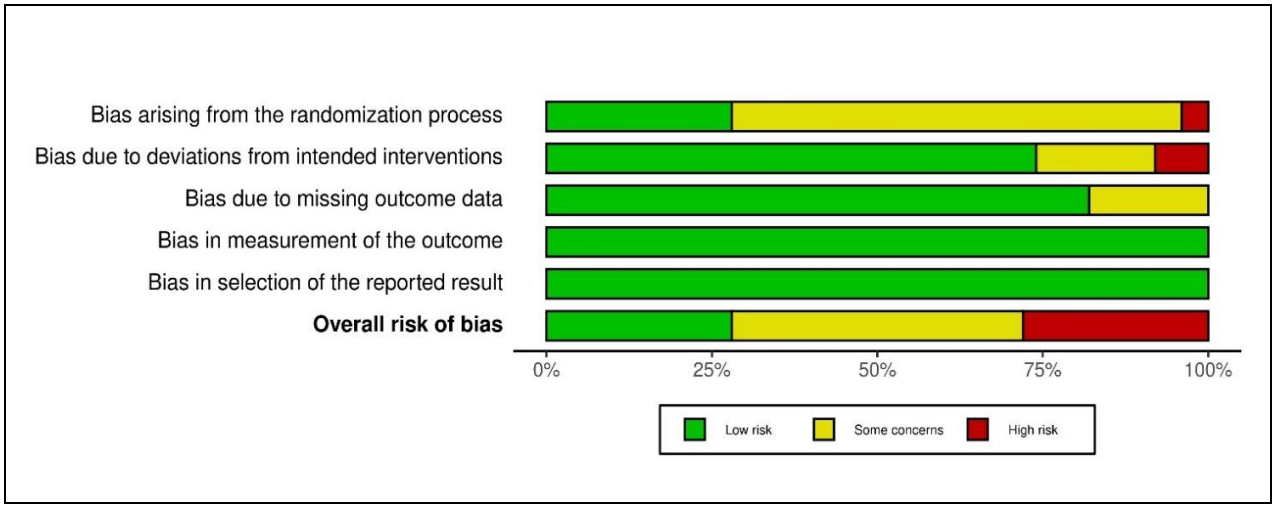

Figure 3.2 Overall risk of bias scores for the specific domains presented as percentages.

Figure 3.3 Risk of bias assessments for included trials.

3.3.3 Results of individual studies, indirect analyses of pooled averages across trials, direct comparisons within and across trials

The pooled average duration to achieve total retraction of the maxillary canines was estimated from indirect meta-analyses at 4.98 months (2 trials; 95% CI=-2.92-12.88 months) (Table 3.2 and Figure 3.4). Pooled average canine tooth movement from baseline (beginning of canine retraction) was 0.97 mm at 1-month (23 trials; 95% CI=0.79-1.16), 1.83 mm at 2-months (20 trials; 95% CI=1.52-2.14), 2.44 mm at 3-months (23 trials; 95% CI=2.10-2.79), 3.49 mm at 4-months (6 trials; 95% CI=1.81-5.17), and 4.25 mm at 5-months (2 trials; 95% CI=0.36-8.14). Furthermore, pooled average canine retraction for each separate month was 0.84 mm for months 1-2 (21 trials; 95% CI=0.68-1.01), 0.73 mm for months 2-3 (17 trials; 95% CI=0.55-0.90) and 0.69 mm for months 3-4 (4 trials; 95% CI=0.08-1.31). As might be expected, substantial heterogeneity across studies was observed for most indirect poolings (I2>95%); thus, the 95% CIs might be more informative than the pooled point estimates (Appendix Figures 1-9).

Direct comparisons between different orthodontic forces, retraction methods, fixed appliances, or treatment adjuncts (including vibration, LLLT, surgically assisted orthodontics, or local injection of platelet-rich fibrin/plasma) were performed both in individual single studies (Appendix Table 3.3) and as meta-analyses of at least two studies (Table 3.3 and Appendix Figures 10-25). Meta-analysis of two trial arms demonstrated a significant reduction in canine retraction duration with surgical-assisted orthodontics (MD=1.11 months less; 95% CI=-2.32-0.10; P=0.05; Table 3.3 and Figure 3.5). Meta-analysis of 8 trial arms indicated that canine tooth movement was greater using LLLT (vs control; 0-3 months; MD=0.53 mm; P=0.05; Table 3.3 and Figure 3.6). Canine tooth movement was greater with surgically assisted orthodontics (vs non-surgical orthodontics) at month 0-1 (n=10; MD=0.52 mm; 95% CI=0.21

to 0.84 mm; P=0.004), months 0-2 (n= 8; MD=0.53 mm; 95% CI=0.06 to 0.97 mm; P=0.04), months 0-3 (n= 8; MD=0.67 mm; 95% CI=0.20 to 1.13 mm; P=0.01), and months 0-4 (n= 3; MD=1.13 mm; 95% CI=0.60 to 1.66 mm; P=0.01) (Table 3.3 and Figures 3.7 and 3.8).

Aside from these meta-analyses, several outcomes were assessed only by single studies and are listed in Appendix Table 3.3. Single study showed that total canine retraction duration was shorter using ClearSnap bracket attachments (vs none; MD=-2.43 months; 95% CI=-2.68 to -2.19 months; P<0.001), while a larger retraction force of 150g was found from a single study to be better both than a 100g force (MD=-0.50 month; 95% CI=-0.98 to -0.02 month; P=0.04) and a 50g force (MD=-1.30 month; 95% CI=-1.99 to -0.61 mm; P<0.001). Canine tooth movement was greater with coil spring (vs laceback; 0-4 months; MD=1.65 mm; 95% CI=0.04 to 3.26 mm; P=0.05) and with combined buccal/palatal MOPs (vs only buccal MOPs; 0-3 months; MD=0.79 mm; 95% CI=0.43 to 1.15 mm; P<0.001). Canine retraction rate was greater with self-ligating brackets (vs conventional-ligation; 0-3 months; MD=0.31 mm/month; P<0.001), and LLLT (vs control; 0-3 months; MD=0.42 mm/month; P<0.001).

Table 3.2 Indirect meta-analyses of pooled averages across the control groups of all studies.

Outcome	Trials	Pooled average (95% CI)	P	tau^2 (95% CI)	I^2 (95% CI)	95% prediction
Total retraction duration (months)	2	4.98 (-2.92, 12.88)	0.08	0.74 (-)	96% (89%, 99%)	-
Retraction month 0-1 (mm)	23	0.97 (0.79, 1.16)	<0.001	0.16 (0.09, 0.33)	99% (98%, 99%)	0.11, 1.83
Retraction month 0-2 (mm)	20	1.83 (1.52, 2.14)	<0.001	0.39 (0.21, 0.89)	98% (97%, 98%)	0.48, 3.17
Retraction month 0-3 (mm)	23	2.44 (2.10, 2.79)	<0.001	0.60 (0.34, 1.21)	99% (99%, 99%)	0.80, 4.08
Retraction month 0-4 (mm)	6	3.49 (1.81, 5.17)	0.003	2.48 (0.89, 14.98)	100% (100%, 100%)	-1.25, 8.23
Retraction month 0-5 (mm)	2	4.25 (0.36, 8.14)	0.05	0.13 (-)	69% (0%, 93%)	-
Retraction month 1-2 (mm)	21	0.84 (0.68, 1.01)	<0.001	0.11 (0.06, 0.25)	96% (95%, 97%)	0.12, 1.57
Retraction month 2-3 (mm)	17	0.73 (0.55, 0.90)	<0.001	0.10 (0.05, 0.25)	96% (95%, 97%)	0.04, 1.41
Retraction month 3-4 (mm)	4	0.69 (0.08, 1.31)	0.04	0.15 (0.04, 1.98)	99% (99%, 99%)	-1.15, 2.54
Retraction month 0-3; averaged (mm/month)	4	0.92 (0.72, 1.12)	<0.001	0.01 (0, 0.25)	65% (0%, 88%)	0.42, 1.42

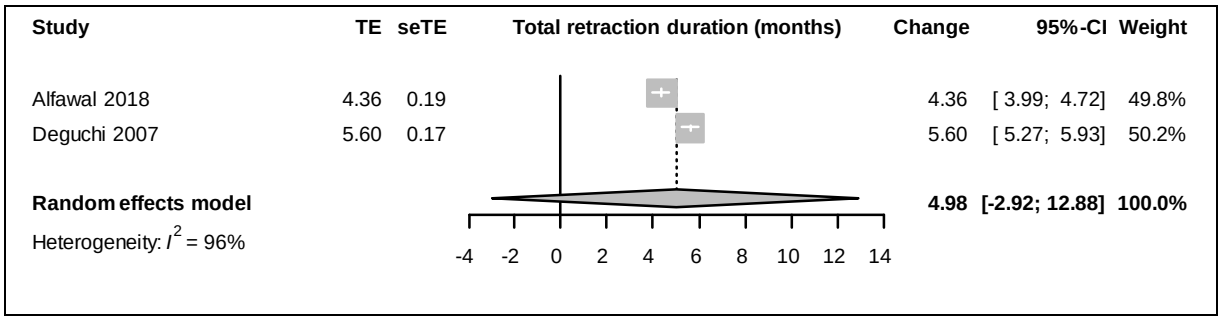

Figure 3.4 Forest plot for the indirect meta-analysis on total retraction duration.

CI, confidence interval; seTE, standard error of the treatment effect; TE, treatment effect.

Table 3.3 Direct meta-analytical comparisons with Mean Differences (MDs) on canine retraction duration and rate

No	Experimental	Reference	Outcome	Trial arms	MD (95% CI)	P value	tau^2 (95% CI)	I^2 (95% CI)	95% prediction
1	150g retraction force	100g retraction force	Retraction month 0-1 (mm)	2	0.03 (-0.15, 0.22)	0.75	0 (-)	0% (-)	-
2	PRP/PRF injection	Control / saline injection	Retraction month 0-1 (mm)	2	-0.06 (3.42, 3.31)	0.87	0 (-)	69% (0%, 93%)	-
3	PRP/PRF injection	Control / saline injection	Retraction month 0-2 (mm)	2	0.18 (-0.84, 1.19)	0.27	0 (-)	0% (-)	-
4	PRP/PRF injection	Control / saline injection	Retraction month 0-3 (mm)	3	0.54 (-0,56, 1,63)	0.17	0.12 (0, 8,93)	49% (0, 85%)	-4,97, 6,04
5	PRP/PRF injection	Control / saline injection	Retraction month 0-4 (mm)	2	0.24 (-3.66, 4.14)	0.58	0 (-)	0% (-)	-
6	PRP/PRF injection	Control / saline injection	Retraction month 0-5 (mm)	2	-0.64 (-7.98, 6.71)	0.47	0.58 (-)	86% (46%, 97%)	-
7	PRP/PRF injection	Control / saline injection	Retraction month 1-2 (mm)	2	0.26 (-2.08, 2.60)	0.39	0.02 (-)	36% (-)	-
8	PRP/PRF injection	Control / saline injection	Retraction month 2-3 (mm)	2	-0.02 (-4.77, 4.73)	0.97	0.24 (-)	84% (32%, 96%)	-
9	PRP/PRF injection	Control / saline injection	Retraction month 3-4 (mm)	2	0.23 (-0.27, 0.73)	0.11	0 (-)	0% (-)	-
10	LLLT	Control	Retraction month 0-1 (mm)	9	0.22 (-0.04, 0.48)	0.09	0.11 (0.04, 0.37)	97% (96%, 98%)	-0.60, 1,04
11	LLLT	Control	Retraction month 0-2 (mm)	9	0.51 (-0.13, 1,15)	0.10	0.67 (0.27, 2.37)	98% (98%, 99%)	-1.53, 2.56
12	LLLT	Control	Retraction month 0-3 (mm)	8	0.53 (0.01, 1.05)	0.05	0.36 (0.14, 1.56)	99% (98%, 99%)	-1.04, 2.10
13	LLLT	Control	Retraction month 1-2 (mm)	9	0.32 (-0.08, 0.72)	0.11	0.27 (0.11, 0.95)	98% (97%, 99%)	-0.97, 1.61
14	LLLT	Control	Retraction month 2-3 (mm)	6	0.19 (-0.08, 0.45)	0.13	0.06 (0.02, 0.36)	97% (96%, 98%)	-0.57, 0.94

15	Self-ligating bracket	Conventional bracket	Retraction month 0-3 (mm)	3	0.59 (-0.45, 1.64)	0.13	0.15 (0.01, 5.73)	77% (24%, 93%)	-5.25, 6.44
16	Adjunct vibration	Control	Retraction month 0-3 (mm)	3	0.31 (-1.11, 1.73)	0.45	0.25 (0.01, 12.81)	77% (26%, 93%)	-7.29, 7.91
17	Surgically-assisted orthodontics	Control	Retraction month 0-1 (mm)	10	0.52 (0.21, 0.84)	0.004	0.17 (0.07, 0.61)	95% (93%, 97%)	-0.47, 1.51
18	Surgically-assisted orthodontics	Control	Retraction month 0-2 (mm)	8	0.53 (0.06, 0.97)	0.04	0.27 (0.10, 1.23)	91% (85%, 95%)	-0.84, 1.90
19	Surgically-assisted orthodontics	Control	Retraction month 0-3 (mm)	8	0.67 (0.20, 1.13)	0.01	0.28 (0.10, 1.22)	94% (90%, 96%)	-0.71, 2.05
20	Surgically-assisted orthodontics	Control	Retraction month 0-4 (mm)	3	1.13 (0.60, 1.66)	0.01	0 (0, 16.90)	46% (0%, 84%)	-0.50, 2.75
21	Surgically-assisted orthodontics	Control	Retraction month 1-2 (mm)	9	0.25 (-0.01, 0.50)	0.05	0.06 (0.02, 0.51)	82% (68%, 90%)	-0.40, 0.89
22	Surgically-assisted orthodontics	Control	Retraction month 2-3 (mm)	8	0.19 (-0.02, 0.40)	0.06	0.05 (0.02, 0.22)	90% (83%, 94%)	-0.41, 0.80
23	Surgically-assisted orthodontics	Control	Retraction month 3-4 (mm)	2	-0.04 (-0.17, 0.08)	0.15	0 (-)	0% (-)	-
24	Surgically-assisted orthodontics	Control	Total retraction duration (months)	2	-1.11 (-2.32, 0.10)	0.05	0 (-)	0% (-)	-

CI, confidence interval; LLLT, low level laser therapy; MD, mean difference; PRF, platelet-rich fibrin; PRP, platelet-rich plasma.

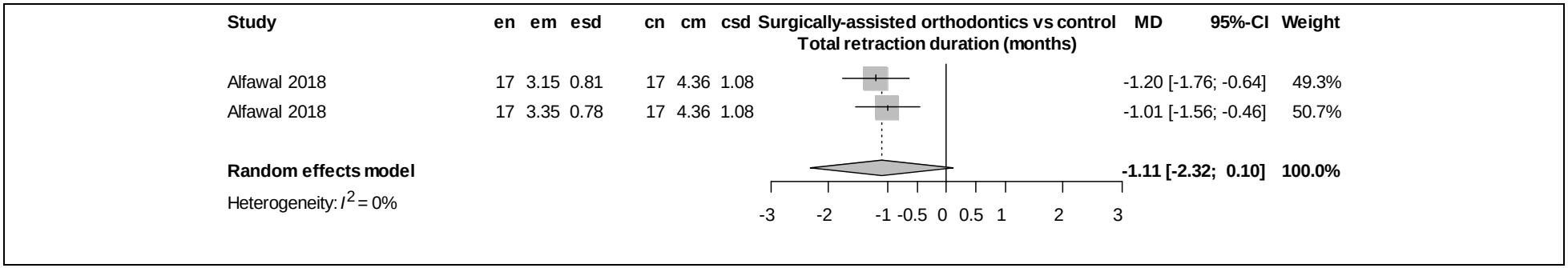

Figure 3.5 Forest plot for the direct meta-analysis on total retraction duration between surgically-assisted group and control group.

en, experimental number; em, experimental mean; esd, experimental standard deviation; cn, control mean; cm, control mean; csd, control standard deviation.

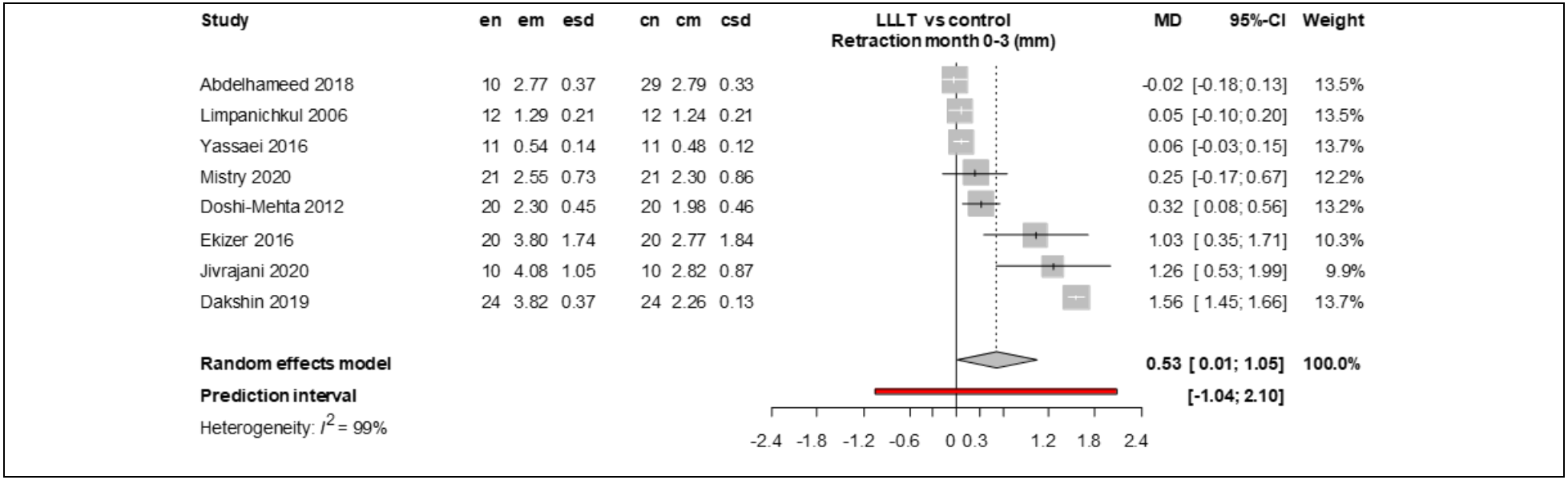

Figure 3.6 Forest plot for the direct meta-analysis on retraction during month 0 to month 3 between LLLT group and control group.

en, experimental number; em, experimental mean; esd, experimental standard deviation; cn, control mean; cm, control mean; csd, control standard deviation.

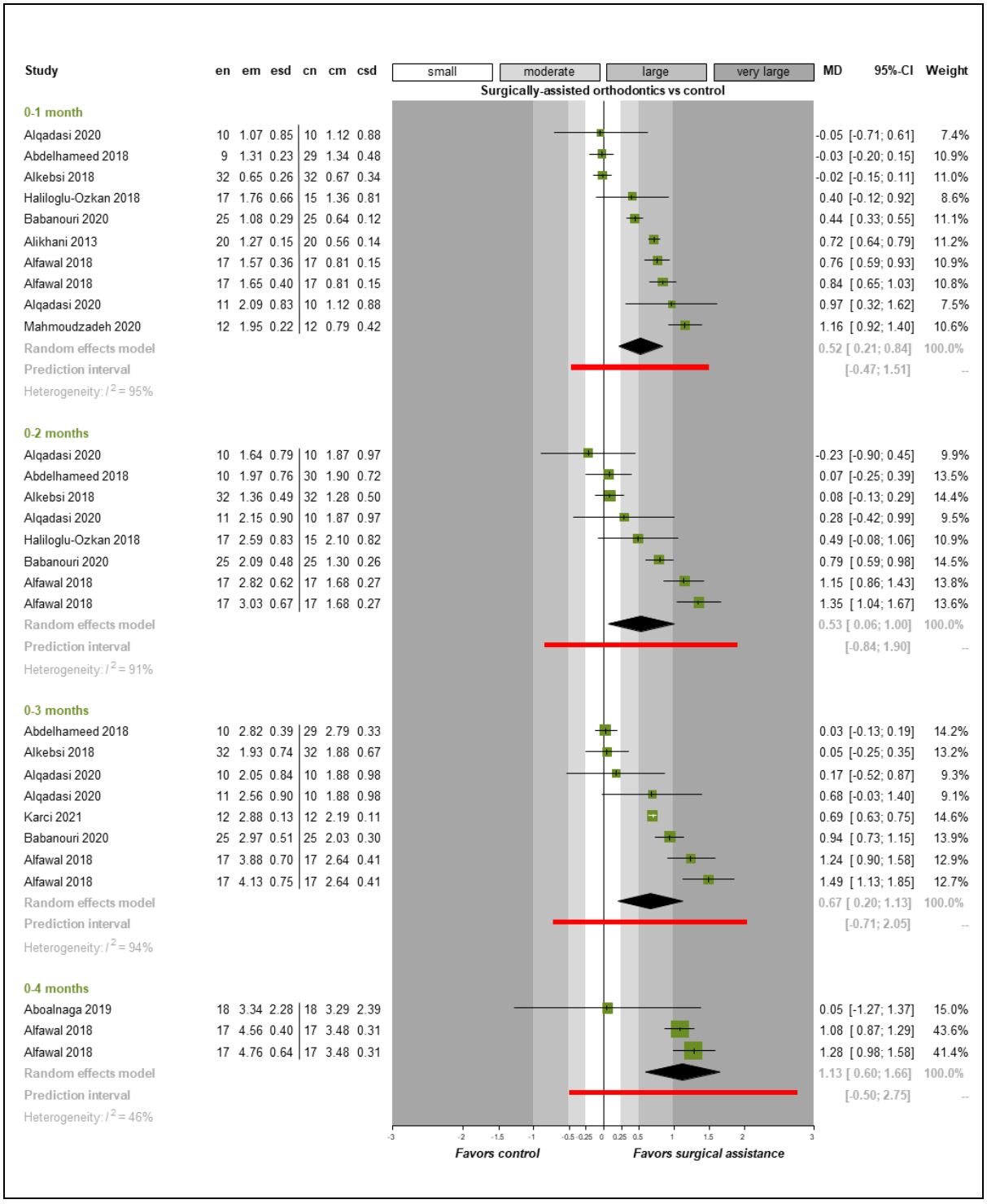

Figure 3.7 Forest plots depicting the effect of surgically-assisted orthodontics vs non-surgically assisted orthodontics on the amount of canine tooth movement at months 0-1, 0-2, 0-3, 0-4 (in millimetres).

en, experimental number; em, experimental mean; esd, experimental standard deviation; cn, control mean; cm, control mean; csd, control standard deviation.

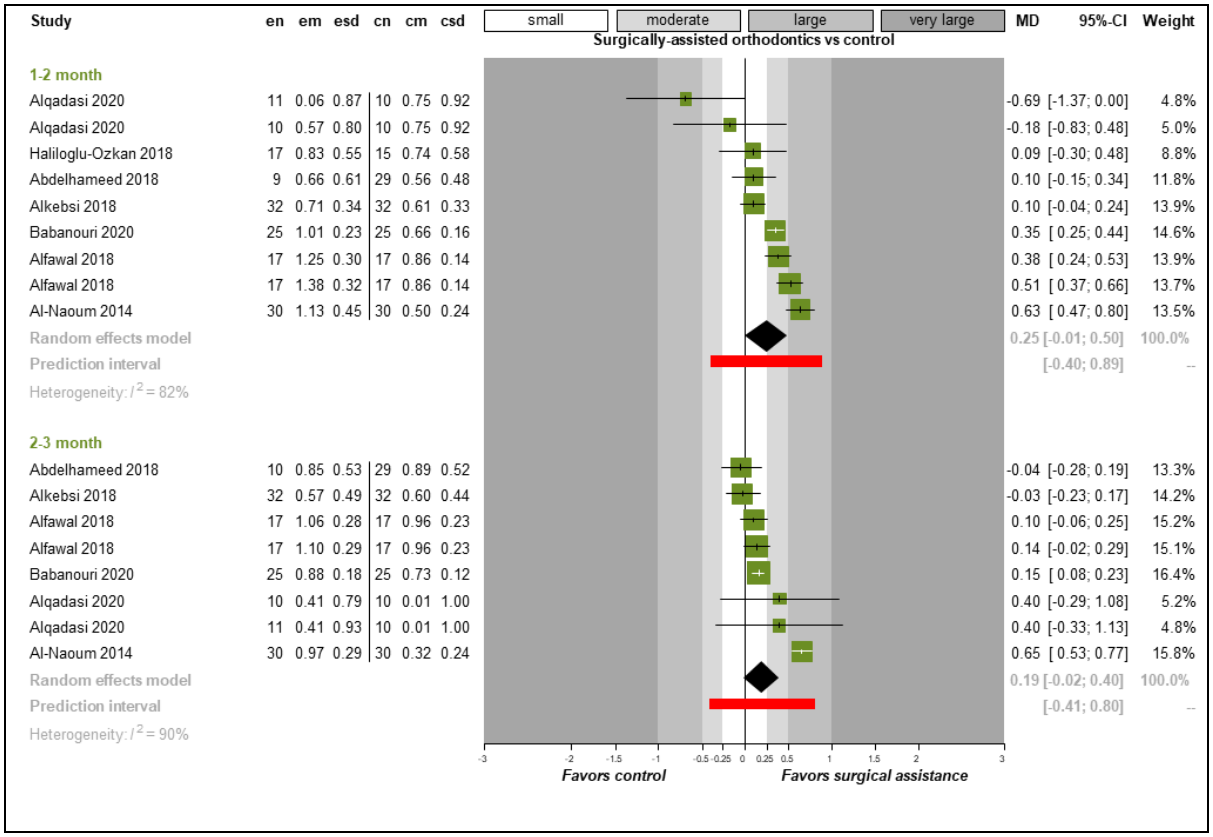

Figure 3.8 Forest plots depicting the effect of surgically-assisted orthodontics vs non-surgically assisted orthodontics on the amount of canine tooth movement at months 1-2 and 2-3 (in millimetres).

en, experimental number; em, experimental mean; esd, experimental standard deviation; cn, control mean; cm, control mean; csd, control standard deviation.

3.3.4 Additional analyses

Substantial heterogeneity was seen for most indirect poolings (I^2>95%); therefore, subgroup and meta-regression analyses were employed to explore potential sources of heterogeneity. In the indirect analysis (Appendix Table 3.4), treatment with 0.018-inch slot brackets was associated with greater canine tooth movement than 0.022-inch slot brackets for months 0-2 (2.24 vs 1.72 mm, respectively; P=0.07), months 0-3 (3.41 vs 2.31 mm, respectively; P=0.003) and months 2-3 (0.96 vs 0.66 mm, respectively; P=0.06).

Subgroup analyses were likewise used to investigate sources of heterogeneity in the direct meta-analyses of MDs among the different modalities of surgically-assisted orthodontics (Table 3.4). Statistically significant subgroup differences were found among the four approaches (corticotomy, LAFC, MOPs, piezocision) for many time points. For the amount of total canine tooth movement in months 0-1, months 0-2 and months 0-3, consistent results were observed, implying laser corticotomy being the most effective, followed by piezocision and MOPs (P value for subgroup differences <0.10 in all instances). Similar results were observed for the monthly rates of canine retraction at months 1-2 or months 2-3, where LAFC or traditional corticotomy likewise proved most effective (P<0.001 amongst subgroups).

Subgroup and meta-regression analyses were employed to explore potential sources of heterogeneity for direct comparisons of MDs between different patient- or treatment-related characteristics (Appendix Table 3.5). Patient gender was significantly associated with the benefit of surgically-assisted orthodontics compared to conventional orthodontics (+0.22 mm per extra 10% males in the sample; P=0.03), which may possibly imply a gender-specific biological response to the former (Appendix Figure 3.25). Anchorage reinforcement with TADs was associated with reduced benefits of added canine retraction owing to surgically-assisted orthodontics compared to TPA-anchored mechanics for months 0-1 (0.33 vs 0.80 mm), months 0-2 (0.28 to 1.24 mm) and months 0-3 (0.43 to 1.36 mm) (P<0.001 in all cases). Nevertheless, these differences may indicate a measurement artefact not necessarily due to increased absolute canine retraction but rather an anchorage loss of the posterior unit that might affect canine movement measurements. Conventional-ligating brackets were associated with greater canine tooth movement than self-ligation (0.30 vs -0.05 mm; P=0.01).

Five indirect meta-analyses and six direct meta-analyses could be evaluated for reporting biases, but Egger's test showed no signs of funnel-plot asymmetry. Sensitivity analyses on the pooled average canine movement amounts (Table 3.5) demonstrated that RCTs

with parallel groups tended to report different canine retraction amounts than split-mouth RCTs, which may indicate artefacts due to different intra-arch configurations. No direct association between the risk of bias and amounts of canine retraction was found. Eventually, evidence of imprecision was found for the indirect analyses, where the most precise trials (i.e., those with probably larger sample sizes) demonstrated much more conservative amounts of retraction than more imprecise (smaller) trials.

For direct meta-analyses of MDs (Table 3.6), no differences were found between parallel groups and split-mouth RCTs or between studies with high risk and with low risk of bias/some concerns. Likewise, no considerable hints of reporting biases were observed with Egger's test. Sensitivity analyses according to study precision also did not detect any overestimation from imprecise studies (small-study effects) since in the two instances with $P<0.10$, the most precise trials showed greater treatment benefits from surgically-assisted orthodontics.

According to the GRADE analysis (Table 3.7), high quality of evidence supported increased canine retraction with surgically-assisted orthodontics, as well as a lack of effect for platelet-rich plasma/fibrin or self-ligating brackets. Moderate quality of evidence supported the finding of no benefit from adjunct use of vibration, due to the high risk-of-bias of one of the included studies.

Table 3.4 Subgroup analyses for the various surgically assisted orthodontics approaches compared to control groups.

	Effect with 95% confidence interval				
Outcome	**Corticotomy**	**Laser corticotomy**	**Micro-osteoperforations**	**Piezocision**	**P**
Retraction month 0-1 (mm)	-	n=2 MD 0.95 (-1.59, 3.49)	n=6 MD 0.27 (-0.08, 0.61)	n=2 MD 0.85 (0.41, 1.29)	<0.001
Retraction month 0-2 (mm)	-	n=1 MD 1.15 (0.86, 1.43)	n=5 MD 0.27 (-0.21, 0.76)	n=2 MD 0.87 (-5.90, 7.64)	<0.001
Retraction month 0-3 (mm)	-	n=1 MD 1.24 (0.90, 1.58)	n=4 MD 0.31 (-0.41, 1.03)	n=3 MD 0.97 (-0.20, 2.13)	0.005
Retraction month 0-4 (mm)	-	n=1 MD 1.08 (0.87, 1.29)	n=1 MD 0.05 (-1.27, 1.37)	n=1 MD 1.28 (0.98, 1.58)	0.16
Retraction month 1-2 (mm)	n=1 MD 0.63 (0.47, 0.80)	n=1 MD 0.39 (0.24, 0.53)	n=5 MD 0.17 (-0.03, 0.36)	n=2 MD -0.04 (-7.65, 7.57)	<0.001
Retraction month 2-3 (mm)	n=1 MD 0.65 (0.53, 0.77)	n=1 MD 0.10 (-0.06, 0.25)	n=4 MD 0.07 (-0.13, 0.28)	n=2 MD 0.15 (-0.53, 0.82)	<0.001
Retraction month 3-4 (mm)	-	n=1 MD -0.03 (-0.12, 0.06)	-	n=1 MD -0.05 (-0.12, 0.02)	0.72
Total retraction duration (months)	-	n=1 MD -1.20 (-1.76, -0.64)	-	n=1 MD -1.01 (-1.56, -0.46)	0.63

MD, mean difference.

Table 3.5 Sensitivity analyses and analyses of reporting biases for indirect meta-analyses from Table 3.2.

		Retraction month 0-1 (mm)			Retraction month 0-2 (mm)			Retraction month 0-3 (mm)			Retraction month 1-2 (mm)			Retraction month 2-3 (mm)		
Factor	**Level**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**
Design	Split-mouth	20	0.91 (0.72, 1.11)	0.01	17	1.69 (1.37, 2.01)	0.001	20	2.31 (1.96, 2.65)	0.03	18	0.79 (0.62, 0.95)	0.10	15	0.69 (0.50, 0.87)	<0.001
	Parallel	3	1.35 (0.70, 1.99)		3	2.53 (1.61, 3.44)		3	3.29 (1.50, 5.08)		3	1.16 (0.24, 2.09)		2	-0.05 (-0.33, 0.22)	
Risk of bias	Low / some concerns	17	0.93 (0.74, 1.13)	0.55	15	1.89 (1.51, 2.28)	0.43	18	2.49 (2.09, 2.87)	0.64	16	0.89 (0.68, 1.09)	0.25	14	0.71 (0.51, 0.91)	0.69
	High	6	1.08 (0.51, 1.64)		5	1.64 (0.91, 2.37)		5	2.29 (1.27, 3.32)		5	0.72 (0.42, 1.02)		3	0.79 (0.05, 1.53)	
Egger's test				0.34			0.13			0.20			0.32			0.63
Precision	Most-precise half	12	0.80 (0.54, 1.06)	0.007	10	1.48 (1.03, 1.92)	<0.001	12	2.17 (1.67, 2.66)	0.04	11	0.68 (0.48, 0.88)	0.002	9	0.65 (0.41, 0.89)	0.23
	Least-precise half	11	1.21 (1.00, 1.41)		10	2.25 (1.97, 2.53)		11	2.78 (2.31, 3.26)		10	1.08 (0.87, 1.29)		8	0.84 (0.55, 1.13)	

Table 3.6 Sensitivity analyses and analyses of reporting biases for direct meta-analyses from Table 3.3.

		LLLT vs control; retraction month 0-1 (mm)			Surgically-assisted orthodontics vs control; retraction month 0-1 (mm)			Surgically-assisted orthodontics vs control; retraction month 0-2 (mm)			Surgically-assisted orthodontics vs control; retraction month 0-3 (mm)			Surgically-assisted orthodontics vs control; retraction month 1-2 (mm)			Surgically-assisted orthodontics vs control; retraction month 2-3 (mm)		
		n	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**
Design	Split-mouth	9			9			7			8			8			8		
	Parallel	-			1			1			-			1			-		
Risk of bias	Low / some concerns	6	0.26 (-0.15, 0.66)	0.58	9	0.54 (0.19, 0.89)	0.66	7	0.53 (-0.02, 1.09)	0.91	8	-	-	8	0.26 (-0.03, 0.55)	0.48	8	-	
	High	3	0.14 (-0.50, 0.77)		1	0.40 (-0.12, 0.92)		1	0.49 (-0.08, 1.06)					1	0.09 (-0.30, 0.48)				
Egger's test		9		0.57	10		0.87	8		0.78	8		0.97	9		0.19	8		0.88
Precision	Most-precise half	4	0.29 (-0.36, 0.95)	0.54	5	0.38 (-0.10, 0.85)	0.20	4	0.83 (-0.06, 1.72)	0.02	4	0.43 (-0.30, 1.16)	0.15	4	0.34 (0.06, 0.61)	0.24	4	0.26 (-0.16, 0.68)	0.08
	Least-precise half	5	0.14 (-0.21, 0.50)		5	0.72 (0.15, 1.30)		4	0.13 (-0.26, 0.52)		4	0.96 (0.04, 1.89)		5	0.07 (-0.51, 0.65)		4	0 (-0.22, 0.22)	

Table 3.7 Summary of findings according to the GRADE approach for the month 0-3.

	Anticipated absolute effects (95% CI)				
Outcome Studies (patients)	**Control group[a]**	**Experimental group**	**Difference in experimental group**	**Quality of the evidence (GRADE)[b]**	**What happens with experimental treatment**
	Control	**PRP/PRF**			
Retraction in 3 months 3 trials (48 patients)	2.96 mm	-	0.5 mm more (0.5 less to 1.6 more)	ÅÅÅÅ high	Little to no difference in canine retraction
	Control brackets	**Self-ligating brackets**			
Retraction in 3 months 3 trials (50 patients)	2.66 mm	-	0.6 mm more (0.5 less to 1.6 more)	ÅÅÅÅ high	Little to no difference in canine retraction
	Control	**Adjunct vibration**			
Retraction in 3 months 3 trials (94 patients)	2.66 mm	-	0.3 mm more (1.1 less to 1.7 more)	ÅÅÅ• moderate[c]	Little to no difference in complete alignment duration
	Control	**Surgically-assisted orthodontics**			
Retraction in 3 months 8 trials (152 patients)	2.28 mm	-	0.7 mm more (0.2 to 1.1 more)	ÅÅÅÅ high[d]	Greater canine retraction

Intervention: orthodontic treatment with fixed appliances with extractions including canine retraction and with/without adjuncts / Population: adolescent and adult patients with crowding / Setting: university clinics, hospitals and private practice (Australia, Brazil, China, Egypt, Iran, Jordan, Malaysia, Syria, Thailand, Turkey, United States of America).

[a] Response in the control group is based on random-effects meta-analysis duration among the control groups.

[b] Starts from "high"

[c] Downgraded by one levels for bias due to the inclusion of one trial with high risk of bias.

[d] Considerable inconsistency observed (tau^2=0.28; I^2=94%), but this does not affect our decision about surgical assisted orthodontics, as the majority of trials were on the same side of the forest plot. However, caution is warranted by the quantification of the actual reduction in alignment duration.

CI, confidence interval; GRADE, Grading of Recommendations Assessment, Development and Evaluation; PRF, platelet rich fibrin; PRP, platelet rich plasma

3.4 Discussion

3.4.1 Summary of evidence

This present systematic review summarises evidence from RCTs on canine retraction duration and rate following maxillary first premolar extractions using full-arch fixed orthodontic appliances. From the initially identified 2259 publications through the literature search, 50 trials were included, with a total of 811 patients. Canine retraction duration was assessed in terms of the time needed to fully retract maxillary canines, and canine retraction rate was determined as the amount of canine tooth movement per unit time.

This review found limited research evaluating our prespecified primary outcome, with only four studies assessing canine retraction duration. Two of the four studies were excluded from data synthesis because of missing data (Ahmad et al., 2020, Jivrajani and Bhad Patil, 2020). Clinical trials have focussed on canine retraction rate as a primary outcome of their interventions, but clinical data concerning complete retraction duration are lacking. The overall pooled average canine retraction duration was 4.98 months. The canine retraction phase is one of the most time-consuming phases of orthodontic treatment and shortening this time may lead to a shorter overall treatment duration (although choosing to retract the maxillary canine teeth as a separate phase of orthodontic treatment might be considered a more time-consuming process when compared with a single stage of en-masse retraction). The results of this review showed that surgically-assisted orthodontics caused a shorter retraction duration than control groups (1.11 months less). This is consistent with two recent reviews demonstrating that corticotomy-facilitated orthodontics results in a shorter treatment duration compared to conventional treatment (Gil et al., 2018, Apalimova et al., 2020). However, these results should be interpreted with caution because only two trials were included in data synthesis, and they might not be representative.

Substantial variation was observed in the amount of canine tooth movement taking place at different treatment-points. There was extreme heterogeneity across trials, explained by differences in clinical settings, patient demographics, type of malocclusion, anchorage reinforcement, fixed appliance type, treatment adjuncts, appointment intervals, and orthodontic mechanics (Mavreas and Athanasiou, 2008, Abbing et al., 2020, Vig et al., 1990, Schubert et al., 2020, Vieira et al., 2018). Furthermore, the timing of canine retraction initiation following premolar extraction varied among included trials. There is limited evidence available in relation to the timing of retraction; however, greater tooth movement at sites of recent extraction has been reported previously (Häsler et al., 1997), which could be related to the immediate tissue inflammatory responses after teeth extraction and RAP (Frost, 1989). Additionally, subgroup analysis indicated that 0.018-inch bracket slot size was associated with greater canine retraction than 0.022-inch. This agrees with previous findings that treatment duration is significantly shorter for 0.018-inch bracket slot size (Amditis and Smith, 2000, Detterline et al., 2010) but contradicts another study and systematic review finding no differences (Vieira et al., 2018, Yassir et al., 2019) and one study finding faster canine tooth movement with 0.022-inch slot size (Cobb et al., 1998).

Direct meta-analyses revealed that surgically-assisted orthodontics resulted in greater canine tooth movement than conventional orthodontics, which has previously been suggested (Fleming et al., 2015, Hoogeveen et al., 2014). High-quality evidence supported greater canine retraction with the surgically-assisted orthodontics at 3 months of treatment (8 trials; 152 patients). Subgroup analyses for the different surgically-assisted techniques showed significant differences. Between LAFC, MOPs and piezocision at months (0-1, 0-2, 0-3), the LAFC subgroup seemed to be the most effective, followed by piezocision and MOPs. Between the 4 subgroups at months (1-2, 2-3), corticotomy was the most efficient. Corticotomy is an invasive technique involving elevating a full thickness mucoperiosteal flap and causing trauma to the

bone, which suggests a scenario where bone injury accelerates all processes involved in healing, inflammation, bone modelling and remodelling- thus accelerating OTM (Frost, 1989, Cano et al., 2012, Wilcko et al., 2001). Previously, it has been demonstrated that corticotomy results in shorter orthodontic treatment duration (Gil et al., 2018, Bhattacharya et al., 2014) and faster OTM (Viwattanatipa and Charnchairerk, 2018, Aboul-Ela et al., 2011, Patterson et al., 2016, Kalemaj et al., 2015, Long et al., 2013). However, LAFC, MOPs, and piezocision are minimally invasive techniques with greater patient acceptance, yet there is limited evidence that these adjuncts may speed up OTM (Alfawal et al., 2016). The above-mentioned findings are consistent with previous results demonstrating that corticotomy was associated with a greater rate of canine tooth movement than piezocision (Viwattanatipa and Charnchairerk, 2018) and piezocision resulted in a greater tooth movement than MOP in a single trial (Alqadasi et al., 2021). Furthermore, piezocision and LAFC were associated in a single study with greater canine tooth movement than control groups (Alfawal et al., 2018). Moreover, after the first month, a reduced amount of canine tooth movement was evident with time, which might be explained by the transient effect of these techniques when carried out only once during orthodontic treatment (Frost, 1989).

Subgroup analyses for the direct meta-analyses showed that anchorage reinforcement methods were associated with amount of canine tooth movement. Orthodontic treatment without TADs or TPA was associated with greater tooth movement compared to TADs or TPA, and treatment with TPA was associated with greater tooth movement than TADs. This might be due to differences in the type of tooth movement, whether bodily or tipping. In trials that used TADs to enhance anchorage, NiTi closing coil springs were put between TADs and power arms; thus, the force passes through the centre of resistance, so bodily movement is anticipated. However, in other trials, NiTi closing coil springs were positioned at the bracket level between the first molar and canine hooks, and some tipping is inescapable. It has been shown previously

that tipping movements are associated with faster OTM rates than bodily movement (Lotzof et al., 1996, Huffman and Way, 1983, Shpack et al., 2008); nonetheless, the duration needed to fully retract the canine was longer because of the need for root uprighting canines retracted with tipping movement (Shpack et al., 2008). Furthermore, it is worth noting that most of the included trials followed up patients for three months after canine retraction, so the greater amount of canine tooth movement in those trials that did not use TADs might be due to tipping. In addition, different reference points were employed to measure the amount of canine tooth movement amongst the included trials, and some of these points are not stable, which may have influenced the estimates.

Finally, parallel groups RCTs tended to report a considerably greater amount of canine tooth movement compared to studies with a split-mouth design. Although a split-mouth trial design can eliminate inter-subject variability from the estimated treatment effect, cross-over effects, spilling of the effects, or contamination of one intervention to another are known drawbacks of this design (Pandis et al., 2013). Moreover, many studies had a small sample size, and most precise studies revealed different results than the least precise studies suggesting a small-study effect that might introduce bias and influence the precision of the estimates (Cappelleri et al., 1996).

3.4.2 Implications for clinical practice and research

Most studies employed a split-mouth design which assumes baseline equivalence between opposite sides of the dental arch and independence to various bilateral interventions, reducing sample size requirements. However, it should be noted that although canine retraction assays using split-mouth approaches are experimentally convenient models for investigating OTM, they have limited implications for understanding methods of reducing treatment duration;

therefore, split-mouth design should be avoided, and randomization be performed at the level of the individual, not the dental arch. Another big concern is that a minority of studies reported on the time taken to achieve complete canine retraction (only 2 studies out of 50 included data on completion of retraction). Multiple studies reported retraction over different time intervals, with many of these only reporting over the short-term, often failing to follow up patients beyond one month. Even for those trials following canine retraction to completion, single canine retraction is only one component of orthodontic treatment in extraction cases, and whilst anchorage-conserving, it is likely to be more time-consuming than en-masse retraction of the six maxillary anterior teeth with sliding mechanics. Indeed, several of these trials use canine retraction against absolute anchorage, which is a sensible experimental model but will rarely be undertaken in routine treatment. In addition, another issue is the frequent reporting of percentage differences in OTM rates, which are mostly clinically meaningless. Future studies should ideally focus on the overall treatment duration, the influence of treatment adjuncts over this period, and the potential need for repeating them during orthodontic treatment. If canine retraction studies are performed, they should investigate complete retraction of the canines and avoid split-mouth designs. Our pooled duration of 5.0 months to achieve complete retraction of the canines presents useful guidance for future sample size calculations.

3.4.3 Strengths and limitations

The strengths of this review include a *priori* registered protocol (Sideri et al., 2018), extensive unrestricted comprehensive literature searching (Papageorgiou et al., 2019, Papageorgiou et al., 2015b), inclusion of RCTs (Langan et al., 2019), use of contemporary statistics, assessing the quality of evidence according to GRADE (Papageorgiou, 2014), and transparent provision of open dataset (Wazwaz et al., 2022a).

However, this systematic review also has some limitations, including methodological issues with the conduct of included studies and the high heterogeneity levels between studies that might influence conclusions. Furthermore, the limited number of trials with limited sample sizes reported on the primary outcome and the relatively short follow-up period after retraction might influence the precision of the effect. Moreover, most meta-analyses were based mainly on trials with split-mouth design and small sample sizes, which might influence the precision of the estimated effects (Cappelleri et al., 1996). Lastly, due to the small number of included studies and incomplete reporting, all pre-planned subgroup and meta-regression analyses could not be undertaken to identify factors associated with the outcome of interest.

3.5 Conclusions

This systematic review identified 50 studies describing 811 patients with a mean age of 19.9 years. Pooled duration to achieve complete retraction of the maxillary canines was approximately 5 months. Substantial heterogeneity was observed across trials which could be explained by the patient or treatment-related characteristics and differences in the included trial design. At 3 months of treatment, high-quality evidence supported greater canine retraction with surgically-assisted orthodontics, while the quality of evidence for the lack-of-benefit was high for both platelet-rich plasma/fibrin and self-ligating brackets and moderate for the adjunct vibration. Further high-quality, well-conducted, and transparently reported parallel-group RCTs with sufficient sample sizes (depending on adequate power calculations) and a more consistent methodology in outcome assessment should be considered to investigate clinically relevant outcomes, which will help identify methods to accelerate OTM. The findings of this systematic review should serve as a basis to inform power calculations for future research.

Chapter 4 The effect of adipokines on the production of inflammatory and ECM remodelling biomarkers in compressed human periodontal and gingival fibroblast: an in vitro cell culture study

4.1 Introduction

Orthodontic tooth movement (OTM) involves changes within the periodontium, which supports the teeth and consists of the periodontal ligament (PDL), gingiva, cementum, and alveolar bone. The PDL is a highly specialised connective tissue that attaches the tooth root cementum to the alveolar bone and is essential for tooth support and the structural integrity of these tissues (Kang et al., 2010, Yang et al., 2015a, Krishnan and Davidovitch, 2009, Krishnan and Davidovitch, 2006). Approximately 50-60% of total PDL cellularity are fibroblasts (Jiang et al., 2016), which are mechanoresponsive and mediate periodontal tissue remodelling and regeneration during OTM (Jiang et al., 2016, Janjic et al., 2018, Krishnan and Davidovitch, 2006, Krishnan and Davidovitch, 2009). Gingival fibroblasts are the predominant cell type in human gingival connective tissue and are responsible for ECM remodelling, bone resorption, secretion of proteases and homeostasis under physiological and pathological conditions (Kong et al., 2018, Nan et al., 2019, Krishnan and Davidovitch, 2009). Upon orthodontic force application, the tooth moves in the periodontal space inducing bone resorption on the compression side and bone deposition on the tension side (Krishnan and Davidovitch, 2006).

Initially, the tooth is displaced in the socket as a result of the force applied to the crown; this causes an immediate aseptic localised inflammatory reaction in the periodontal tissues, which is mediated by a variety of inflammatory cytokines, prostaglandins, proteases, and

others, followed by extracellular matrix remodelling of the periodontium and ultimately tooth movement occurs (Meeran, 2012, Li et al., 2018, Krishnan and Davidovitch, 2006). IL-1 is a cytokine produced and released locally by mechanically stimulated cells as early as an hour and peaks at 24 hours, mediating the formative and resorptive stages of connective tissue remodelling during OTM (Meikle, 2006, Kapoor et al., 2014, Kumar et al., 2015). IL-1 has two isoforms, IL-1α and IL-1β, which have been demonstrated to have similar biological actions, both systemically and locally (Krishnan and Davidovitch, 2006, Kumar et al., 2015). Matrix metalloproteinases (MMPs) which play a crucial role in ECM remodelling of the periodontium during OTM, are a group of zinc-dependent proteases divided according to substrate specificity into six groups (Meeran, 2012, Behm et al., 2021a).

Obesity, which is on the rise in children and adults worldwide, is characterised by chronic low-grade inflammation (Fantuzzi, 2005, Weihrauch-Blüher and Wiegand, 2018). It has been demonstrated that the adipose tissue of obese individuals significantly increases the release of TNF-α, IL-6, and IL-8 (Fantuzzi, 2005, Ouchi et al., 2011). Adiponectin and leptin are adipokines mainly secreted by adipocytes (Ouchi et al., 2011), and obesity is associated with decreased adiponectin levels (Ouchi et al., 1999, Kadowaki and Yamauchi, 2005) and increased leptin levels (Considine et al., 1996). Leptin has a pro-inflammatory effect and is known to regulate the immune system and inflammatory reactions (Fantuzzi, 2005). Moreover, leptin is considered a cytokine whose receptor has a similar structure and functions with various long-chain helical cytokines such as IL-6 (Meeran, 2012, Williams et al., 2016, Ouchi et al., 2011). It has been reported previously that leptin increased the release of IL-6, IL-8, and MMPs in human periodontal fibroblasts (hPDLFs) and human gingival fibroblasts (hGFs) in vitro (Yun-Jung et al., 2013, Williams et al., 2016).

Adiponectin exerts anti-inflammatory effects (Iwayama et al., 2012). Low levels of adiponectin in patients with severe periodontal disease was reported previously (Zimmermann

et al., 2013), and local injections of adiponectin reduced OTM in rats (Haugen et al., 2017). AdipoRon, an adiponectin receptor agonist, is an orally active substance that binds and activates adiponectin receptors (AdipoR1 and AdipoR2) and mimics some of the positive effects of adiponectin (Wu et al., 2019, Bhat et al., 2020). AdipoRon is widely known to have potent anti-obesity and anti-diabetic characteristics, making it a suitable candidate for the treatment of many disorders (Wu et al., 2019, Bhat et al., 2020).

Obesity and overweight pose a risk for the development and deterioration of periodontal diseases (Keller et al., 2015); also, obese and normal-weight patients respond differently to periodontal therapy (Papageorgiou et al., 2015a). Although there are very scarce studies in the literature on the association between obesity and OTM (Consolaro, 2017), some in vivo studies demonstrated that leptin levels in GCF considerably changed during OTM, indicating that leptin has a vital biological role in this process (Sar et al., 2019, Srinivasan et al., 2019, Saloom et al., 2017). Additionally, obesity impacts craniofacial and pubertal growth, bone metabolism and psychosocial well-being, which might affect orthodontic treatment of obese individuals (Neeley and Gonzales, 2007). Moreover, increased rates of OTM were observed in obese patients compared to normal weight-patients (Saloom et al., 2017).

OTM is a complex biological process involving several tissues, cells, and structures in vivo. Hence, in vitro models have evolved to simulate different forces during OTM using different cell types to investigate responses to forces at the cellular level. Various models have been used, including centrifugation, hydrostatic pressure, Flexercell tension system, fluid flow, vibration, and weight approaches (Janjic et al., 2018, Yang et al., 2015a, Schröder et al., 2020b, Long et al., 2002). The weight method has been considered the most appropriate approach to simulate orthodontic force generated on the compression side during OTM (Yang et al., 2015a, Janjic et al., 2018), which involves placing a weight over the cells to apply compressive force (Kanzaki et al., 2002).

Upon orthodontic force application, mechanical signals are transduced to the mechanosensory cells, and intracellular signalling pathways are activated. Several signalling pathways were reported to play a vital role in mechanical signal transduction and OTM (Li et al., 2021b, Fu et al., 2016, Janjic et al., 2018, Kang et al., 2010). The mechanistic target of rapamycin (mTOR) signalling pathway is a vital pathway that regulates cell growth, homeostasis, metabolism, proliferation, differentiation, and protein synthesis (Wang et al., 2019, Laplante and Sabatini, 2012). mTOR is a serine/threonine kinase and belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family (Xia et al., 2017, Laplante and Sabatini, 2012, Dancey, 2010). It interacts with several proteins to form two physically and functionally distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) (Laplante and Sabatini, 2012, Ma and Blenis, 2009). mTORC1 controls protein synthesis and responds to different stimuli, including growth factors, amino acids, energy, oxygen, and stress (Laplante and Sabatini, 2012). The upstream Protein kinase B (AKT/PKB) activates mTORC1, which in turn can induce protein synthesis by directly phosphorylating two downstream proteins, S6K1 (ribosomal protein S6 kinase 1) and 4E-BP1 (the eukaryotic translation initiation factor 4E binding protein 1) (Qi and Zhang, 2014, Laplante and Sabatini, 2012, Dancey, 2010). Moreover, mTORC1 regulates cellular metabolism positively and autophagy negatively (Blawat et al., 2020, Laplante and Sabatini, 2012). Scarce information about mTORC2 is available; it directly activates Akt by phosphorylation and regulates cell survival and cytoskeletal organisation (Dancey, 2010, Laplante and Sabatini, 2012). In mTOR signalling, AKT is crucial as it is an upstream activator of mTORC1 as well as a downstream effector of mTORC2 (Dancey, 2010) (Figure 4.1). PI3K/AKT/mTOR/P70S6K signalling pathway was previously found to have a pivotal role in the remodelling of the periodontium and to be involved in OTM (Xu et al., 2017). Autophagy is considered a protective catabolic mechanism that involves degrading cellular organelles, cytoplasm, lipids, or aberrant proteins

triggered by stress or starvation (Li et al., 2021b, Rabinowitz and White, 2010). mTOR signalling is directly involved in autophagic vesicle formation, and mTOR inhibition promotes autophagy (Pattingre et al., 2008, Jiang et al., 2022, Li et al., 2021a). Autophagy was demonstrated to be induced by orthodontic loading of mice molars and was correlated with inflammatory biomarkers (Li et al., 2021a). Moreover, autophagy was increased in hPDLFs subjected to compressive forces (Huang et al., 2021, Chen et al., 2019).

It has been demonstrated that mTOR signalling pathway activity is triggered by hyperglycaemia or hyperlipidaemia (Pal China et al., 2018). Adiponectin shows cytoprotective effects and has been found to inhibit the mTOR signalling pathway (Choi et al., 2020) and induce autophagy (Pal China et al., 2018).

Cultured hPDLFs have been extensively used in vitro to study the biochemical responses of the cells to different stimuli due to their pivotal role in OTM and periodontal disease. However, hGFs have received scant attention, albeit of their importance in the overall response to orthodontic forces. Thereby, hPDLFs and hGFs will be investigated in this in vitro study using weight loading approach to simulate orthodontic forces. As described above, orthodontic forces induce inflammation and ECM remodelling of the periodontium, and obesity-related biomarkers have anti-inflammatory (adiponectin) and pro-inflammatory (leptin) effects. Therefore, this study aimed to measure the effect of adipokines on the expression of inflammation and ECM remodelling markers in compressed hPDLFs and hGFs cells. Specifically, to measure the effect of AdipoRon and leptin on IL-1α-induced expression of inflammation (IL-6 and IL-8) and ECM remodelling markers (MMPs) in the presence or absence of compressive force. And finally, to investigate the mechanism of compressive force and adipokines through the phosphorylation of mTOR upstream and downstream proteins in hPDLFs and hGFs cells.

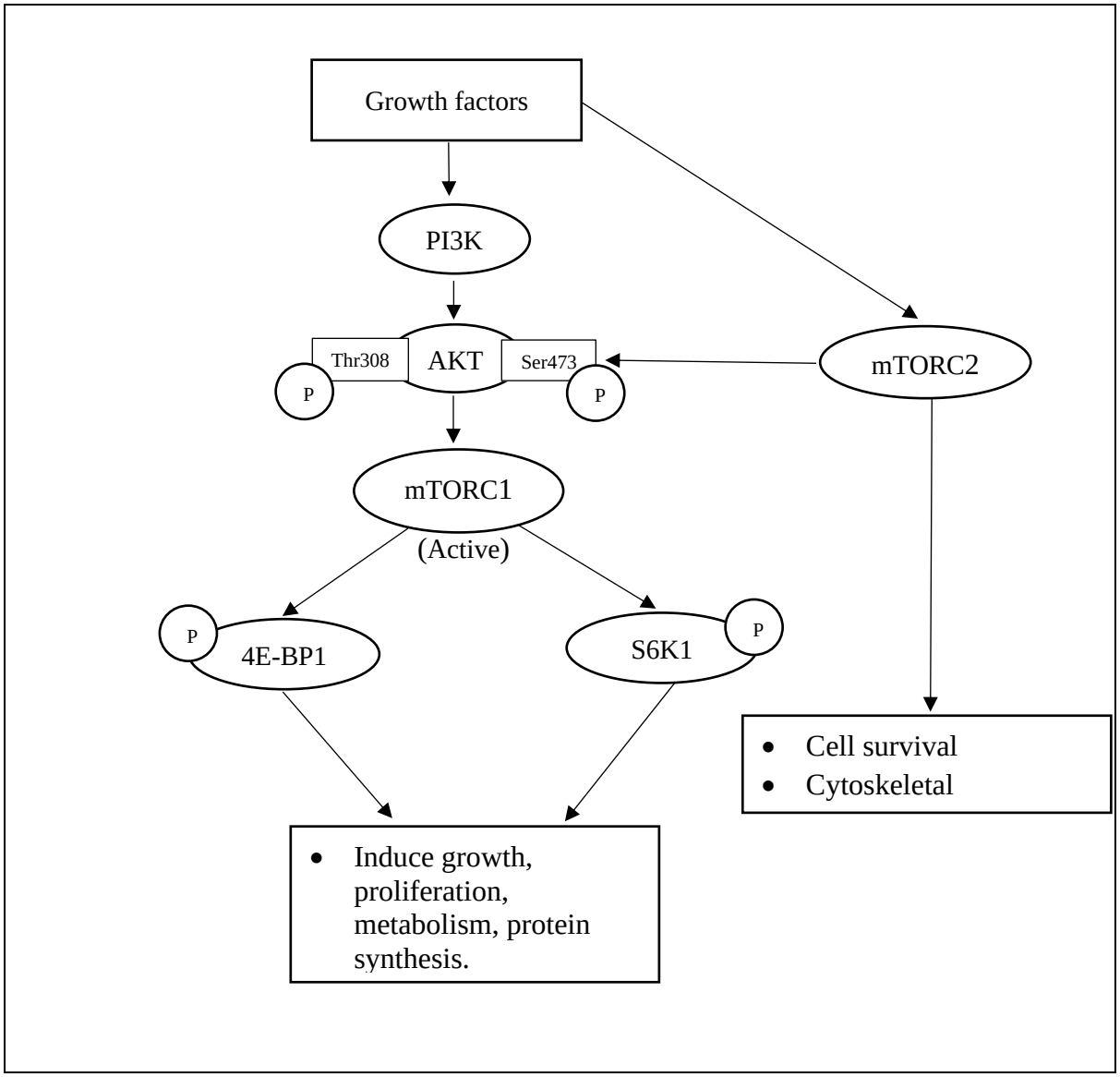

Figure 4.1 Overview of mTOR signalling pathway.

PI3K activates AKT by phosphorylation, then AKT activates mTORC1, which in turn directly phosphorylates S6K1 and 4E-BP1 to promote growth, proliferation, metabolism, and protein synthesis and inhibits autophagy. mTOR2 directly activates AKT via phosphorylation and regulates cell survival and cytoskeletal organisation. PI3K, phosphoinositol-3 kinase; p, phosphorylated; Thr; threonine; Ser, serine; AKT, protein kinase B; mTOR, mechanistic target of rapamycin; S6K1, ribosomal protein S6 kinase 1; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1 (Laplante and Sabatini, 2012, Gao et al., 2012, Ma and Blenis, 2009, Blawat et al., 2020, Dancey, 2010).

4.2 Materials and Methods

4.2.1 Cell culture

Primary hGFs were derived from healthy gingival tissues isolated from third molar extraction sites, and primary hPDLFs were derived from the periodontal tissue around the mid-third of extracted lower third molars roots from healthy, non-smoking patients aged between 20-35 years old. Ethical approval was obtained from Kent NHS Research Ethics Committee (Reference No: 11/LO/0259), and informed written consent was obtained from all the patients. HPDLFs and hGFs were characterized by their spindle-shaped morphology (Figure 4.2); their isolation and characterization was performed by the periodontal group and have been previously described (Garna et al., 2022).

HGFs and hPDLFs were grown in Dulbecco's Modified Eagles Medium (DMEM)–high glucose (4500 mg/L glucose) (D6429-500ML, Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS) (F9665-100ML, Sigma-Aldrich), and 1% penicillin-streptomycin (P4333-100ML, Sigma-Aldrich). Cells were incubated at 37°C in a 5% CO2 humidified incubator in T75 flasks (Greiner Bio-one), and they were split when approximately 80-90% confluent using 0.25 % trypsin-EDTA (1X) (25200056, Gibco). Cells from passages 4 to 8 were randomly seeded at a density of $3x10^5$ cells/well in 6-well culture plates (StarLab) for all conducted experiments and grown overnight in a humidified incubator at 37 ºC with 5 % CO2. After overnight incubation, cells were stimulated with IL-1α (12778-2UG, Sigma-Aldrich), Recombinant human Leptin (398-LP-05M, Biotechne), and AdipoRon (5096/10, Tocris, Biotechne). The chosen concentrations used in this study have been published and reported to be efficient for similar experiments with hPDLFs and hGFs. Cells were stimulated with leptin (10µg/ml) (Yun-Jung et al., 2013, Schröder et al., 2021, Williams et al., 2016), IL-1α (0.1 ng/ml) (Williams et al., 2016), and AdipoRon (40 µM) (Wu et al., 2019), and compressive

forces alone or in combination, for 24, 48, and 72 hours. Each experiment was repeated three times, and unstimulated cells at each time point served as a control.

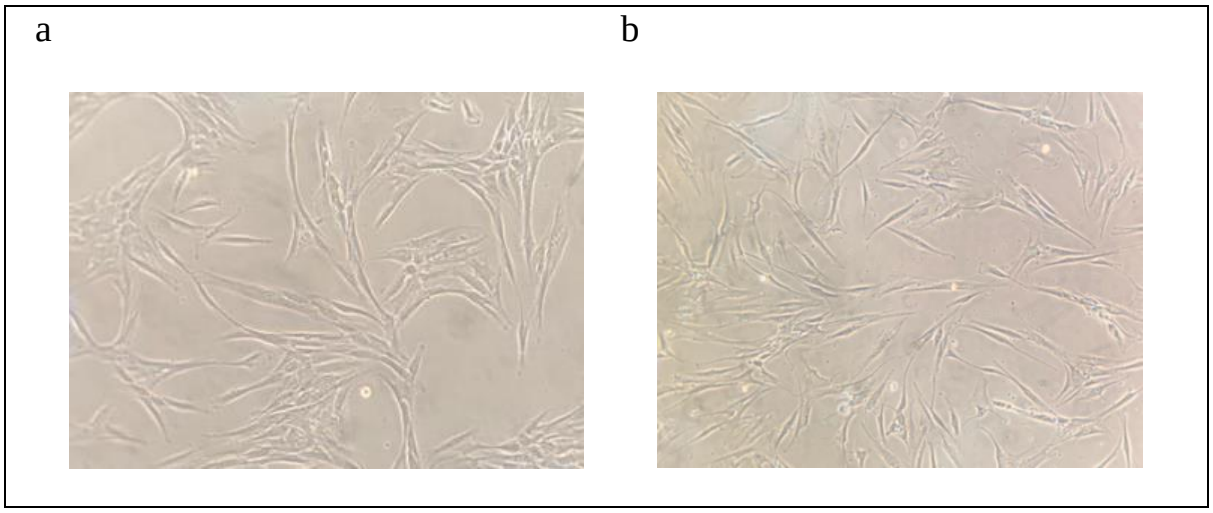

Figure 4.2 The appearance of hPDLFs and hGFs under the light microscope.

hPDLFs **(a)** and hGF **(b)** were seeded at a density of $3x10^5$ cells/well in 6-well culture plates and were characterised by their spindle-shaped morphology as observed under the light microscope.

4.2.2 Compressive force application

To reproduce orthodontic forces generated during orthodontic tooth movement in vitro, a static continuous compressive force was generated using a weight placed onto confluent hGFs, and hPDLFs cell layer in a way that gravity elicits a static, compressive, and unidirectional force according to the method developed by Kanzaki et al. (Kanzaki et al., 2002). In brief, a glass cylinder (30 mm in diameter) has been placed over a confluent cell layer in the well of a 6-well culture plate (Figure 4.3). The weight was adjusted by adding metal parts to generate a compressive force of 2 gm/cm^2, as previously used with the same method simulating the forces that periodontal ligament cells experience when they are compressed between the alveolar bone and the root on the compression side during OTM (Niederau et al., 2020, Ullrich et al., 2019, Janjic et al., 2018).

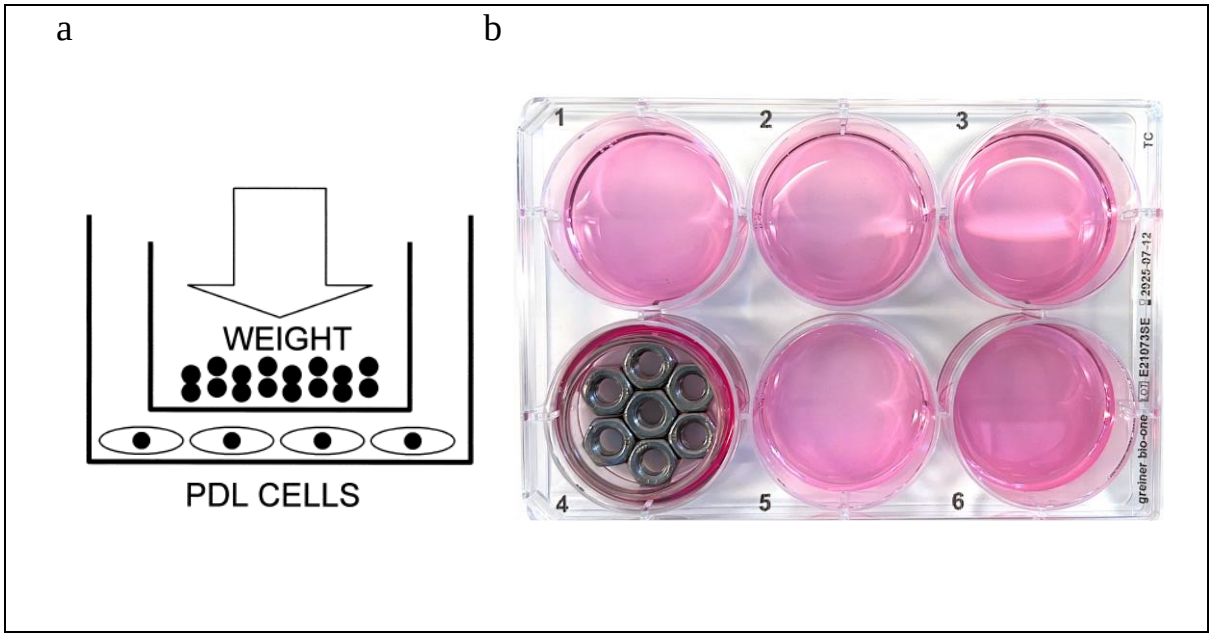

Figure 4.3 The method used to simulate orthodontic forces in vitro.

(a) Schematic diagram depicting the method of compressive force application, adapted from (Kanzaki et al., 2002). **(b)** Compressive force was generated using a weight placed onto confluent hGFs, and hPDLFs cell layer. A glass cylinder (30 mm in diameter) was placed over a confluent cell layer in the well of a 6-well culture plate, and the weight was adjusted by adding metal parts to induce a compressive force of 2 gm/cm^2.

4.2.3 Total protein analysis

Total protein concentration in the samples was quantified using the Thermo Scientific^{TM} Pierce^{TM} Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific, USA) according to the manufacturer's protocol. The protein concentration was measured with reference to bovine serum albumin (BSA), which was used to prepare a duplicate set of diluted standards. Samples were diluted in deionized water (1:10) before the BCA working reagent was added. Then, the plate was incubated at 37°C in the incubator for 30 minutes. The absorbance was then measured at 540 nm using a Thermo Scientific multiskan FC plate reader. The known concentration of diluted BSA standard and their absorbance values were used to create a standard curve to calculate the unknown sample protein concentration.

4.2.4 Cell viability assay

The MTT (3-(4,5- dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to assess cell metabolic activity under the various experimental conditions according to the manufacturer's protocol (M5655, Sigma-Aldrich). MTT (3-(4,5- dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide) is reduced to purple formazan granules in the mitochondria of living cells. In brief, hPDLFs and hGFs were seeded in 6-well culture plates and were stimulated with IL-1α (0.1 ng/ml), leptin (10 µg/ml), AdipoRon (40 µM), and compressive force (2 gm/cm^2) alone or in combination. After 24, 48, and 72 hours 2000 µl DMEM medium containing 200 µl MTT solution (5 mg/ml MTT in PBS) was added, and the culture plates were incubated at 37°C for 4 hours in the dark. Next, the MTT solution was carefully removed to avoid disturbing the formazan granules, and then an equal volume of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) was added to dissolve the granules. The absorbance of the purple-coloured solution was measured at 590 nm with a reference filter of 620 nm using a Thermo Scientific multiskan FC plate reader. Data were obtained from three experiments, and cell viability was expressed as a fold change relative to the unstimulated control.

4.2.5 Cytotoxicity assay

Lactate dehydrogenase (LDH) is an enzyme found almost within the cytoplasm of all cells. When cells die, the plasma membrane is damaged, and the contents of the cytosol are released into the cell culture supernatant. This is a crucial feature of cell death, and the amount of LDH in cell supernatants is directly proportional to the number of dead cells (Kumar et al., 2018). LDH assay was performed to assess cytotoxicity for the different experimental conditions using Cytotoxicity Detection Kit^{PLUS} (LDH) (4744926001, Roche) according to according to the manufacturer's protocol by measuring LDH activity released from the cytosol of damaged cells. One hundred µl cell supernatants were mixed with 100 µl freshly prepared LDH reaction

mixture and incubated for 25 minutes at room temperature in the dark. After that, 50 mL of stop solution was added and shaken for 10 seconds, and then absorbance was measured at 490 nm, subtracting background absorbance at 690 nm using a Thermo Scientific multiskan FC plate reader. Data were obtained from three experiments and are presented as a fold change relative to the unstimulated control.

4.2.6 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) (R&D systems) was used to assess total human MMP-1 (DY901B-05), total MMP-3 (DY513-05), total MMP-8 (DY908), and total IL-8 (DY208-05) (active and pro-active forms) levels in hPDLFs and hGFs supernatants according to the manufacturer's protocol. For each ELISA, 96-well microplates (ThermoFisher Scientific, UK) were coated with capture antibody diluted in Dulbecco's Phosphate Buffered Saline (PBS) (D8537-100ml, Sigma-Aldrich) to the working concentration and incubated overnight at room temperature. The microplates were then aspirated and washed with wash buffer (0.05% Tween-20 in PBS) three times. The plates were then blocked by adding reagent diluent (1 % Bovine Serum Albumin (BSA) (Sigma-Aldrich, UK) in PBS) for 1 hour at room temperature. In the meantime, 2-fold serial dilutions of the standard in reagent diluent were carried out to produce a seven-point standard curve (MMP-1 top standard: 10,000 pg/ml, MMP-3 top standard: 4,000 pg/ml, IL-8 top standard: 4,000 pg/ml, IL-24 top standard: 4 ng/ml). Next, the microplates were aspirated and washed as above, and then standards, samples (diluted in reagent diluent), and blanks (reagent diluent) were added in duplicate and incubated for 2 hours at room temperature. After that, the microplates were aspirated and washed as above, then the detection antibody diluted in reagent diluent to the working concentration was added and incubated for 2 hours at room temperature. Afterward, microplates were aspirated and washed as above, and then a streptavidin-horseradish peroxidase (HRP) solution, diluted

in reagent diluent to the working concentration, was added and incubated for 20 minutes at room temperature in the dark. Later, the microplates were aspirated and washed as above before adding a substrate solution (SureBlue™ TMB 1-Component Microwell Peroxidase Substrate, Seracare) for 20 minutes at room temperature in the dark. Finally, a stop solution was added to the microplates to stop colour development. The optical density of each well was determined immediately at 450 nm using a Thermo Scientific multiskan FC plate reader. The blank and standard concentrations mean readings were used to generate a four-parameter logistic (4-PL) curve-fit (Appendix Figure 4.1). The standard curve was then used to calculate the sample concentrations, which were then multiplied by any dilution factors. Data were obtained from three experiments, and protein levels were expressed as a fold change in relation to the unstimulated control.

4.2.7 Quantitative real-time polymerase chain reaction (RT-qPCR) analysis

Total RNA extraction was performed using RNeasy Micro Kit (74004, Qiagen according to the manufacturer's protocol. Briefly, hGFs and hPDLFs supernatants were collected and stored at – 80°C for later analysis. Direct lysis of the cells was performed using lysis buffer supplemented with 1% 2-mercaptoethanol (Sigma-Aldrich, UK). The lysates were then collected and processed immediately. An equal volume of 70 % ethanol was added to the cell lysates and vortexed for 30 seconds. Next, the samples were transferred to an RNeasy MinElute spin column and centrifuged at 10000 g for 15 seconds. The spin columns were then washed before DNase solution was added and kept for 15 minutes at room temperature. Afterward, the spin columns were washed and centrifuged several times before RNase-free water (Qiagen, UK) was added directly to the centre of the spin column membranes to elute RNA into a clean collection tube by centrifugation at 14000 for 1 minute. RNA yield and the A260/280 and A260/230 ratios were then measured with the NanoDrop Spectrophotometer (Thermo Fischer

Scientific, UK). Readings of more than 1.8 for both ratios were regarded as high purity and used for cDNA synthesis. Two hundred ng of extracted RNA were reverse transcribed using iScript™ cDNA Synthesis kit (1708841, Bio-Rad) according to the manufacturer's instructions. 20 µl of reverse transcription reaction was incubated in a thermal cycler as follows: 5 minutes at 25°C to promote primer annealing, 20 minutes at 46 °C to promote reverse transcription, 1 minute at 95 °C to inhibit the activity of the enzyme, and the reaction was ceased by transferring the tubes to ice (4 °C); cDNA was then diluted 10-fold with nuclease-free water (Bio-Rad, UK), aliquoted, and stored at - 20 °C. RT-qPCR reactions were prepared by adding 2 µl cDNA, 0.5 µl of each forward and reverse primers (10 µM) (Eurofins, UK), 5 µl SsoAdvanced ™ Universal SYBR Green Supermix (1725272, Bio-Rad), and 2 µl nuclease-free water to give a final volume of 10 µl per reaction. The qPCR technique consists of three basic steps: denaturation, annealing, and extension. At high temperatures (90–97 °C), the double-stranded DNA is first denatured into two single strands of DNA. Primers bind to the DNA template strands in the second stage, which takes place at a lower temperature (50–60 °C), to prime extension. In the third step, extension is carried out by a DNA polymerase at the end of the annealed primers to produce a new complimentary strand of DNA (Joshi and Deshpande, 2011). These steps are repeated 40 times (cycles). RT-qPCR was carried out using Corbett Rotor-Gene 6000 System (Qiagen, UK) according to the protocol for Corbett Rotor-Gene in Table 4.1.

The design for COX-2, IL-6, MMP-8, TIMP-1, and GAPDH primers were obtained from (Grimm et al., 2020), IL-8 (Wu et al., 2011), MMP-1 and MMP-3 (Gotoh et al., 2013), MMP-2 and MMP-9 (Liu et al., 2018), and MMP-7 (Cury et al., 2007). Primer specificity was checked by melt curve analysis, and the Melting curves of all primers showed a single peak indicating an absence of primer dimers (Appendix Figure 4.2); primers used and their sequences are listed in Table 4.2, and GAPDH was used as the housekeeping gene.

Relative gene quantification of RT-qPCR experiments was assessed according to the $2^{-\Delta\Delta CT}$ method; the data are displayed as the fold change in the target gene expression normalized to the housekeeping gene and relative to the unstimulated control (Livak and Schmittgen, 2001). Data were obtained from three experiments, and cDNA from each experiment was analysed in duplicate.

Table 4.1 Corbett Rotor-Gene thermal cycling protocol

Setting/Mode	**Polymerase Activation and DNA Denaturation**	**Amplification**			**Melt curve analysis**
		Denaturation	**Annealing/Extension At 60 °C**	**Cycles**	
Fast	2 minutes at 98 °C	15 seconds	30 seconds	40	65-95 °C 0.5 °C increment 2-5 seconds /step

Table 4.2 List of primers and sequences used for RT-qPCR

Gene	**Forward 5'−3'**	**Reverse 5'−3'**
COX-2	TTG AAG ATTATG TGC AAC AC	ATA GAG TGC TTC CAA CTC TG
IL-6	AGC CCT GAG AAA GGA GAC AT	CTT TTT CAG CCATCT TTG GA
Il-8	CAAACCTTTCCACCCCAAAT	CTCAGCCCTCTTCAAAAACT
MMP-1	TGGGCTTGAAGCTGCTTAC	TCGATATGCTTCACAGTTCTAGG
MMP-2	CTCATCGCAGATGCCTGGAA	CAGCCTAGCCAGTCGGATTTG
MMP-3	TGTCTCAAGATGATATAAATGGCATTC	TGCTGACAGCATCAAAGG
MMP-7	AAAGAGATCCCCCTGCATTT	GTGAGCATCTCCTCCGAGAC
MMP-8	GTT CAG CAA GCATTT TCG TT	CAC GGA GGA CAG GTA GAATG
MMP-9	ACGCACGACGTCTTCCAGTA	CCACCTGGTTCAACTCACTCC
TIMP-1	TGG ACT CTT GCA CAT CAC TAC CTGC	AGG CAA GGT GAC GGG ACT GGAA
GAPDH	AAA AAC CTG CCA AAT ATG AT-3′	CAG TGA GGG TCT CTC TCT TC-3′

4.2.8 Western Blotting

HPDLFs and hGFs were seeded in 6-well plates as described previously (Section cell culture). Cells were stimulated with IL-1α (0.1 ng/ml), Leptin (10 μg/ml), AdipoRon (40 μM), compressive force (2 gm/cm^2), and Torin 1 (200 nM) (Tocris Bioscience, UK) for 24 hours. The supernatants were discarded, then ice-cold PBS was used to wash the cells. After aspirating PBS, cells were lysed using ice-cold RIPA buffer (Sigma-Aldrich, Poole, Dorset, UK) supplemented with protease inhibitor cocktail (Roche, UK). Cells were then scraped, collected, and kept on ice. Next, the lysates were centrifuged at 10,000 g for 5 minutes at 4°C, and supernatants were collected, aliquoted, and stored at - 80°C for later use.

Samples were prepared by mixing with 5% NuPage lithium dodecyl sulphate sample buffer (LDS, Invitrogen, UK), 10% 0.5 M DTT (dithiothreitol), and boiled at 100°C for 5 minutes. Next, samples were separated on precast 4-2% SDS-PAGE gel (NuPAGE® Novex™ Bis-Tris Gels, Invitrogen, UK) at 125 mA and 200 volts for 32 minutes in Xcell vertical electrophoresis unit (Invitrogen, UK) with 25 ml running buffer (NP0002, Novex) and 475 ml deionized water. Proteins were then transferred onto the surface of a 0.22 μm pore–size nitrocellulose membrane (1620097, Bio-Rad, UK). The transfer was performed with a nitrocellulose membrane placed on top of the gel and sandwiched between filter paper and a sponge soaked with transfer buffer (25ml transfer buffer (NP0006, Novex), 50ml of Methanol, and 425ml of deionized water) at 30 volts constant and 170 mA for 60 minutes. The membrane was then blocked with 5% bovine serum albumin (BSA) in Tris Buffered Saline with Tween-20 (TBST) (50 mM TRIS, 150 mM NaCl, 0.1% Tween-20, PH 7.5) for 1 hour at room temperature. After that, the membrane was washed three times with TBST, followed by incubation with the proper working dilution of the primary antibody overnight at 4°C. The membrane was then washed three times with TBST and incubated with the recommended dilution of conjugated secondary antibody in TBST for 1 hour at room temperature. Next, the

membrane was washed thoroughly with TBST 3 times, 5 minutes each (primary and secondary antibodies used, and their dilutions are listed in Table 4.3). A mixture of ECL Clarity Western Peroxide solution and Luminal/Enhancer solution (1:1) (Bio-Rad, UK) was used to visualize protein bands using ChemiDoc™ MP System (Bio-Rad, UK). The membranes were re-probed using an internal control antibody (β-actin) to normalise protein levels detected. Each experiment was repeated four times, and unstimulated cells served as a control. Data are presented as the fold change relative to the unstimulated control. Band quantification was carried out using ImageJ (Fiji).

Table 4.3 List of antibodies used for western blotting.

Antibody	Host	Working dilution
Phospho-Akt (Ser473) (9271, Cell Signalling Technology)	Rabbit	1:1000
Akt (pan) (C67E7) (4691, Cell Signalling Technology)	Rabbit	1:1000
Phospho-4E-BP1 (T37/46) (236B4, Cell Signalling Technology)	Rabbit	1:1000
4E-BP1 (9452, Cell Signalling Technology)	Rabbit	1:1000
β-actin (A2228-200µl, (Sigma Aldrich, UK)	Mouse	1 µg/mL
Polyclonal Goat Anti-Mouse Immunoglobulins- HRP (P0447, Dako)	Goat	1:3000
Polyclonal swine anti-rabbit Immunoglobulins-HRP (P0217, Dako)	Swine	1:2000

4.2.9 Statistical analysis

Data were tested for normality using Shapiro-Wilk test. One-way ANOVA was used to analyse the normally distributed data followed by correction for multiple testing using Dunnett's or Tukey's multiple comparisons tests. Key significant values related to the aims of the study were reported. The significant differences between the control and the following variables were reported: compressive force, IL-1α, leptin, AdipoRon, force+leptin, force+IL-1α, force+AdipoRon, leptin+IL-1α, AdipoRon+IL-1α, leptin+IL1α+force, and AdipoRon+IL-1α+force. Also, the significant differences between IL-1α stimulated cells and the following:

force+IL-1α, leptin+IL-1α, AdipoRon+IL-1α, leptin+IL1α+force, and AdipoRon+IL-1α+force. Statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, California USA). The differences were considered statistically significant if $p<0.05$.

4.3 Results

4.3.1 Cell viability of human periodontal and gingival fibroblasts

The MTT assay was used to assess cell viability of hPDLFs and hGFs after stimulation with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), leptin (10 µg/ml) and AdipoRon (40 µM) alone or in combination for 24, 48, and 72 hours.

For hPDLFs, the MTT assay showed that the metabolic activity of all the experimental stimulants was comparable to unstimulated cells after 24 hours ($P>0.05$). After 48 and 72 hours, IL-1α, leptin, AdipoRon, leptin+IL-1α, and AdipoRon+IL-1α had no effect on cell viability compared to unstimulated cells ($P>0.05$). However, the compressive force (2 gm/cm^2) significantly reduced the metabolic activity of hPDLFs after 48 and 72 hours when applied alone ($p<0.001$; $p<0.0001$) or combined with IL-1α (both $p<0.01$), leptin ($p<0.01$; $p<0.001$), AdipoRon ($p<0.001$; $p<0.0001$), leptin+IL-1α ($p<0.001$; $p<0.01$), and AdipoRon+IL-1α ($p<0.001$; $p<0.0001$) (Figure 4.4a-c).

For hGFs, metabolic activity of all the experimental stimulants was comparable to unstimulated cells after 24 hours stimulation ($P>0.05$). After 48 and 72 hours, cell viability of hGFs stimulated with IL-1α, leptin, or AdipoRon was comparable to unstimulated cells ($P > 0.05$). Nevertheless, when leptin or AdipoRon was combined with IL-1 α, a significant increase in metabolic activity of hGFs was observed after 72 hours ($p<0.05$) but not after 48 hours ($p>0.05$). On the other hand, the compressive force (2 gm/cm^2) caused a significant reduction

in cell viability of hGFs when applied alone ($p<0.0001$) or combined with IL-1α ($p<0.001$), leptin ($p<0.05$), AdipoRon ($p<0.0001$), leptin+IL-1α ($p<0.001$), and AdipoRon+IL-1α ($p<0.0001$) (Figure 4.4d-f).

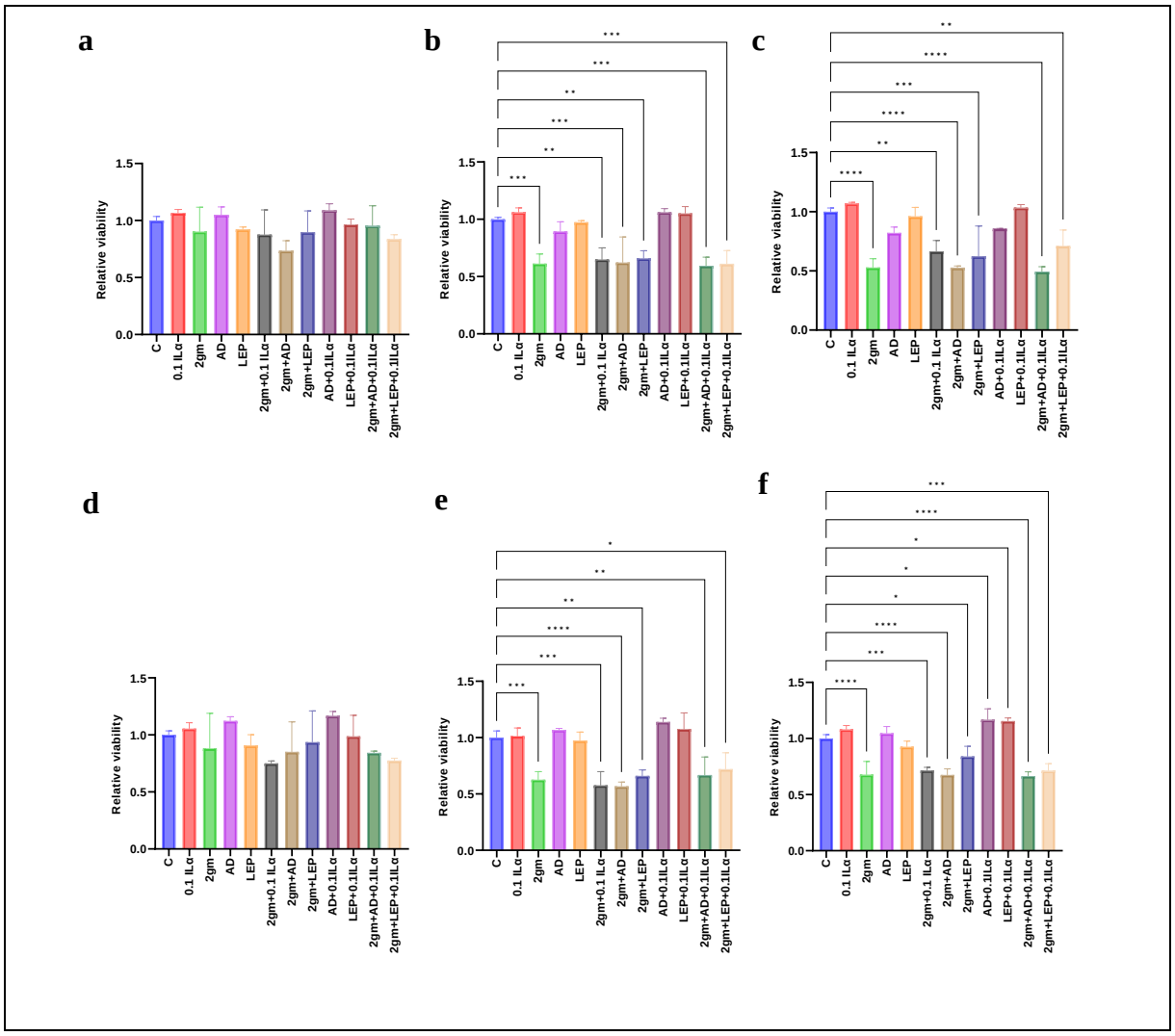

Figure 4.4 The effect of IL-1α, leptin, AdipoRon, and force on cell viability of hPDLFs and hGFs.

hPDLFs (a-c) and hGFs (d-f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), leptin (10 µg/ml), and AdipoRon (40 µM) alone or in combination. Cell viability was assessed using MTT assay after 24 (a & d), 48 (b & e), and 72 hours (c & f) of stimulation. Cell viability was expressed as a fold change relative to the unstimulated cells. C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; AD, AdipoRon; LEP, leptin;2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Dunnett-corrected). * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$.

4.3.2 Cytotoxicity of IL-1α, compressive force, leptin, and AdipoRon

LDH activity was assessed in the supernatants of hPDLFs and hGFs. Cells were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), leptin (10 μg/ml), and AdipoRon (40 μM) alone or in combination for 24, 48, and 72 hours. LDH analysis revealed no significant differences in the cytotoxicity of all the different experimental conditions compared to the unstimulated cells at 24, 48, and 72 hours for both hGFs and hPDLFs, indicating no cytotoxic effects for any cell stimulation applied (Appendix Figure 4.3).

4.3.3 IL-6 expression

IL-6 gene expression was assessed by RT-qPCR. Compressive force (2 gm/cm^2), AdipoRon (40 μM), and leptin (10 μg/ml) had no significant effect on IL-6 mRNA levels in hPDLFs and hGFs after 24-, 48-, and 72-hour stimulation.

For hPDLFs, IL-1α (0.1 ng/ml) significantly increased IL-6 mRNA expression compared to the unstimulated cells at 24, 48, and 72 hours (Figures 4.5 and 4.6). However, when hPDLFs were stimulated with both IL-1α (0.1 ng/ml) and AdipoRon (40 μM), AdipoRon reduced IL-6 mRNA levels induced by IL-1α at 24 hours (47.63 folds), 48 hours (36 folds), and 72 hours (5 folds); with significance at 48 hours compared to IL-1α-stimulated hPDLFs ($p<0.01$) (Figure 4.5a-c). In contrast, the compressive force significantly increased IL-6 mRNA levels induced by IL-1α in force+IL-1α stimulated hPDLFs at 24 (98.3 folds; $p<0.05$), 48 (39.7 folds; $p<0.01$), and 72 hours (12.4 folds; $p<0.05$) compared to IL-1α stimulated hPDLFs. However, this increase was diminished by AdipoRon in Adiporon+IL-1α+force stimulated hPDLFs, and IL-6 mRNA levels were comparable to those of IL-1α stimulated hPDLFs at 24, 48, and 72 hours (Figure 4.5a-c). On the other hand, leptin and IL-1α synergised to significantly increase IL-6 mRNA levels in leptin+IL-1α stimulated hPDLFs at 24 hours (106.4 folds;

$p<0.001$) compared to IL-1α stimulated hPDLFs but were not significant at 48 and 72 hours ($P>0.05$). Furthermore, IL-6 mRNA levels were further enhanced in leptin+IL1α+force stimulated hPDLFs at 24 hours (143.3 folds; $p<0.0001$ compared to IL-1α stimulated hPDLFs) (Figure 4.6a-c).

For hGFs, IL-1α (0.1 ng/ml) significantly increased IL-6 mRNA expression compared to the unstimulated cells at 24, 48, and 72 hours. However, AdipoRon attenuated IL-6 mRNA expression induced by IL-1α in Adiporon+IL-1α stimulated hGFs by 42.9, 21.6, and 14.1 folds at 24, 48, and 72 hours, respectively, but without showing any significance compared to IL-1α stimulated hPDLFs (Figure 4.5d-f). On the contrary, the compressive force enhanced IL-6 mRNA expression induced by IL-1α in force+IL-1α stimulated hGFs by 43.8, 8.8, and 16.5 folds at 24, 48, and 72 hours, respectively, though, without any significance compared to IL-1α stimulated hGFs ($p<0.05$) (Figures 4.5 and 4.6). However, this increase in IL-6 mRNA expression was reduced in Adiporon+IL-1α+force stimulated hGFs at all time points (Figure 4.5d-f). On the other hand, IL-6 mRNA levels induced by IL-1α were enhanced in the presence of leptin by 26.2, 9.2, and 20 folds and were further enhanced in the presence of both leptin and force by 122.9, 29.9 18.6 folds at 24, 48, and 72 hours, respectively showing a significant increase only at 24 hours in leptin+IL1α+force stimulated hGFs compared to IL-1α stimulated hGFs ($p<0.01$) (Figure 4.6d-f).

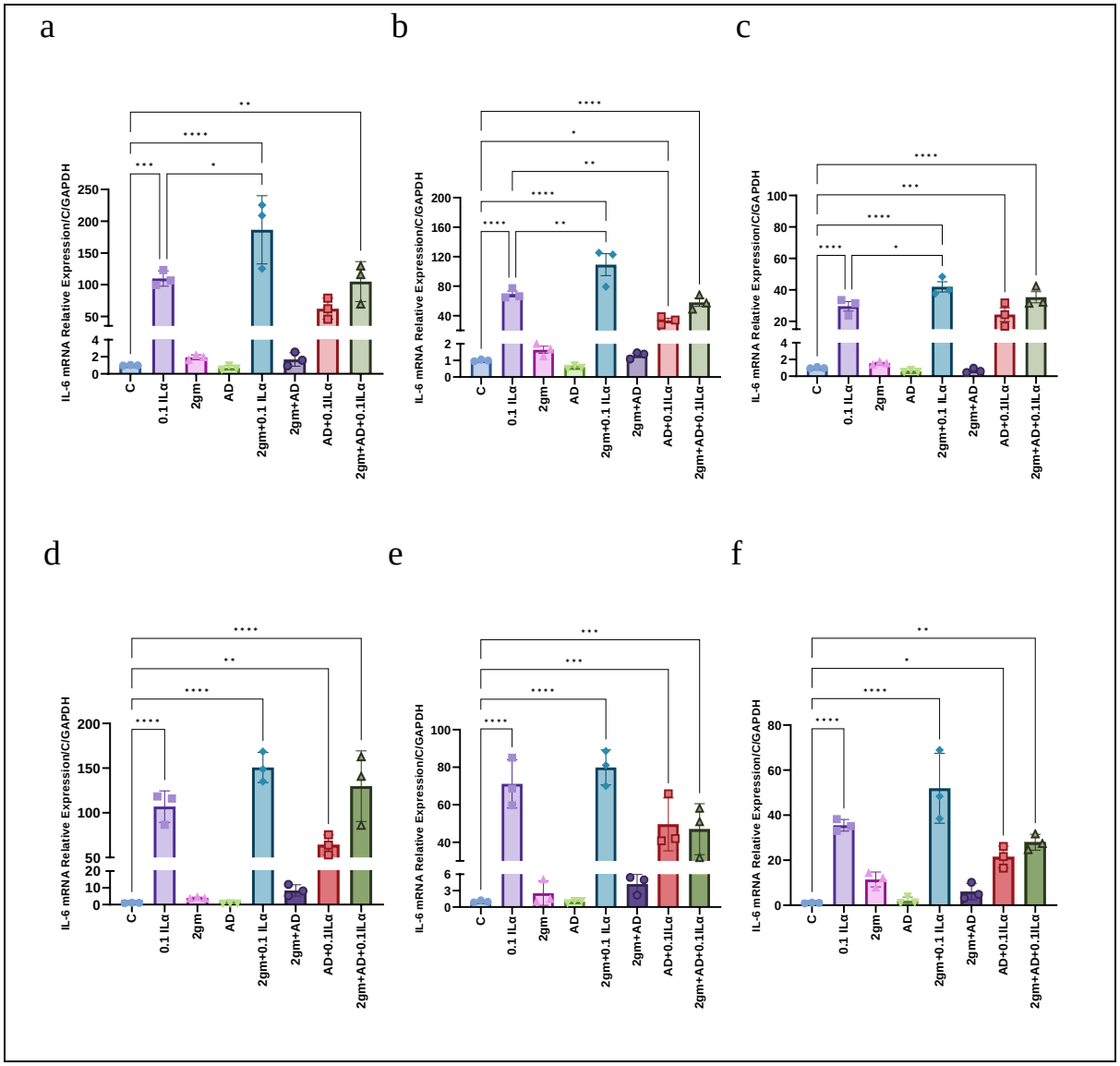

Figure 4.5 Relative IL-6 mRNA expression in hPDLFs and hGFs.

hPDLFs (a-c) and hGFs (d-f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and AdipoRon (40 µM) alone or in combination for 24 (a & d), 48 (b & e), and 72 (c & f) hours. IL-6 mRNA expression was assessed using RT-qPCR and measured using the 2^{-ddCt} method using GAPDH as the housekeeping gene and relative to the unstimulated cells. C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; AD, AdipoRon; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

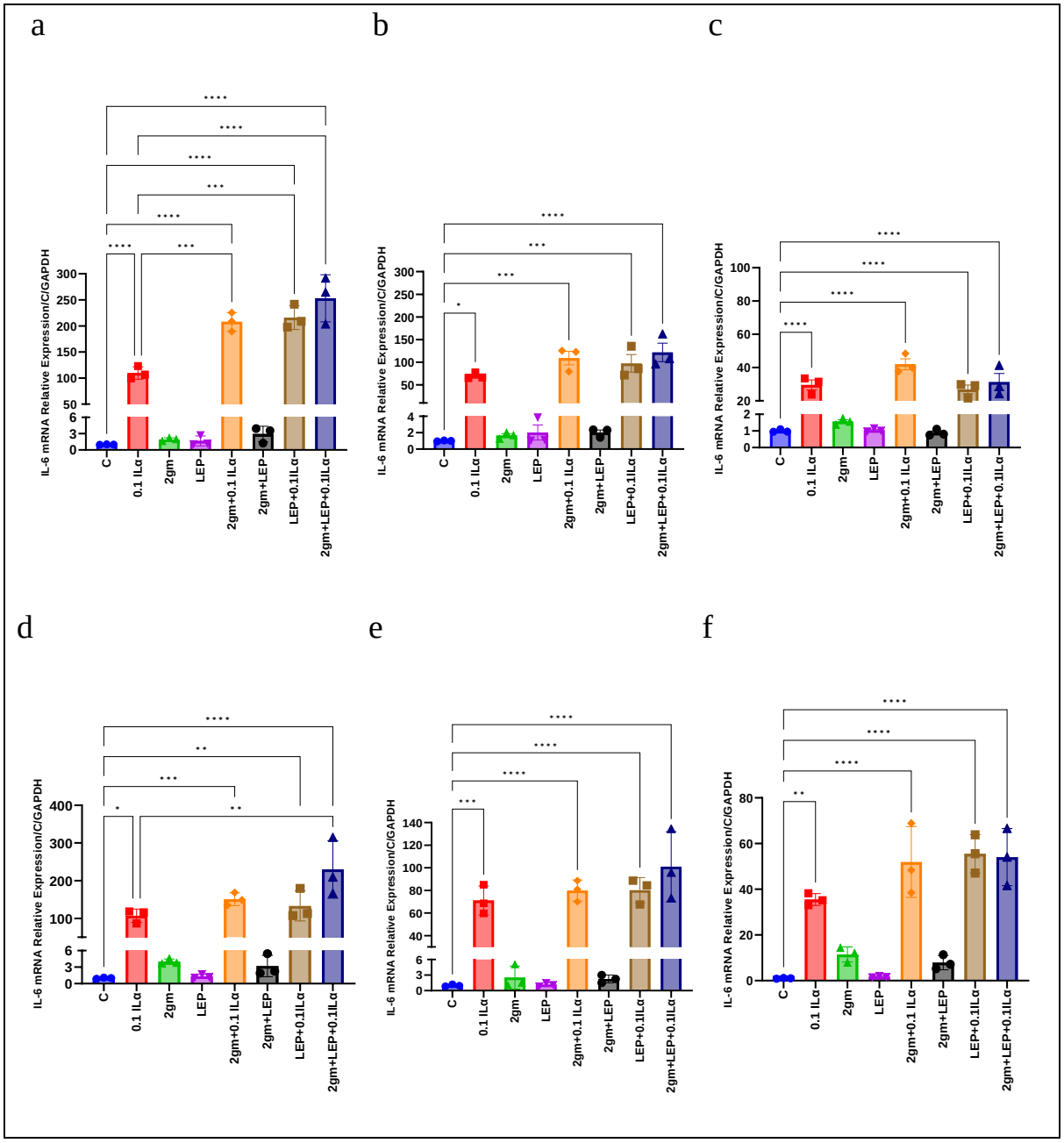

Figure 4.6 Relative IL-6 mRNA expression in hPDLFs and hGFs.

hPDLFs (a-c) and hGFs (d-f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and leptin (10 µg/ml) alone or in combination for 24 (a & d), 48 (b & e), and 72 (c & f) hours. IL-6 mRNA expression was assessed using RT-qPCR and measured using the 2^{-ddCt} method using GAPDH as the housekeeping gene and relative to the unstimulated cells. C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; LEP, leptin; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$; **** = $p<0.0001$.

4.3.4 IL-8 expression

IL-8 gene expression was assessed by RT-qPCR, and IL-8 protein concentration in the supernatant was assessed by ELISA (Figures 4.7 and 4.8).

For hPDLFs, compressive force (2 gm/cm^2), AdipoRon (40 μM), and leptin (10 μg/ml) did not significantly change IL-8 mRNA and protein levels in hPDLFs after 24-, 48-, and 72-hour stimulation. IL-1α (0.1 ng/ml) caused a significant increase in IL-8 mRNA and proteins levels compared to the unstimulated cells at 24, 48, and 72 hours (Figures 4.7 and 4.8). When hPDLFs were stimulated with AdipoRon+IL-1α, AdipoRon significantly decreased IL-8 mRNA levels induced by IL-1α at 24 (1634 folds; $p<0.0001$) and 48 hours (2444 folds; $p<0.001$) compared to IL-1α-stimulated hPDLFs. At 72 hours, IL-8 mRNA levels increased to a comparable level to IL-1α stimulated hPDLFs ($p>0.05$) (Figure 4.7a-c). To determine whether AdipoRon had a similar effect on IL-8 protein secretion induced by IL-1α, IL-8 concentrations in the supernatants were assessed using ELISA. AdipoRon attenuated IL-1α-induced IL-8 protein secretion in hPDLFs at all time points but was statistically significant at 48 hours (53.1 folds; $p<0.0001$) and 72 hours (24.6 folds; $p<0.05$) compared to IL-1α stimulated hPDLFs (Figure 4.7d-f). Conversely, force+IL-1α significantly increased IL-8 mRNA levels induced by IL-1α by 1454 folds (24 hours; $p<0.001$), 2683 folds (48 hours; $p<0.05$) and 4203 folds (72 hours; $p<0.01$) compared to IL-1α stimulated hPDLFs. Moreover, IL-8 protein levels in force+IL-1α stimulated hPDLFs were enhanced by 16.7, 7.8, 13.2 folds at 24, 48, and 72 hours, respectively, compared to IL-1α stimulated hPDLFs, though this increase was not significant. Nevertheless, AdipoRon, in the presence of compressive force, reduced IL-1α-induced IL-8 mRNA levels by 1308 folds (24 hours; $p<0.001$), 1762 folds (48 hours, $p<0.05$), and1509 folds (72 hours; $p>0.05$), and IL-8 protein levels by 24.9 folds (24 hours; $p>0.05$), 35.3 folds (48 hours; $p<0.001$), and 18.7 folds (72 hours; $p>0.05$), compared to IL-1α stimulated hPDLFs (Figure 4.7a-f). On the other hand, IL-8 mRNA levels induced by

IL-1α were increased in leptin+IL-1α stimulated hPDLFs at all time points, showing a significant increase at 24 hours ($p<0.0001$) (Figure 4.8a-c). IL-8 protein levels were also increased at all timepoints but were not statistically significant compared to IL-1α stimulated hPDLFs (Figure 4.8d-f). Additionally, the combination of leptin and force caused a further increase in IL-1α-induced IL-8 mRNA and protein levels; IL-8 mRNA levels were increased at 24 (3208 folds; $p<0.0001$), 48 (3231 folds; $p<0.05$), and 72 hours (5471 folds; $p<0.01$) compared to IL-1α stimulated hPDLFs (Figure 4.8a-c). Also, IL-8 protein levels were further enhanced at 24 hours (28.32 folds; $p<0.01$), 48 hours (24.46 folds; $p>0.05$), and 72 hours (37.2 folds; $p<0.01$) compared to IL-1α stimulated hPDLFs (Figure 4.8d-f).

For hGFs, AdipoRon (40 μM) and leptin (10 μg/ml) did not significantly change IL-8 mRNA and protein levels after 24-, 48-, and 72-hour stimulation. However, compressive force increased IL-8 mRNA levels at 24 hours (2 folds), 48 hours (4 folds); and 72 hours (267 folds), but this increase was not statistically significant compared to unstimulated cells. Also, force resulted in a similar increase in IL-8 protein levels but was statistically significant at 72 hours compared to unstimulated cells (18.28 folds; $p<0.01$). IL-1α (0.1 ng/ml) significantly increased IL-8 mRNA and protein levels compared to the unstimulated cells at all time points (Figures 4.7 and 4.8). AdipoRon decreased IL-1α-induced IL-8 mRNA and protein levels in AdipoRon+IL-1α stimulated hGFs at all time points, with IL-8 mRNA showing a significant decrease at 24 hours (678.7 folds; $p<0.05$) and 48 hours (292.2 folds; $p<0.001$) (Figure 4.7g-i), and IL-8 protein levels showing a significant decrease at 48 hours (41.1 folds; $p<0.0001$) compared to IL-1α stimulated hGFs (Figure 4.7j-l). In contrast, IL-8 mRNA and protein levels in force+IL-1α stimulated hGFs were increased at all time points; however, with significance at 48 (182 folds; $p<0.05$) and 72 hours (752.8 folds; $p<0.0001$) for mRNA levels and at 48 hours (38.4 folds; $p<0.0001$) for protein levels, compared to IL-1α stimulated hGFs (Figure 4.7g-l). However, AdipoRon diminished IL-1α-induced IL-8 mRNA and protein upregulation

with compressive force in Adiporon+IL-1 α+force stimulated hGFs, with only IL-8 protein levels being significantly reduced at 48 hours ($p<0.0001$) compared to IL-1α stimulated hGFs (Figures 4.7g-l). On the other hand, leptin+IL-1α increased IL-8 mRNA levels by 543, 108, and 184 folds at 24, 48, and 72 hours, respectively, however this increase was not statistically significant compared to IL-1α stimulated hGFs ($p>0.05$) (Figure 4.8g-i). Also, IL-8 protein levels were significantly increased at 48 hours (29.2 folds; $p<0.001$) in comparison to IL-1α stimulated hGFs (Figure 4.8j-l). In addition, IL-1α-induced IL-8 mRNA levels were significantly enhanced in the presence of compressive force and leptin by 1378 folds (24 hours; $p<0.001$), 367.6 folds (48 hours; $p<0.01$), and 1022 folds (72 hours; $p<0.0001$) compared to IL-1α stimulated hGFs (Figure 4.8g-i). Moreover, Il-8 protein levels were also significantly increased by 10.18 (24 hours; $p<0.05$) and 56.7 folds (48 hours; $p<0.0001$) compared to IL-1α stimulated hGFs (Figure 4.8j-l).

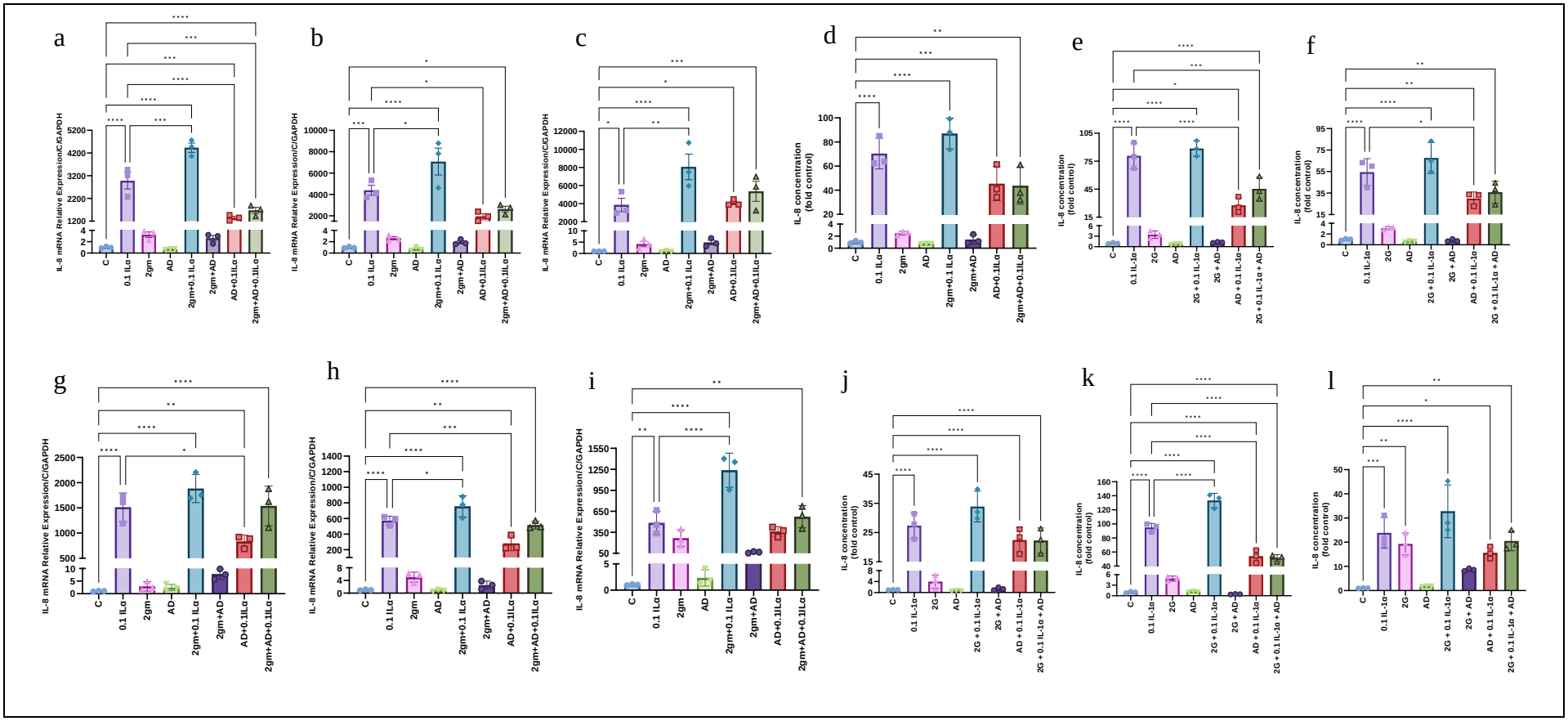

Figure 4.7 Relative IL-8 mRNA and protein expression in hPDLFs and hGFs.

hPDLFs (a-f) and hGFs (g-l) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and AdipoRon (40 μM) alone or in combination for 24 (a, d, g, j), 48 (b, e, h, k), and 72 (c, f, i, l) hours. IL-8 mRNA expression was assessed using RT-qPCR for hPDLFs (a-c) and hGFs (g-i). IL-8 protein secretion was assessed using ELISA and measured as fold change relative to the unstimulated cells for hPDLFs (d-f) and hGFs (j-l). C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; AD, AdipoRon; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

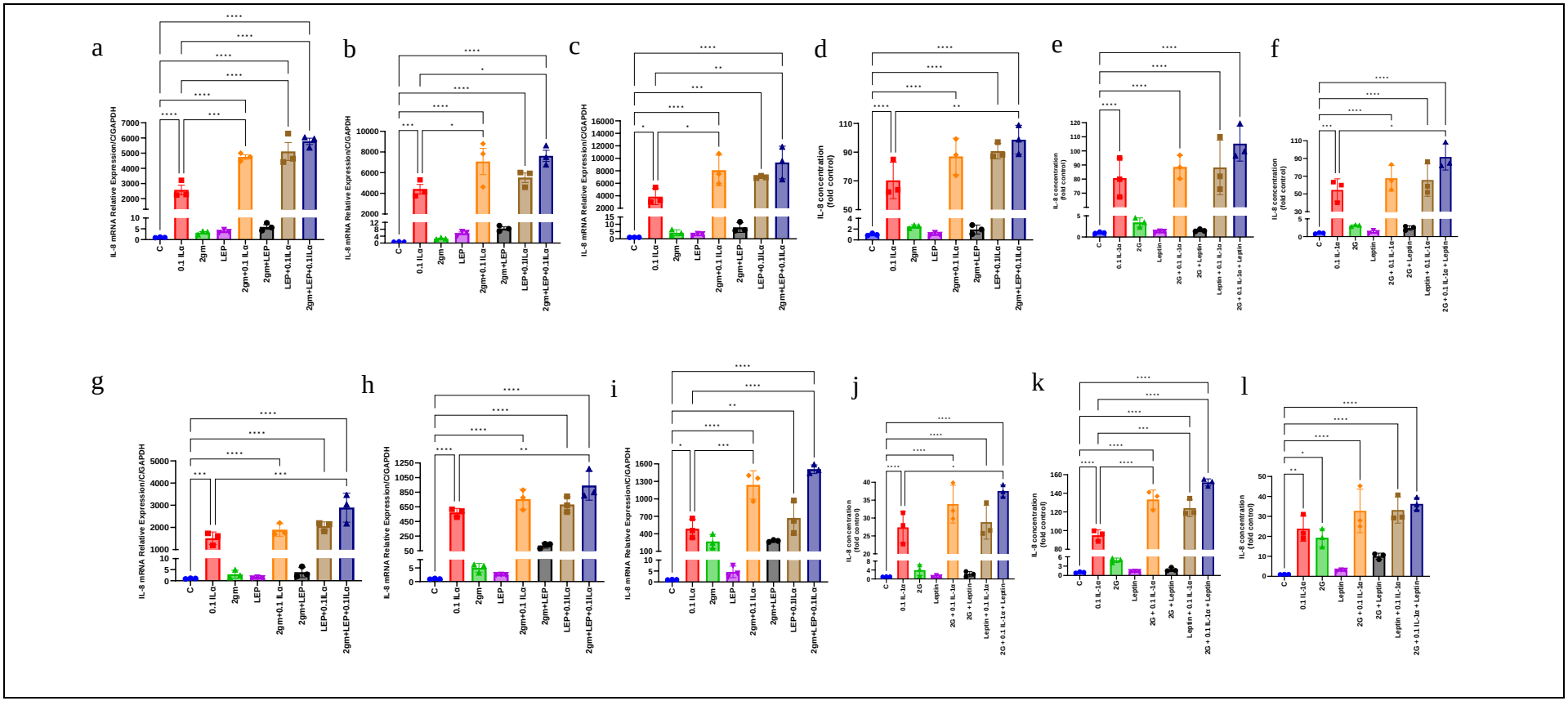

Figure 4.8 Relative IL-8 mRNA and protein expression in hPDLFs and hGFs.

hPDLFs (a-f) and hGFs (g-l) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and leptin (10 µg/ml) alone or in combination for 24 (a, d, g, j), 48 (b, e, h, k), and 72 (c, f, i, l) hours. IL-8 mRNA expression was assessed using RT-qPCR for hPDLFs (a-c) and hGFs (g-i). IL-8 protein secretion was assessed using ELISA and measured as fold change relative to the unstimulated cells for hPDLFs (d-f) and hGFs (j-l). C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; LEP, leptin; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

4.3.5 MMP-1 expression

MMP-1 gene expression was assessed by RT-qPCR, and MMP-1 protein concentrations in the supernatants were assessed by ELISA (Figures 4.9 and 4.10).

For hPDLFs, compressive force (2 gm/cm^2), AdipoRon (40 μM), and leptin (10 μg/ml) did not significantly affect MMP-1 mRNA and protein levels after 24-, 48-, and 72-hour stimulation compared to unstimulated cells. IL-1α significantly increased MMP-1 mRNA and protein levels at all time points compared to unstimulated cells (Figures 4.9 and 4.10). However, in AdipoRon+IL-1α stimulated hPDLFs, MMP-1 mRNA and protein levels were significantly reduced at all time points, though only showing a significant decrease at 48 (6.9 folds; $p<0.01$) and 72 hours (4.348 folds; $p<0.01$) for MMP-1 mRNA levels, and at 24 (3 folds; $P<0.05$) and 48 hours (2.2 folds; $p<0.05$) for MMP-1 protein levels, compared to IL-1α stimulated hPDLFs (Figures 4.9a-f). Conversely, compressive force enhanced IL-1α-induced MMP-1 mRNA levels in force+IL-1α stimulated hPDLFs at 24 (5.5 folds; $p<0.05$) and 48 hours (2 folds; $p>0.05$) compared to IL-1α stimulated hPDLFs (Figure 4.9a-c). IL-1α-induced MMP-1 protein levels were also enhanced in the presence of compressive force at all time points; however, the increase was not significant compared to IL-1α stimulated hPDLFs ($p>0.05$) (Figure 4.9d-f). Nevertheless, the increase in IL-1α-induced MMP-1 mRNA and protein levels caused by compressive force was abolished in the presence of AdipoRon in Adiporon+IL-1α+force stimulated hPDLFs (Figure 4.9a-f). On the contrary, Leptin enhanced IL-1α-induced MMP-1 mRNA (24 and 48 hours) and protein levels (all time points), however without any significance compared to IL-1α stimulated hPDLFs. Furthermore, IL-1α-induced MMP-1 mRNA and protein levels were significantly enhanced in the presence of compressive force and leptin at 24 ($p<0.01$, $p<0.05$) and 48 hours (both compared to IL-1α stimulated hPDLFs ($p>0.05$) (Figure 4.10a-f).

For hGFs, AdipoRon (40 μM) and leptin (10 μg/ml) did not significantly affect MMP-1 mRNA and protein levels after 24-, 48-, and 72-hour stimulation compared to unstimulated cells. Compressive force increased MMP-1 mRNA and protein levels at all time points, showing significance only at 72 hours (both $p<0.01$) compared to unstimulated cells. Moreover, IL-1α significantly increased MMP-1 mRNA and protein levels at all time points compared to unstimulated cells (Figures 4.9 and 4.10). However, IL-1α-induced MMP-1 mRNA and protein levels were reduced in the presence of AdipoRon at all time points, though without any significance compared to IL-1α stimulated hGFs (Figure 4.9g-l). In contrast, compressive force in the presence of IL-1α increased MMP-1 mRNA and protein levels at all time points but were only statistically significant at 72 hours (6 folds; $p<0.0001$) for MMP-1 mRNA levels, compared to IL-1α stimulated hGFs (Figures 4.9 and 4.10). However, this increase in IL-1α-induced MMP-1 mRNA and protein levels caused by compressive force was suppressed in the presence of AdipoRon in AdipoRon+IL-1α+force stimulated hGFs (Figure 4.9g-l). On the other hand, MMP-1 mRNA and protein levels significantly increased at 24 hours ($p<0.0001$) in leptin+IL-1α stimulated hGFs. In addition, IL-1α-induced MMP-1 mRNA levels were further enhanced in the presence of compressive force and leptin at 24 (50 folds; $p<0.0001$), 48 (6 folds; $p>0.05$), and 72 hours (10 folds; $p<0.001$) compared to IL-1α stimulated hGFs. Whereas MMP-1 protein levels were significantly increased at 24 hours (9 folds; $p<0.0001$) compared to IL-1α stimulated hGFs (Figure 4.10g-l).

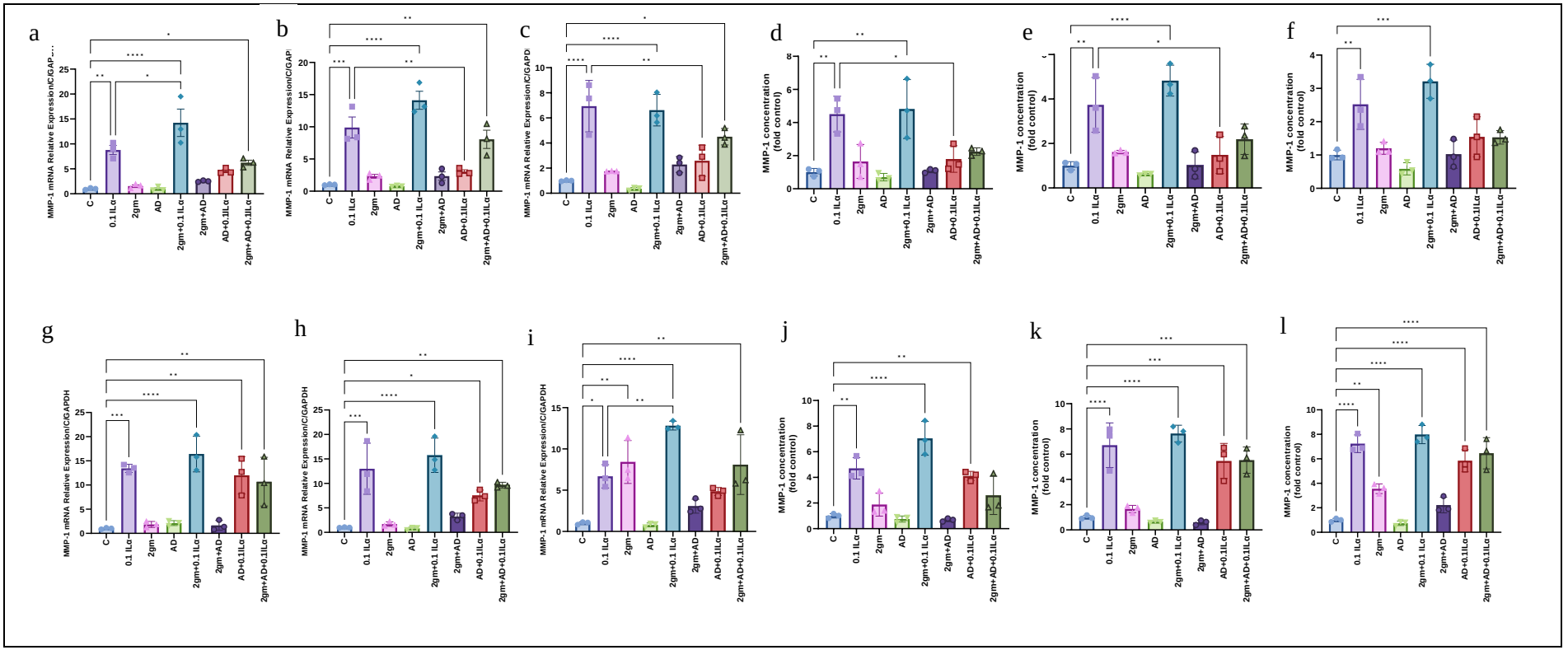

Figure 4.9 Relative MMP-1 mRNA and protein expression in hPDLFs and hGFs.

hPDLFs (a-f) and hGFs (g-l) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and AdipoRon (40 μM) alone or in combination for 24 (a, d, g, j), 48 (b, e, h, k), and 72 (c, f, i, l) hours. MMP-1 mRNA expression was assessed using RT-qPCR for hPDLFs (a-c) and hGFs (g-i). IL-8 protein secretion was assessed using ELISA and measured as fold change relative to the unstimulated cells for hPDLFs (d-f) and hGFs (j-l). C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; AD, AdipoRon; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

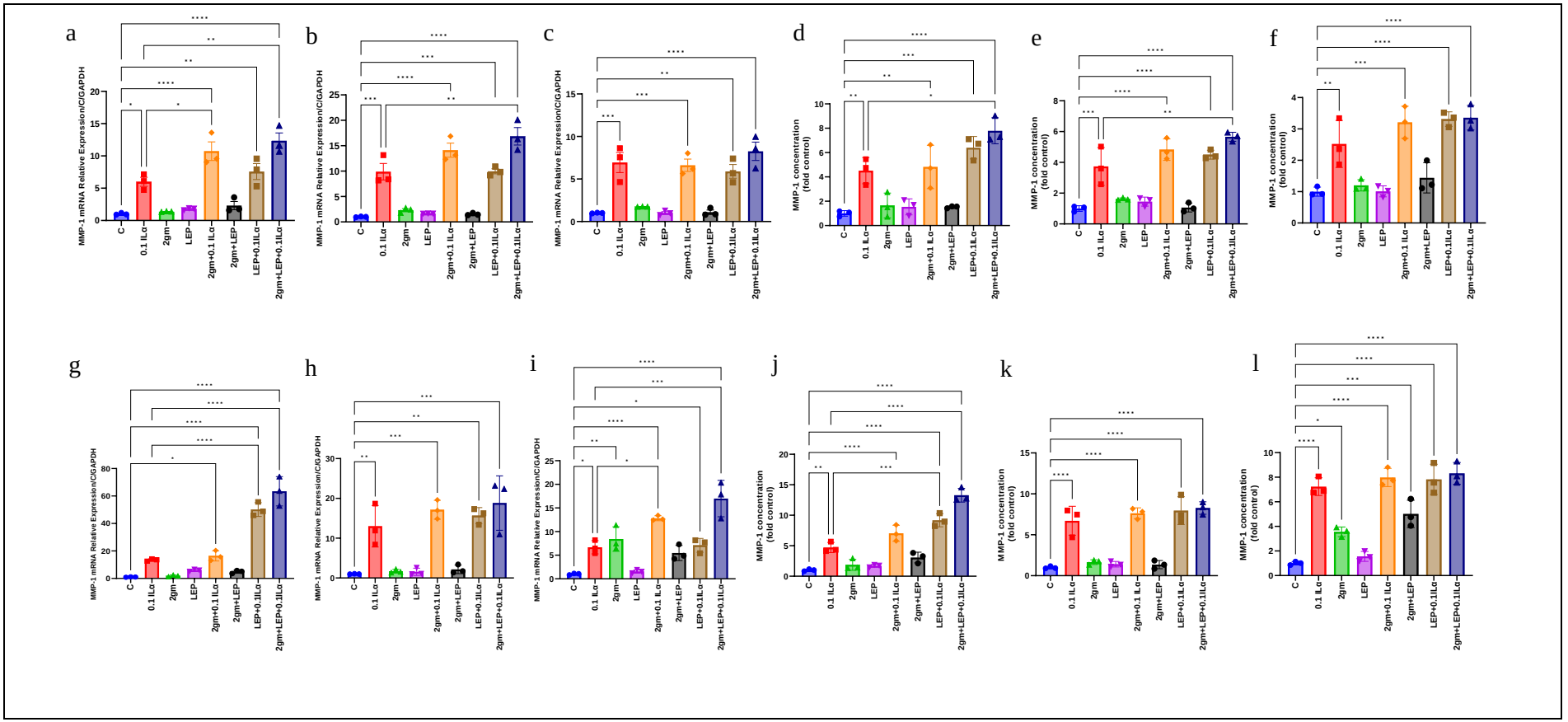

Figure 4.10 Relative MMP-1 mRNA protein expression in hPDLFs and hGFs.

hPDLFs (a-f) and hGFs (g-l) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and leptin (10 µg/ml) alone or in combination for 24 (a, d, g, j), 48 (b, e, h, k), and 72 (c, f, i, l) hours. MMP-1 mRNA expression was assessed using RT-qPCR for hPDLFs (a-c) and hGFs (g-i). IL-8 protein secretion was assessed using ELISA and measured as fold change relative to the unstimulated cells for hPDLFs (d-f) and hGFs (j-l). C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; LEP, leptin; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

4.3.6 MMP-2 expression

MMP-2 gene expression was assessed by RT-qPCR, and even though MMP-2 was expressed constitutively, no significant differences in MMP-2 mRNA levels were detected in both hPDLFs and hGFs stimulated with compressive force (2 gm/cm^2), AdipoRon (40 μM), and leptin alone or in combination at all time points compared to the unstimulated cells. In addition, AdipoRon and leptin had no significant effect on IL-1α-induced MMP-2 mRNA expression in the presence or absence of compressive force at all time points (Appendix Figures 4.4 and 4.5).

4.3.7 MMP-3 expression

MMP-3 mRNA and protein levels were assessed using RT-qPCR and ELISA, respectively. The force (2 gm/cm^2), AdipoRon (40 μM), and leptin (10 μg/ml) did not significantly affect MMP-3 mRNA and protein levels in hPDLFs and hGFs after 24-, 48-, and 72-hour stimulation compared to unstimulated cells ($p>0.05$) (Figures 4.11 and 4.12).

For hPDLFs, IL-1α significantly increased MMP-3 mRNA and protein levels at all time points compared to unstimulated cells. AdipoRon significantly attenuated MMP-3 mRNA and protein levels induced by IL-1α in AdipoRon+IL-1α stimulated hPDLFs at 24 (37.8 and 2 folds, respectively; $p<0.001$, $p<0.05$), 48 (59.3 and 26 folds, respectively; $p<0.05$, $p<0.001$), and 72 hours (231 and 30.3 folds, respectively; $p<0.05$, $p<0.0001$) compared to IL-1α stimulated hPDLFs (Figure 4.11a-f). Conversely, compressive force enhanced IL-1α-induced MMP-3 mRNA levels at 48 and 72 hours; however, without any significance compared to IL-1α stimulated hPDLFs, whereas IL-1α-induced MMP-3 proteins levels were significantly increased at 48 ($p<0.05$) and 72 hours ($p<0.01$) compared to IL-1α stimulated hPDLFs (Figures 4.11 and 4.12). However, this increase in IL-1α-induced MMP-3 mRNA and protein levels caused by compressive force was abolished in the presence of AdipoRon at all time points, showing a significant decrease at 24 hours ($p<0.01$) for MMP-3 mRNA levels and at all time

points for MMP-3 proteins compared to IL-1α stimulated hPDLFs ($p<0.0001$) (Figure 4.11a-f). Leptin increased MMP-3 mRNA and protein levels in leptin+IL-1α stimulated hPDLFs at all time points, though without any significance compared to IL-1α stimulated hPDLFs. Moreover, the presence of compressive force and leptin further enhanced IL-1α-induced MMP-3 mRNA and protein levels at all time points, showing a significant increase at 48 hours (120 and 23 folds, respectively; $p<0.05$) compared to IL-1α stimulated hPDLFs (Figure 4.12a-f).

For hGFs, IL-1α significantly increased MMP-3 mRNA and protein levels at all time points compared to unstimulated cells. However, AdipoRon significantly attenuated IL-1α-induced MMP-3 mRNA and protein levels at 24 (6 and 3 folds, respectively; both $p<0.01$) and 48 hours (46 and 4.4 folds; $p<0.01$, $p<0.05$), compared to IL-1α stimulated hGFs (Figure 4.11g-i). On the contrary, IL-1α-induced MMP-3 mRNA and protein levels were increased in the presence of compressive force at 48 and 72 hours; however, only showing a significant increase at 48 hours ($P<0.05$) for MMP-3 mRNA levels, compared to IL-1α stimulated hGFs. On the other hand, IL-1α-induced MMP-3 mRNA levels were significantly attenuated at 24 (5.3 folds; $p<0.01$) and 48 hours (45 folds; $p<0.001$), whereas MMP-3 protein levels were reduced but without any significance at all time points in Adiporon+IL-1α+force stimulated hGFs compared to IL-1α stimulated hGFs (Figure 4.11g-l). On the contrary, IL-1α-induced MMP-3 mRNA and protein levels were increased at 24 hours in leptin+IL-1α stimulated hGFs compared to IL-1α stimulated hGFs, showing only significance for mRNA levels ($p<0.001$). In addition, IL-1α-induced MMP-3 mRNA levels were increased more in the presence of compressive force and leptin at all time points but were statistically significant at 24 hours (13 folds; $p<0.0001$) compared to IL-1α stimulated hGFs. Likewise, leptin+IL1α+force induced more MMP-3 protein levels at all time points; however, without any significance compared to IL-1α stimulated hGFs (Figure 4.12g-l).

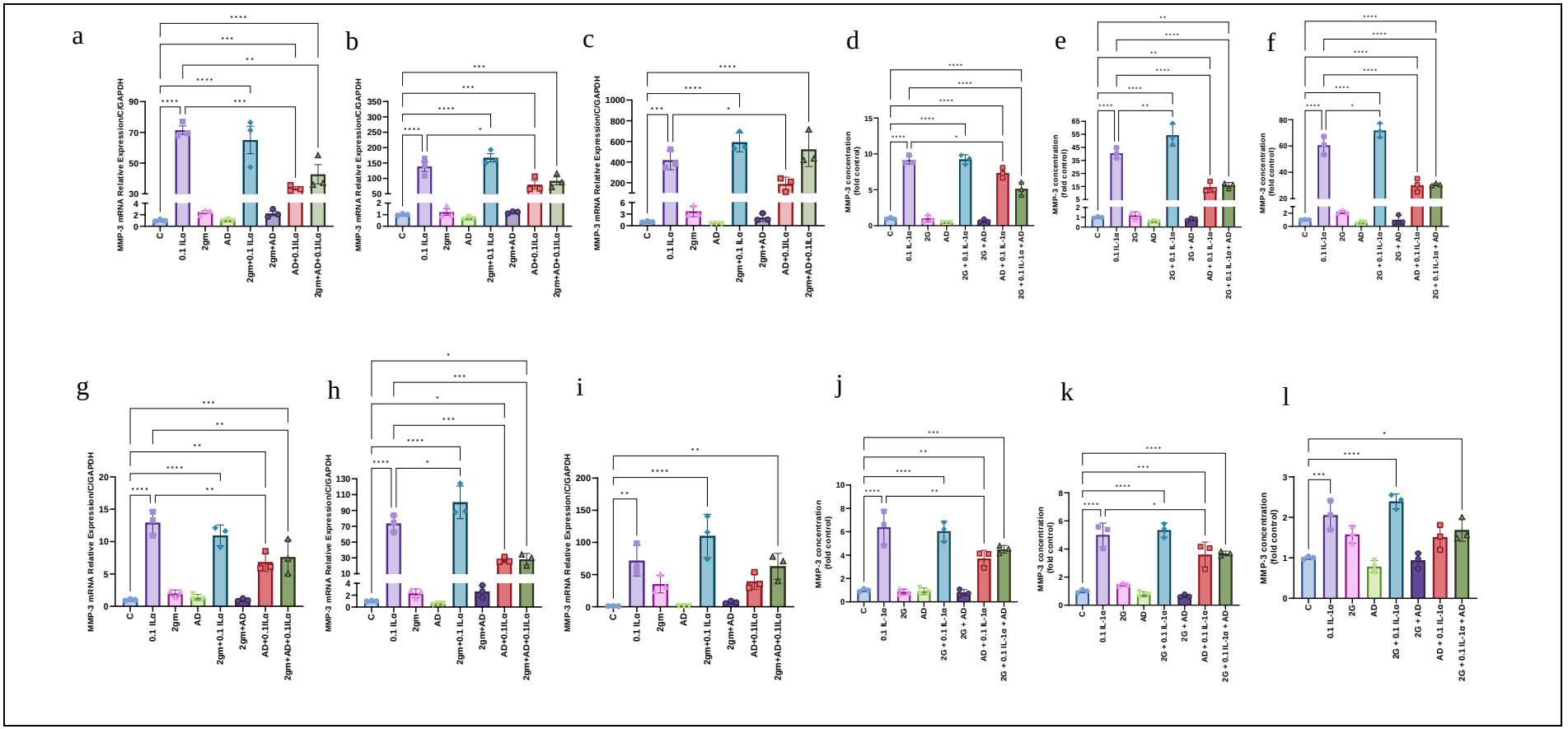

Figure 4.11 Relative MMP-3 mRNA and protein expression in hPDLFs and hGFs.

hPDLFs (a-f) and hGFs (g-l) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and AdipoRon (40 μM) alone or in combination for 24 (a, d, g, j), 48 (b, e, h, k), and 72 (c, f, i, l) hours. MMP-3 mRNA expression was assessed using RT-qPCR for hPDLFs (a-c) and hGFs (g-i). IL-8 protein secretion was assessed using ELISA and measured as fold change relative to the unstimulated cells for hPDLFs (d-f) and hGFs (j-l). C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; AD, AdipoRon; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

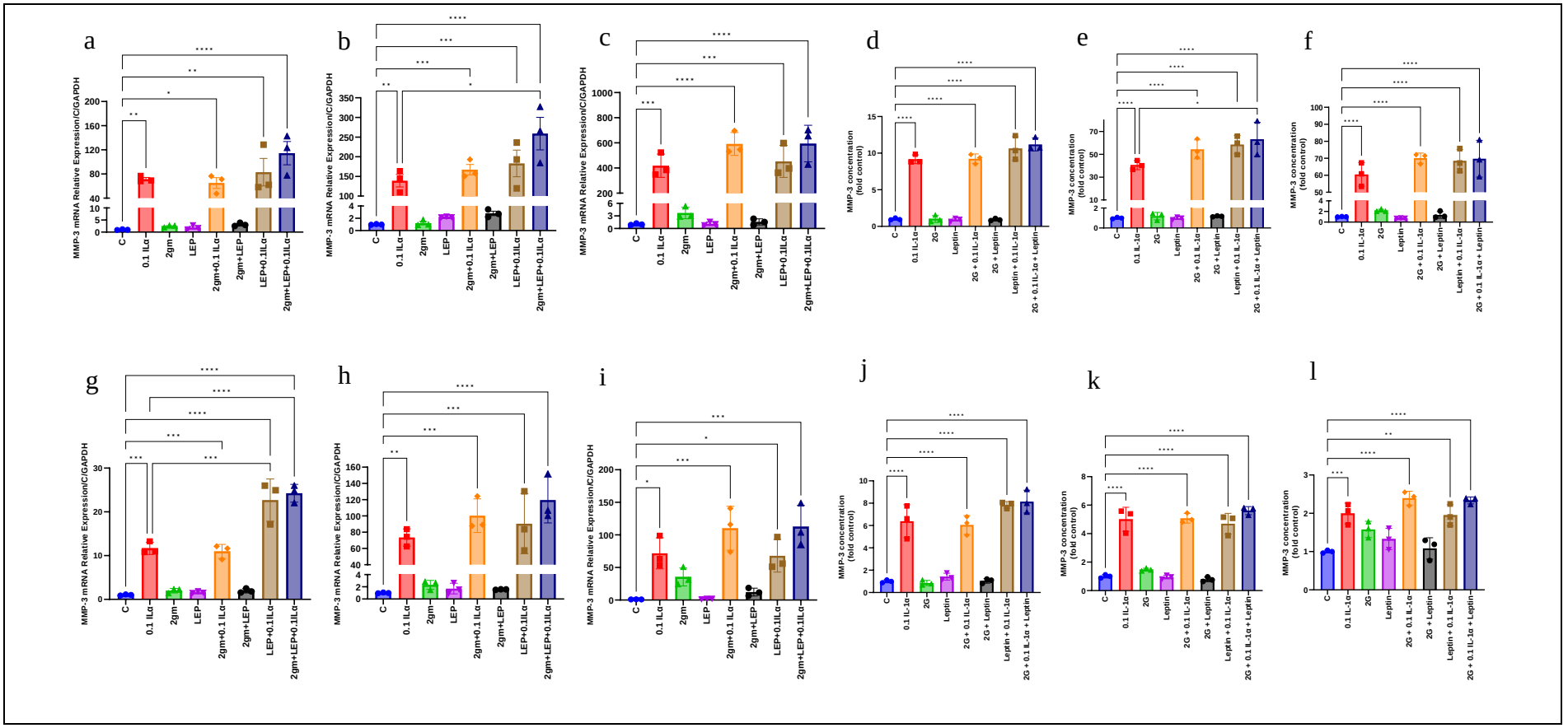

Figure 4.12 MMP-3 mRNA protein expression in hPDLFs and hGFs.

hPDLFs (a-f) and hGFs (g-l) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and leptin (10 µg/ml) alone or in combination for 24 (a, d, g, j), 48 (b, e, h, k), and 72 (c, f, i, l) hours. MMP-3 mRNA expression was assessed using RT-qPCR for hPDLFs (a-c) and hGFs (g-i). IL-8 protein secretion was assessed using ELISA and measured as fold change relative to the unstimulated cells for hPDLFs (d-f) and hGFs (j-l). C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; LEP, leptin; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = p<0.05; ** = p<0.01; *** = p<0.001; **** = p<0.0001.

4.3.8 MMP-8 expression

MMP-8 mRNA levels were assessed using RT-qPCR. AdipoRon and leptin did not significantly affect MMP-8 mRNA levels in hPDLFs and hGFs after 24-, 48-, and 72-hour stimulation compared to unstimulated cells ($p>0.05$) (Figures 4.13 and 4.14).

For hPDLFs, compressive force enhanced MMP-8 mRNA expression at all time points but was significant at 24 hours (3.3 folds; $p<0.01$) when applied alone and at 24 and 48 hours (4.9 and 2.1 folds, respectively; $p<0.05$) in the presence of leptin, compared to unstimulated cells (Figure 4.14). IL-1α significantly increased MMP-8 mRNA levels at 24 hours (2.6 folds; $p<0.05$). IL-1α-induced MMP-8 mRNA levels were attenuated in the presence of AdipoRon at 24 hours ($p<0.05$) compared to IL-1α stimulated hPDLFs. On the other hand, compressive force led to an increase in IL-1α-induced MMP-8 mRNA levels at 24 and 48 hours ($P<0.05$ compared to unstimulated cells). However, this increase in IL-1α-induced MMP-8 mRNA expression caused by compressive force was abolished in the presence of AdipoRon (Figure 4.13a-c). In contrast, leptin enhanced IL-1α-induced MMP-8 mRNA levels at 48 hours. Still, the greatest increase in IL-α-induced MMP-8 mRNA levels was in the presence of compressive force and leptin at 24 ($p<0.05$ compared to IL-1α stimulated hPDLFs) and 48 hours ($p<0.0001$) compared to unstimulated cells (Figure 4.14a-c).

For hGFs, force significantly increased MMP-8 mRNA levels at 24 hours ($p<0.001$) compared to unstimulated cells. IL-α significantly increased MMP-8 mRNA expression at 24, 48 and 72 hours compared to unstimulated cells (Figure 4.13). Compressive force enhanced IL-1α-induced MMP-8 mRNA levels at 24 hours but without significance compared to IL-1α stimulated hGFs. Conversely, AdipoRon attenuated IL-1α-induced MMP-8 mRNA in the absence or presence of compressive force at all time points; though only significant at 48 ($P<0.05$) and 72 hours ($p<0.010$) compared to IL-1α stimulated hGFs (Figure 4.13d-f).

However, leptin enhanced IL-1α-induced MMP-8 mRNA levels at all time points; however, without any significance compared to IL-1α stimulated hGFs. Furthermore, IL-1α-induced MMP-8 mRNA levels were further significantly enhanced in the presence of leptin and compressive force at 24 hours (6 folds; $p<0.0001$) and 48 hours (4.4 folds; $p<0.05$) compared to IL-1α stimulated hGFs (Figure 4.14d-f).

MMP-8 protein was not detected in the supernatants of unstimulated hPDLFs and hGFs or after stimulation with force, AdipoRon, leptin, and IL-1α when assessed using ELISA.

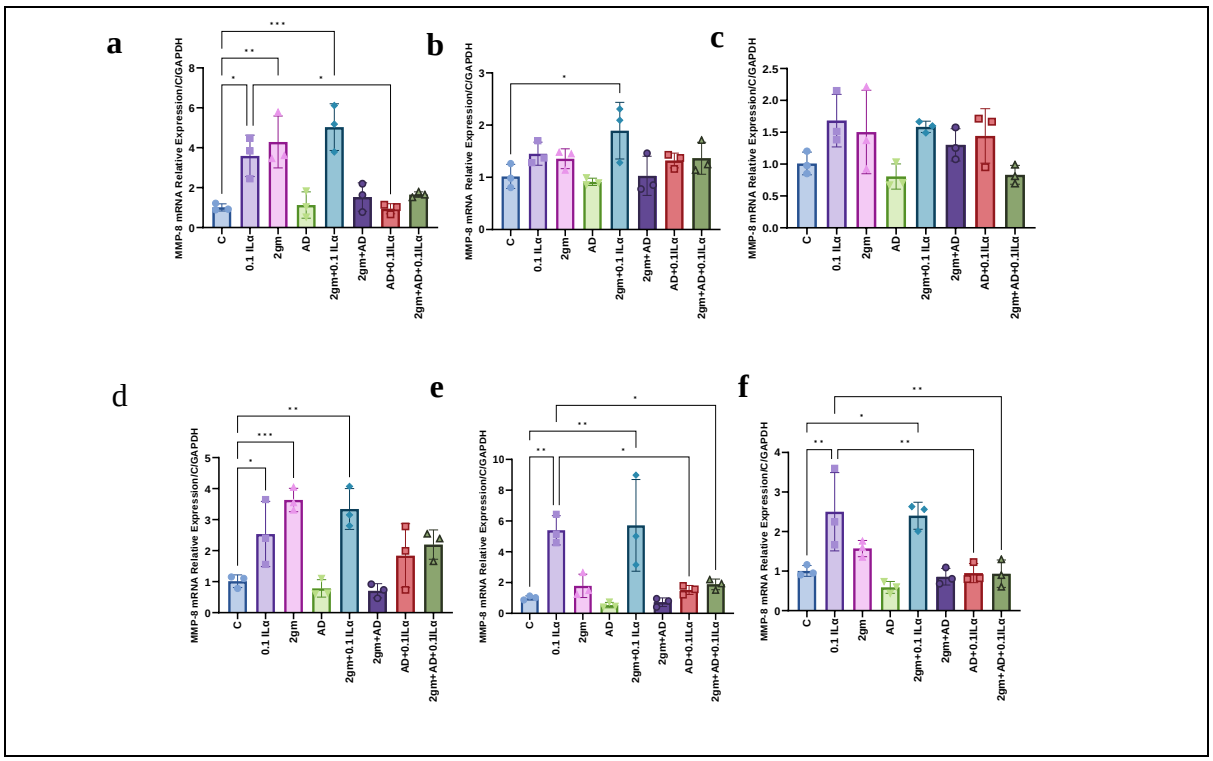

Figure 4.13 Relative MMP-8 mRNA expression in hPDLFs and hGFs.

hPDLFs (a-c) and hGFs (d-f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and AdipoRon (40 µM) alone or in combination for 24 (a & d), 48 (b & e), and 72 (c & f) hours. MMP-8 mRNA expression was measured using the 2^{-ddCt} method using GAPDH as the housekeeping gene and relative to the unstimulated cells. C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; AD, AdipoRon; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

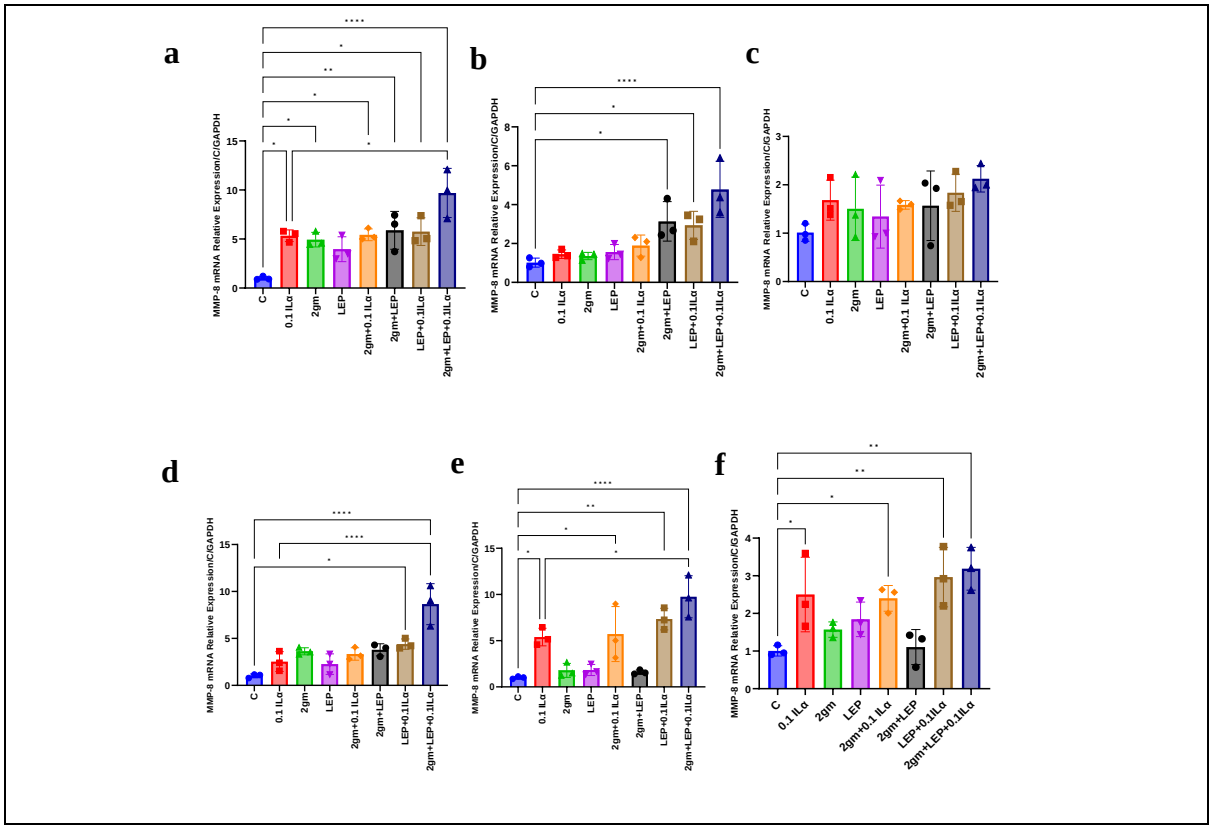

Figure 4.14 MMP-8 mRNA expression in hPDLFs and hGFs.

hPDLFs (a-c) and hGFs (d-f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and leptin (10 µg/ml) alone or in combination for 24 (a & d), 48 (b & e), and 72 (c & f) hours. MMP-8 mRNA expression was measured using the 2^{-ddCt} method using GAPDH as the housekeeping gene and relative to the unstimulated cells. C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; LEP, leptin; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected). * = $p<0.05$; ** = $p<0.01$; **** = $p<0.0001$.

4.3.9 TIMP-1 expression

No significant differences in TIMP-1 mRNA levels were detected in both hPDLFs and hGFs stimulated with compressive force (2 gm/cm^2), AdipoRon (40 µM), and leptin (10 µg/ml) alone or in combination at all time points. In addition, AdipoRon and leptin had no significant effect on IL-1α-induced TIMP-1 mRNA levels in the presence or absence of compressive force at all time points ($p>0.05$) (Appendix Figures 4.6 and 4.7).

4.3.10 Other MMPs

MMP-7 and MMP-9 mRNA expression were not detected in hPDLFs and hGFs basally or after stimulation with force, AdipoRon, leptin, and IL-1α alone or in combination.

4.3.11 Effect on mTOR pathway

HGFs and hPDLFs were stimulated with IL-1α, compressive force, AdipoRon (40 μM), leptin (10 μg/ml) or Torin 1 (200 nM) for 24 hours. Western blotting analysis was used to assess the effect of those stimulants on upstream and downstream proteins of the mTOR pathway by assessing the alteration in the phosphorylation of AKT and 4E-BP1, respectively. In addition, the levels of corresponding total AKT and 4E-BP1, and the internal standard β-actin were assessed in the same samples using specific antibodies for these proteins. PAKT, total AKT, P4E-BP1 and total 4E-BP1 were normalized to β-actin, and the levels of PAKT and P4E-BP1 were calculated using the ratio of PAKT to total AKT and P4E-BP1 to total 4E-BP1, respectively. Torin 1 was used as a positive control, and it abolished the phosphorylation of AKT and 4E-BP1 in hPDLFs and hGFs, indicating inhibition of the mTOR pathway and that the western blotting system was successful.

No change in total AKT and 4E-BP1 was observed in all samples. Compared to the control, the compressive force significantly reduced PAKT and P4E-BP1 levels in hPDLFs and hGFs ($p<0.05$). In addition, this analysis showed that PAKT and P4E-BP1 levels were significantly decreased in hPDLFs and hGFs stimulated with AdipoRon compared to the control ($p<0.05$). On the other hand, both IL-1α and leptin did not affect PAKT and P4E-BP-1 levels in both hPDLFs and hGFs ($p>0.05$) (Figures 4.15 and 4.16). Our findings suggest that the inhibitory effect of compressive force and AdipoRon resembled those of Torin 1, a known inhibitor of both mTORC1 and 2 (Park et al., 2016), indicating that compressive force and AdipoRon may play a role in mTORC1 and 2 inhibitions.

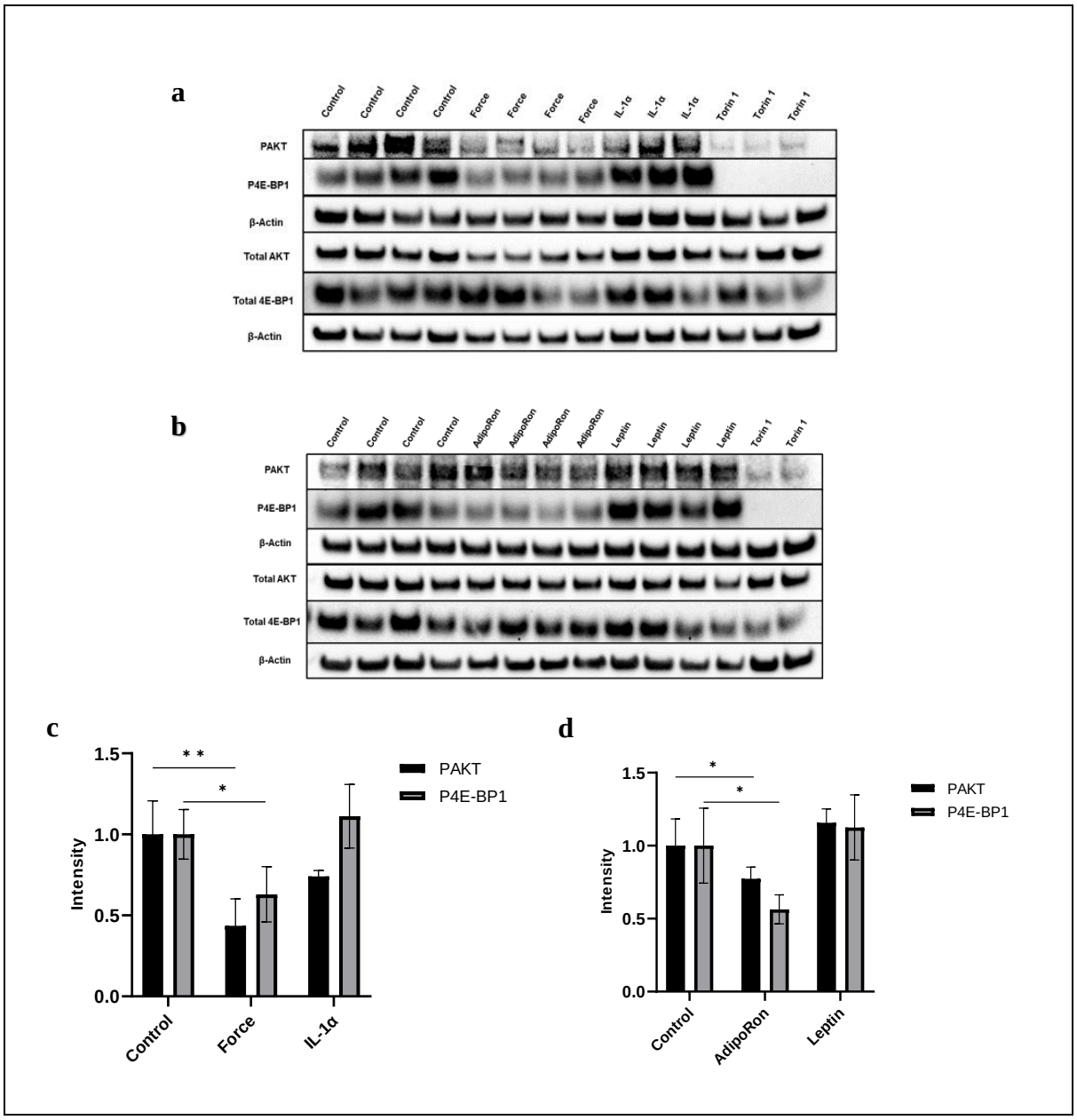

Figure 4.15 Effects of force, IL-1α, AdipoRon, or leptin on PAKT and P4E-BP1 expression in hPDLFs.

hPDLFs were stimulated with IL-1α (0.1 ng/ml) or compressive force (2 gm/cm^2) **(a, c),** AdipoRon (40 μM) or leptin (10 μg/ml) **(b, d)** for 24 hours. The intensity of each protein band was quantified, and each value was calculated as follows: PAKT, total AKT, P4E-BP1, and total 4E-BP1 were normalized to β-actin, and PAKT/β-actin and P4E-BP1/β-actin were divided by total Akt/ β-action and total 4E-BP1, respectively. Data are presented as the fold change relative to the control and expressed as mean ± SD. n=4. One-way ANOVA followed by Dunnett's multiple-comparisons test. * = $p<0.05$; ** = $p<0.01$.

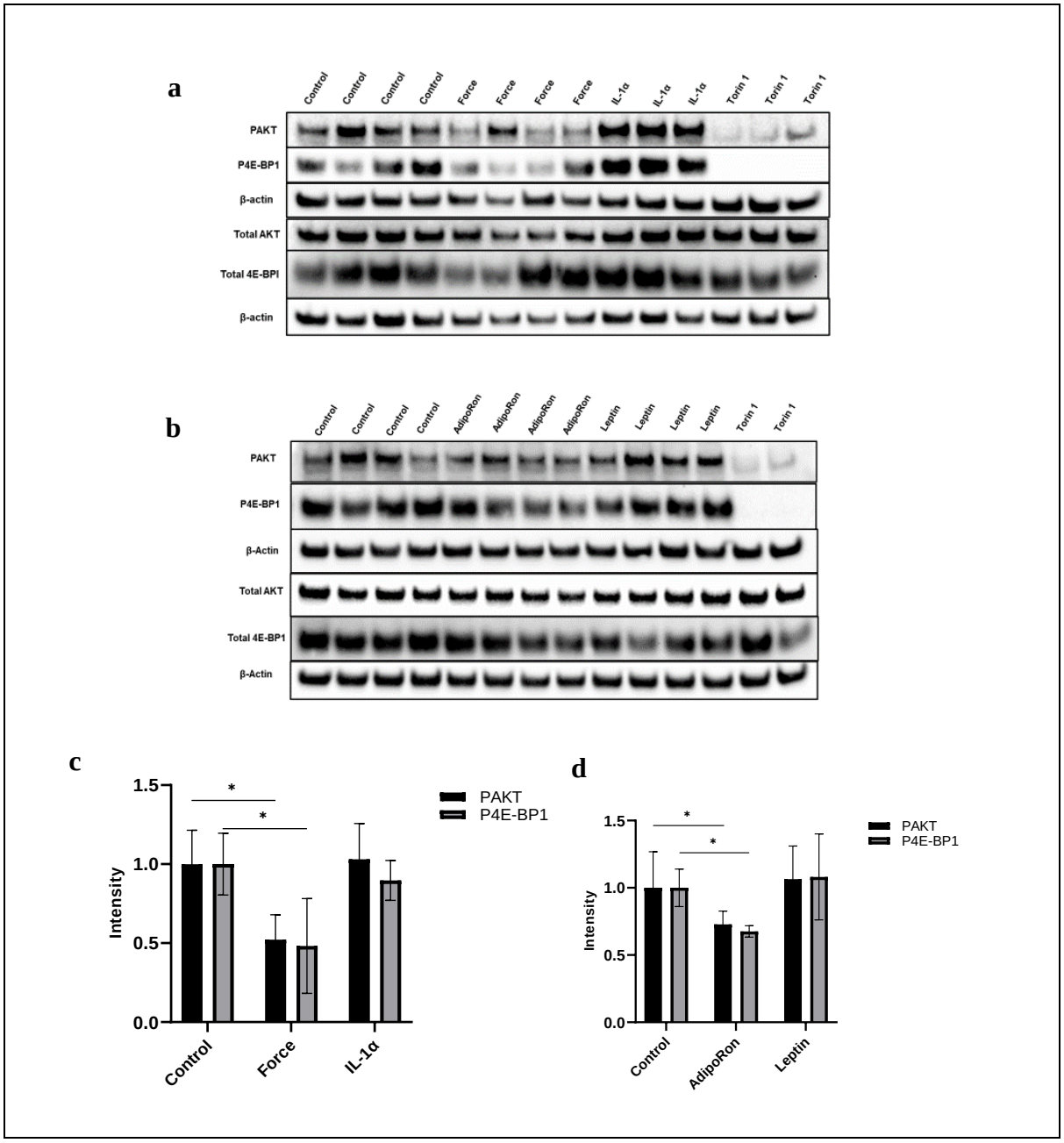

Figure 4.16 Effects of force, IL-1α, AdipoRon, or leptin on PAKT and P4E-BP1 expression in hGFs.

hGFs were stimulated with IL-1α (0.1 ng/ml) or compressive force (2 gm/cm^2) **(a, c),** AdipoRon (40 μM) or leptin (10 μg/ml) **(b, d)** for 24 hours. The intensity of each protein band was quantified, and each value was calculated as follows: PAKT, total AKT, P4E-BP1, and total 4E-BP1 were normalized to β-actin, and PAKT/β-actin and P4E-BP1/β-actin were divided by total Akt/ β-action and total 4E-BP1, consecutively. Data are presented as the fold change relative to the unstimulated cells (control) and expressed as mean ± SD. n=4. One-way ANOVA followed by Dunnett's multiple-comparisons test. * = $p<0.05$.

4.4 Discussion

Obesity is increasing worldwide and is a risk factor for many diseases, including diabetes, cardiovascular diseases, and periodontitis (Guh et al., 2009, Nishida et al., 2005). Adipokines, mainly leptin and adiponectin, contribute to periodontal inflammation and healing, and have been extensively studied because of their vital role in immune responses. Leptin activates the innate and adaptive immune cells to induce a pro-inflammatory effect, and its serum levels are increased in patients with periodontitis (Li et al., 2015). On the other hand, Adiponectin serum levels are decreased, with anti-inflammatory properties (Wang et al., 2021b). Recently, it has been demonstrated in a prospective clinical cohort study that levels of leptin differed significantly between obese and normal weight patients before and during orthodontic treatment, with higher rates of OTM in obese patients (Saloom et al., 2017). There are barely any available data relating these adipokines to OTM; hence, it is crucial to explore their biological effects. To our knowledge, this is the first study to report the effect of simulated static compressive forces and adipokines on IL-1α-induced expression of MMPs in hPDLFs and hGFs. We demonstrated that leptin and AdipoRon enhanced and attenuated, respectively, IL-1α-induced expression of inflammatory and MMPs in compressed hPDLFs and hGFs. This suggests the pro- and ant-inflammatory local effects of leptin and AdipoRon, respectively.

Orthodontic forces induce an aseptic immediate inflammatory reaction, characterised by releasing IL-1 and TNFα as early as 1 hour (Jayaprakash et al., 2019, Gujar et al., 2019, Bletsa et al., 2006), which in turn, induce the release of other inflammatory mediators, such as IL-6, IL-8 and MMPs (Gujar et al., 2019). Hence, it is essential to have a closer look at IL-1-induced expression of inflammatory and ECM remodelling biomarkers. Thus, to simulate these inflammatory conditions in vitro, hPDLFs and hGFs were incubated with IL-1α. To simulate orthodontic forces that the cells are subjected to on the compression side of the tooth during OTM, a compressive force was applied using the weight method. A compressive force

magnitude of 2 gm/cm^2 was used in this study because it is the most used one and has been demonstrated to induce the best cellular responses (Yang et al., 2015a, Janjic et al., 2018, Kang et al., 2010, Mitsuhashi et al., 2011, Roth et al., 2022, Huang et al., 2021); however, forces with higher magnitudes (4 gm/cm^2) caused partial damage and induced death of hPDLFs (Kanzaki et al., 2002, Blawat et al., 2020).

Although several in vitro studies have been performed to find the association between mechanical forces and inflammatory and ECM remodelling biomarkers, inconsistent findings were observed in the literature. Our results showed that compressive force by itself caused a trivial increase in IL-6, IL-8, MMP-1, and MMP-3 expression in hPDLFs; however, this increase was not statistically significant. Similarly, compressive force increased the expression of those biomarkers in HGFs, but with significant increases for IL-8 and MMP-1. Multiple studies reported an increase in IL-6 and IL-8 production in hPDLFs by compressive forces (Brockhaus et al., 2021, Schröder et al., 2021, Schröder et al., 2019, Asano et al., 2011, Phusuntornsakul et al., 2018). However, others reported that IL-6 expression did not change over time in compressed hPDLFs (Grimm et al., 2020) and the paradental tissues of mice after force loading (Li et al., 2021a). Another study reported that compressive forces decreased and increased IL-6 production in healthy and diseased hPDLFs, respectively (El-Awady et al., 2013). Several studies showed that compression of hPDLFs increased MMP-1 and MMP-3 expression (Redlich et al., 2004, Hacopian et al., 2011, El-Awady et al., 2013, Lisboa et al., 2013); however, other studies reported that compression had no effect on MMP-2, and TIMP-1 expression in hPDLFs (Lisboa et al., 2013). Another study reported that compressive forces decreased MMP-1 expression in three-dimensional culture of hGFs (Nan et al., 2019). This inconsistency in the literature regarding the effect of mechanical forces on inflammatory and ECM remodelling biomarkers could be related to differences in force types, force levels, cell

types, cultivation methods, stimulation times, assays used for biochemical analyses, individual variability, and the effects of other variables.

Increased MMP-8 levels in GCF of patients during orthodontic treatment were observed (Ingman et al., 2005, Apajalahti et al., 2003, Zhang et al., 2020). Our findings revealed that compressive force increased MMP-8 expression in hPDLFs and hGFs. This agrees with multiple studies reporting that compressive forces (Nettelhoff et al., 2016, Grimm et al., 2020) and tensile forces (Jacobs et al., 2014, Jacobs et al., 2018) increased MMP-8 production by hPDLFs. However, other studies reported that tensile forces decreased (Saminathan et al., 2012, Ma et al., 2015) or had no effect (Schröder et al., 2020a) on MMP-8 expression in hPDLFs. This indicates that MMP-8 plays a crucial role in ECM remodelling of the periodontium during OTM.

Our data demonstrated that compressive forces enhanced IL-1α-induced expression of IL-6, IL-8, MMP-1, MMP-3, and MMP-8 in hPDLFs and hGFs. This agrees with two previous studies; the first study reported that cyclic compressive forces enhanced IL-1β-induced IL-6 and MMP-8 expression in hPDLFs (Grimm et al., 2020). The second study showed that tensile forces enhanced IL-1β- induced MMP-1 and MMP-2 expression in hPDLFs (Behm et al., 2021a). This indicates that compressive forces alone have a minor role and that the acute aseptic inflammatory reaction induced by orthodontic forces is a prerequisite for OTM and is the pivotal key for ECM remodelling in the periodontium.

Leptin receptors (long and short forms) are expressed in hPDLFs and hGFs (Yun-Jung et al., 2013, Li et al., 2015), indicating that these cells are sensitive and responsive to leptin. Our results demonstrated that leptin had no significant effect on IL-6, IL-8, MMP-1, MMP-3, and MMP-8 in the presence or absence of compressive force in hPDLFs and hGFs (except for MMP-8, whose levels increased by leptin in compressed hPDLFs). These findings disagree with two previous studies reporting that leptin increased IL-6 and IL-8 expression in hPDLFs

and hGFs (Yun-Jung et al., 2013) and MMP-1, MMP-3, and MMP-8 in hGFs (Williams et al., 2016). One study reported that response to leptin is dose-dependent, with high leptin levels enhanced IL-6 expression with compressive force in hPDLFs, but low leptin levels had no effect (Schröder et al., 2021). However, in this specific study, leptin with and without compressive force increased IL-6 expression by 3 and 1 folds, respectively, which was comparable to our results. On the other hand, a recent study reported that leptin reduced IL-1β and IL-6 expression with tensile or compressive forces in macrophages (Paddenberg et al., 2022). These inconsistent results may be due to differences in leptin structure, sources, concentrations, cell types, and cultivation methods. Variability in MMPs expression between individuals has also been described previously in studies of hGFs (Sukkar et al., 2007, Williams et al., 2016); hence, ECM remodelling could be inherently different between donors.

The effect of leptin on the acute inflammatory reaction induced by orthodontic forces within the periodontium and the underlying cellular mechanism is still ambiguous. We found for the first time that leptin synergised with IL-1α and enhanced IL-6, IL-8, MMP-1, MMP3, and MMP-8 in hPDLFs and hGFs. This is consistent with the findings of previous studies, which reported that leptin synergised with IL-1α to increase MMP-1 and MMP-3, and MMP-8 production in chondrocytes and human osteoarthritic cartilage (Hui et al., 2012, Koskinen et al., 2011), and hGFs (Williams et al., 2016). This suggests that leptin with compressive force substantially enhances ECM remodelling during OTM under inflammatory conditions by promoting the production of cytokines and MMPs, which might explain the reported increased OTM rates observed in obese patients with higher leptin levels compared to normal-weight patients (Saloom et al., 2017).

There is very limited evidence available relating to the effect of adiponectin or its analogues on OTM. Adiponectin receptors, AdipoR1 and AdipoR2, are expressed constitutively in hPDLFs and hGFs and exert anti-inflammatory effects (Iwayama et al., 2012,

Wang et al., 2021b). Our results showed that AdipoRon had no significant effect on the expression of the inflammatory and ECM biomarkers studied in the presence or absence of compressive force. This finding is consistent with a previous study, which reported that globular adiponectin did not change IL-6 and IL-8 expression in hPDLFs and hGFs (Park et al., 2011). Still, it contradicts other studies reporting decreased production of proinflammatory biomarkers in diseased gingival tissues by AdipoRon (Wu et al., 2019) and rheumatoid arthritis synovial fibroblasts by adiponectin (Lee et al., 2008).

Additionally, we found that AdipoRon attenuated IL-1α-induced expression of IL-6, IL-8, MMP-1, MMP-3, and MMP-8 in the presence or absence of compressive force, indicating an anti-inflammatory effect. This agrees with previous studies which reported that Adiponectin reduced IL-6 and IL-8 expression in IL-1β-stimulated hGFs (Iwayama et al., 2012), decreased LPS-induced IL-1β, IL-6, IL-8, MMP-1, and MMP-3 expression in human gingival epithelial cells (Kraus et al., 2012), and attenuated LPS-induced inflammatory biomarkers production in hPDLFs (Wu et al., 2021). From the above, it can be postulated that adiponectin might influence OTM by supressing the inflammation associated with it, and this might explain the finding of a previous study that revealed reduced rates of OTM in rates after local injection of adiponectin (Haugen et al., 2017).

MMP-7 and MMP-9 were not detected by RT-qPCR basally or after stimulation with any other stimulant in our study. This is consistent with previous studies that reported no detection of these MMPs in hPDLFs and hGFs (Williams et al., 2016, Zhou and Windsor, 2006, Bolcato-Bellemin et al., 2000). This could be explained by the fact that MMP-7 is mainly produced by epithelial cells (Nagase et al., 2006, Löffek et al., 2011), whereas MMP-9 is primarily produced by neutrophils (Restaíno et al., 2007) or gingival keratinocytes (Mäkelä et al., 1994), rather than fibroblasts.

Western blotting analysis was used to measure the phosphorylation of key upstream and downstream regulatory proteins of the mTOR signalling pathway to investigate whether this signalling pathway is affected by the compressive force or adipokines. Our findings suggest an impact of compressive force and AdipoRon on the mTOR signalling pathway manifested by downregulating the expression of phosphorylated AKT and 4EPB1 proteins. However, leptin and inflammatory stimulation had no significant effect on this pathway.

Our results showed that the compressive force decreased phosphorylated levels of AKT and 4E-BP1 proteins in hPDLFs and hGFs. This was consistent with a previous study which reported that stimulation hPDLFs with 2 gm/cm^2 compressive force led to a decrease in the phosphorylated levels of 4E-BP1 and AKT; however, overloading hPDLFs by 4 and 8 gm/cm^2 compressive forces increased their phosphorylated levels (Blawat et al., 2020). In addition, another study reported that PI3K/AKT signalling pathway was significantly affected in hPDLFs subjected to 2 gm/cm^2 compressive force and that phosphorylated AKT was significantly decreased (Huang et al., 2021). On the other hand, this contradicts the finding of another study that found an increase in the phosphorylated levels of AKT in human periodontal tissues during OTM (Xu et al., 2017). However, those investigators measured levels of phosphorylated AKT in human periodontal tissue from extracted premolars after three days of orthodontic force application, which might explain the differences in the results.

mTOR signalling pathway is a vital pathway that integrates a variety of environmental signals to regulate cell growth, metabolism, haemostasis, proliferation, differentiation, protein synthesis, and autophagy (Wang et al., 2019, Laplante and Sabatini, 2012). It has been reported that moderate stress intensity can suppress mTOR activity to promote protective responses and facilitate a faster adaptation to stress, whilst abnormally increased mTOR activity during stress can be harmful to the cell (Aramburu et al., 2014). Several mechanisms inactivate mTORC1 kinase activity to maintain haemostasis and induce autophagy to enable stress adaptation and

survival (Rabinowitz and White, 2010). The two studies mentioned above (Blawat et al., 2020, Huang et al., 2021) that reported that 2 g/cm^2 reduced phosphorylated AKT demonstrated that this compressive force exerted a protective effect and activated autophagy. Hence, autophagy is considered a protective biological mechanism degrading organelles, cytoplasm, and proteins. Autophagy (a crucial process during OTM) is activated by compression and hypoxia (Chen et al., 2019, Li et al., 2021b, Memmert et al., 2019) and inhibited by mTORC1 activity (Jiang et al., 2022). Moreover, compressive forces increased autophagy in hPDLFs (Huang et al., 2021, Chen et al., 2019) and cementoblasts (Liu et al., 2019). A previous study demonstrated that orthodontic loading of mouse molars increased autophagy and inflammatory biomarkers during OTM. Moreover, those investigators administered rapamycin (an autophagy inducer and mTORC1 inhibitor) to the orthodontically loaded molars and found that OTM was reduced and autophagy was activated, implying that it may decrease inflammation during OTM (Li et al., 2021a). As a result, our findings suggest an influence of the 2 gm/cm^2 compressive force on the mTOR signalling pathway, which may be through inducing autophagy to protect cells and induce haemostasis. However, overloading the cells with higher magnitudes of forces might activate MTORC1 and inhibit autophagy leading to uncontrolled inflammatory responses and ECM remodelling of the periodontium.

mTOR signalling pathway is important for the modulation of innate and adaptive immunity (Thomson et al., 2009, Jiang et al., 2022), and dysregulation is linked to autoimmune disorders (Yang et al., 2015b). Adiponectin showed cytoprotective effects and was found to inhibit mTOR (Choi et al., 2020); also, AdipoRon was found to inhibit mTOR (Bhat et al., 2020, Pal China et al., 2018) and exhibit anti-inflammatory effects (Bhat et al., 2020). This is consistent with our findings, which demonstrated that AdipoRon inhibited the mTOR signalling pathway, and is also supported by our RT-qPCR and ELISA results, which showed that AdipoRon had an anti-inflammatory effect. It has been reported that AdipoRon could be a

potentially promising therapy to treat periodontitis associated with diabetes via regulating hyperglycaemia, repressing inflammation, improving bone regeneration, and inhibiting bone loss (Wu et al., 2019); it may also be beneficial for tooth stabilization and relapse prevention after orthodontic treatment.

Our results demonstrated that IL-1α did not affect phosphorylated AKT and 4E-BP1 levels. This result disagrees with a previous study, which reported reduced and increased phosphorylated AKT levels with low and high concentrations of IL-1β, respectively. This specific study also reported the same trend with 4E-BP1, suggesting that the effect on mTOR is dose-dependent (Blawat et al., 2020), which could explain the difference between that study and ours; thus, the concentration of the inflammatory mediator in our study was not enough to activate or inactivate the mTOR signalling pathway.

mTOR has been shown to play important roles in leptin signalling to regulate inflammation. Leptin induced phosphorylation of 4E-BP1, and was found to activate the mTOR signalling pathway in macrophages (Maya-Monteiro and Bozza, 2008). Our results showed leptin caused an increase in phosphorylated AKT and 4E-BP1 levels after 24 hours of stimulation; however, this increase was not significant. Another study demonstrated that phosphorylation of AKT increased at 15 minutes after leptin stimulation in hPDLFs, then decreased at 30 and 60 minutes (Yun-Jung et al., 2013). This inconsistency could be related to different concentrations, duration of stimulation, and cell types.

Our results in this study showed a significant decrease in cell viability in hPDLFs and hGFs subjected to compressive force, consistent with multiple studies (Kang et al., 2010, Kanjanamekanant et al., 2013, Ullrich et al., 2019, Schröder et al., 2019). On the other hand, our LDH cytotoxicity assay results confirmed no cytotoxic effect of the 2 gm/cm^2 compressive force. This agrees with a previous study demonstrating that a compressive force of 2 g/cm^2 did

not cause cell death but exerted a protective effect, but 4 and 8 gm/cm^2 compressive forces caused cell death in hPDLFs. (Blawat et al., 2020)

Gingival tissue has a fundamental role in OTM and has been reported to be modified clinically and histologically during OTM (Redlich et al., 1999). Both hPDLFs and hGFs play a vital role in OTM by the secretion of inflammatory and ECM remodelling mediators; therefore, both cell lines were investigated in this study. hPDLFs and hGFs are sensitive to forces and respond to mechanical stresses and regulate ECM remodelling during OTM (Jiang et al., 2016, Krishnan and Davidovitch, 2009). Our results demonstrated that both hPDLFs and hGFs reacted similarly to all stimulants regarding the expression of the inflammatory cytokines and MMPs, albeit at different levels, apart from the significant effect of compressive force on IL-8 and MMP-1 expression in hGFs. These results agree with a previous study showing that hPDLFs and hGFs reacted to mechanical stretching by inducing a similar pattern of MMPs and TIMPs expression (Bolcato-Bellemin et al., 2000). The same results were also observed in another study showing a similar response of both hPDLFs and hGFs to leptin regarding IL-6, IL-8, and leptin receptors expression (Yun-Jung et al., 2013). A previous study reported substantial heterogeneity between different gingival fibroblast cell lines, with one cell line expressing hPDLFs-related marker genes and exhibiting hPDLFs-like properties. This specific paper explains these differences by indicating that the anatomical site from which the cells were derived might impact their characteristics (Garna et al., 2022). Moreover, it is worth noting that in cell culture, each cell type works on its own and responds separately to any stimulant; however, in vivo, it is a complex process with many interacting signals and factors affecting how each cell type responds to mechanical forces or any other stimulants; thus, this could have influenced their responses as well.

4.5 Conclusions

The results of this chapter demonstrated that leptin showed pro-inflammatory properties by selectively enhancing IL-1α-induced expression of inflammatory and ECM biomarkers in compressed hPDLFs and hGFs. Whereas AdipoRon exhibited anti-inflammatory properties by attenuating these biomarkers under similar conditions. This suggests that the extent of periodontal and gingival fibroblast-mediated ECM remodelling during OTM depends, in part, on the combination of compressive forces and adipokines in the presence of inflammation. Moreover, compressive forces and AdipoRon had regulatory effects on crucial target proteins of mTOR signalling pathway.

Overall, compression modulates inflammatory pathways, which can be further affected by adipokines. This response could be a mechanistic link between OTM and obesity.

Chapter 5 Salivary peptidome analysis and protease prediction during orthodontic treatment with fixed appliances: a retrospective study

5.1 Introduction

Whole mouth saliva (WMS) is a complex fluid that comprises secretions from the major and minor salivary glands, constituents from GCF, oral microbiome, and contains abundant proteins, peptides, and enzymes. Saliva bathes the oral hard and soft tissues, and its contents are responsible for vital functions, including food taste and digestion, along with protecting the oral mucosa from pathogenic bacteria to keep a healthy oral environment (Carpenter, 2013). Saliva collection is simple, easy, safe, convenient, and non-invasive, making it an ideal diagnostic bio-medium and a great alternative to other bodily fluids for research purposes. Recently, proteomic/peptidomic non-targeted approaches have become a recent focus in research and have shown that saliva analysis can detect the presence or absence of various biomarkers, which can serve as possible indicators for the early detection, progression monitoring, or response to many oral and systemic disorders treatments (Zhang et al., 2012, Pappa et al., 2021, Pfaffe et al., 2011, Castagnola et al., 2011).

Multiple enzymes have been identified in the WMS, including carbonic anhydrase, amylase, catalase, and proteases (Castagnola et al., 2011). More than 500 proteases are encoded in the human genome, making them the largest family of proteins. Proteases, which can be classified according to their mode of action into aspartic, glutamic, metalloproteases, cysteine, serine, and threonine proteases, are involved in all biological processes such as cell cycle progression, cell proliferation, differentiation, migration, morphogenesis, tissue remodelling, wound healing, angiogenesis, and apoptosis (Mulkern et al., 2020, Magalhães et al., 2018).

Proteases regulate the initiation, progression, and resolution of inflammation and ECM remodelling (Marshall et al., 2017),

There has been a tremendous interest in understanding the role of proteases during OTM. Therefore, several studies using targeted approaches have evaluated the presence or activity of specific proteases during orthodontic treatment. It has been reported that cathepsins and matrix metalloproteinases (MMPs) are involved in ECM remodelling of the alveolar bone and PDL during OTM (Li et al., 2018, Henneman et al., 2008, Waddington and Embery, 2001). Among MMPs, MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, and MMP-14 levels have all been found to increase in GCF during orthodontic treatment (Garlet et al., 2007, Bildt et al., 2009, Zhang et al., 2020, Apajalahti et al., 2003, Behm et al., 2021b, Cantarella et al., 2006). Furthermore, salivary MMP-8, MMP-9, and MMP-12 concentrations are increased during OTM (Xu et al., 2020).

WMS comprises many low molecular weight proteins, the salivary peptidome, which accounts for roughly 40-50% of total secreted proteins, as well as peptides produced by proteolysis of proteins from various sources. Proteases in saliva modulate several salivary proteins' structure and function, resulting in low molecular weight proteins and peptides. Hence, the characterisation of the salivary peptidome and identification of salivary proteases are crucial steps toward a molecular understanding of the oral microenvironment homeostasis and proteolysis-linked inflammatory disorders, allowing for the invention of molecular tools for disease diagnosis, precise prognosis, and follow-up on existing disease therapy. Therefore, attempts have been made to characterise the salivary peptidome, identifying approximately 2000 peptides, only 400-600 of which are produced from salivary glands, implying a considerable qualitative peptide input from other sources. Earlier research on saliva has shown that most peptides belong to the proline-rich proteins (PRPs) family, the histatin family, and statherin (Trindade et al., 2015a, Cabras et al., 2014, Amado et al., 2010).

Proteolysis processes are the primary source of peptides, and significant efforts have been undertaken to identify the resultant fragments, cleavage sites, and implicated proteases. A peptidomic approach, by mass spectrometry and bioinformatics, has been used to investigate the profile of naturally occurring peptides derived from proteins in several biological fluids, including urine, saliva, cerebrospinal fluid, wound fluid, and serum. In silico prediction of proteases using the open-source software- Proteasix (www.proteasix.org) has been conducted to predict proteases potentially implicated in the generation of these peptides in patients with periodontitis (Trindade et al., 2015b), wound infection (Hartman et al., 2021), cardiorenal syndrome (Petra et al., 2021), and diabetic nephropathy (Krochmal et al., 2017). Proteasix uses information about naturally occurring peptides (corresponding protein UniProt ID and start/stop amino acid position) as identified by mass spectrometry, to predict potential cleaving proteases; it retrieves information about cleavage sites from protease databases (MEROPS, BRENDA), resulting in a generated list of predicted proteases (Klein et al., 2013). The analysis of cleavage site specificity by Proteasix software can predict the likelihood of a large set of endogenous proteases being responsible for the generation of salivary peptides, and the obtained peptidome-protease profile can be beneficial to elucidate the peptidome dynamics and the proteolytic events underlying physiological and pathological processes taking place within the oral cavity (Trindade et al., 2018, Amado et al., 2010).

To the best of our knowledge, the natural peptidome generated in saliva during OTM has not previously been investigated. Hence, this retrospective longitudinal study aimed to use the peptidomic approach, supplemented by mass spectrometry and bioinformatics to predict the profile and activity pattern of proteases responsible for salivary peptide generation, identify susceptible protein targets, and to assess time-dependent changes in the salivary peptidome and predicted proteases during the alignment stage of orthodontic treatment with fixed appliances.

Moreover, targeted approaches using zymography and ELISA were used to validate the prediction results.

5.2 Materials and methods

5.2.1 Study design and participants

This retrospective longitudinal study assessed WMS derived from 16 participants during the alignment phase of orthodontic treatment with fixed appliances. Participants fulfilled the following inclusion criteria: underwent fixed appliance orthodontic treatment (with or without tooth extractions); 12-18 years old at treatment start; mandibular arch incisor irregularity of 4-12 mm; medically fit and healthy; taking no prescription medication and normal-weight body mass index.

Ethical approval of this project was obtained from the United Kingdom National Research Ethics Service, NRES Committee foundation (14/LO/0769), and written informed consent was obtained from all parents, guardians, and children before collecting the samples. All methods were conducted following the approved guidelines and regulations. Fixed appliances (Victory-APC 0.022-inch brackets, MBT prescription; 3M-Unitek) were placed for all participants at the Department of Orthodontics, Faculty of Dentistry, Oral & Craniofacial Sciences, King's College London (Guy's and St Thomas; NHS Foundation Trust). A particular archwire sequence was followed (0.014-inch nickel-titanium; 0.018-inch nickel-titanium; 0.017 x 0.025-inch nickel titanium and 0.019 x 0.025-inch stainless steel). Participants were reviewed every six weeks and were followed up to completion of alignment between January 2015 and June 2016.

The sample size was calculated based on a previous study that looked at time-dependent changes in MMP-8 and MMP-9 levels in saliva during orthodontic treatment. Differences in

the levels of those salivary biomarkers between time points were identified in this investigation, with a mean effect size of 0.87 (Sioustis et al., 2021). Using G*Power 3.1.9.7 software (Faul et al., 2007), a sample size of 13 was estimated to be sufficient to detect a significant difference in salivary biomarker levels between the different time points (assuming a significance level of 0.05 and power of 0.80). However, to compensate for power underestimation between the biomarkers, a sample size of 16 was used.

5.2.2 Saliva collection and processing

Unstimulated WMS was collected at four time points: (T1) start of treatment; (T2) 1 hour and (T3) 1 week following fixed appliance placement; and (T4) end of alignment stage (0.019 x 0.025-inch stainless steel rectangular archwire placed in the lower arch). Participants were asked to passively drool in a sterile plastic tube for 5 minutes. Samples were centrifuged at 9200 g for 5 minutes, aliquoted into small tubes (1000 µl capacity), labelled, and stored at – 80 °C. Samples were defrosted on ice, and total protein concentration was measured using Thermo Scientific™ Pierce™ Bicinchoninic Acid (BCA) Protein Assay (Thermo Scientific, USA) according to the manufacturer's protocol as detailed in section (4.2.3) in Chapter 4 of this thesis.

For all participants, plaque levels and gingival health were measured at T1, T3, and T4 using established validated plaque and gingival indices. The thickness of dental plaque adjacent to the gingival margin of the tooth was measured using Silness and Löe criteria (Silness and Loe, 1964), which assigns a score from 0 to 3 to each of the four surfaces of the tooth; these scores were then added and divided by four to yield the plaque index of the tooth. The plaque index for the individual was then computed by summing the scores from all teeth examined

and dividing them by the number of teeth examined. The gingival index was calculated using the same approach following Löe and Silness criteria (Löe and Silness, 1963).

5.2.3 Separation of naturally occurring peptides from saliva

Naturally occurring peptides were collected from WMS samples of 5 participants at each time point (20 samples). Ten kDa cut-off Spin filters (Amicon; Merck- Millipore, UK) were washed and conditioned with 500 µL of ammonium bicarbonate (10 mM) solution by centrifugation at 4000 rpm, for 25 min. Afterward, spin filters were loaded with 1 ml saliva and centrifuged at 4000 rpm for 25 minutes; then, the resulting filtrate peptides were collected and sent for mass spectrometry analysis (Cambridge Institute for Medical Research Proteomics Centre, UK).

5.2.4 Mass spectrometry

100 µL of salivary peptide sample was dried down using a Savant SpeedVac Concentrator (Thermo Scientific). The dried-down sample was solubilized in a 50 µL loading solvent containing 3% acetonitrile (MeCN) and 0.1% trifluoroacetic acid and 1 µL was analyzed by LC-MS/MS using a Q Exactive Plus coupled to an RSLCnano3000 (Thermo Scientific). Peptides were resolved on a 50 cm EASY-spray column (Thermo Scientific) using a gradient rising from 3 to 40 % solvent B (80 % MeCN, 0.1 % formic acid) by 90 minutes. MS spectra were acquired at 70,000 (fwhm) between m/z 400 to 1500. The resulting filtrate peptides were analyzed by LC-MS/MS using a Q Exactive Plus coupled to an RSLCnano3000 (Thermo Scientific). Peptides were resolved on a 50 cm EASY-spray column (Thermo Scientific) using a gradient rising from 10 to 40 % solvent B (80 % MeCN, 0.1 % formic acid) by 42 minutes. The S-lens FR level was set to 50.0. MS spectra were acquired at 70,000 (fwhm) between m/z 200 to 2000. Data were processed using PEAKS Studio (version X, Bioinformatics Solutions Inc.) with the following measures: no enzyme; Human database (UniProt reference proteome

downloaded on 18 Dec 2018 containing 21066 proteins) or bacterial database (Uniprot proteome downloaded 5 Apr 2019 containing 161286 proteins) with further contaminant database (containing 246 common contaminants); oxidation (M), carbamidomethylation (C) as variable modifications at the PEAKS DB stage, LFQ was performed using PEAKS LFQ using normalization by total protein intensity. Protein and protein-peptide data were exported from PEAKS Studio.

5.2.5 Zymography

Samples were analysed using 10% zymogram gelatin gels electrophoresis (Novex, Life Technologies, UK). Equal amounts of non-reducing Tris-Glycine SDS sample buffer (2X) (Novex, Life Technologies, UK), and samples (10µg) were loaded into the wells of the gel, and the gel was run at 125 volts constant for 90 minutes with 10X Tris-Glycine SDS running buffer (100 ml 10X Tris-Glycine SDS running buffer to 900 ml deionized water) (Novex, Life Technologies, UK). After that, the gel was placed in zymogram renaturing buffer (Novex, Life Technologies, UK) for 30 minutes. The gel was then incubated in zymogram developing buffer (Novex, Life Technologies, UK) for another 30 minutes, which was later replaced with a fresh developing buffer, and the gel was incubated overnight at room temperature. Then, the gel was stained with Coomassie Brilliant Blue and de-stained. Protease digestion appeared as clear bands against a darkly stained blue background. Zymogram gel was scanned with ChemiDoc™ MP Imaging System (Bio-Rad, UK). Densitometric analysis was carried out with Image J (Schneider et al., 2012).

5.2.6 Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) (DuoSet ELISA, R&D systems) was used to assess total human MMP-8 (DY908) and MMP-9 (DY911-05) (active and pro-active forms) according to the manufacturer's instructions following the methodology described in section (4.2.6) in Chapter 4 of this thesis. The blank and standard concentrations mean readings were used to generate a four-parameter logistic (4-PL) curve fit (Appendix Figure 4.1).

5.2.7 Bioinformatic analysis

Protease prediction was performed using the Proteasix tool in function of the peptides identified by mass spectrometry. Proteasix is an open-source peptide-centric tool that can be used to predict in silico the proteases involved in generating naturally occurring peptides and returns all possible proteases at a cleavage site. It is available online at http://www.proteasix.org (Klein et al., 2013). Briefly, a spreadsheet was created with four columns from the list of peptides identified by mass spectrometry (peptide ID, protein accession number, position of the start amino acid, and position of the end amino acid). On the Proteasix website (http://www.proteasix.org), the input list was pasted from the spreadsheet into the appropriate section, and the analysis was then run until detailed results were produced. Thereafter, the whole output list was selected and pasted into an Excel spreadsheet where the number of cleavage events was counted using the Excel function "COUNTIF". Then the percentage of cleavage from the total cleavage events was calculated (Trindade et al., 2018) (Figure 5.1).

Jvenn (http://bioinfo.genotoul.fr/jvenn/example.html) is an online Venn diagram tool used to find unique proteins at each time point and those common to T1, T2, T3, and T4.

5.2.8 Statistical analysis

Data were checked for normality using Shapiro-Wilk and Kolmogorov–Smirnov normality tests. Repeated measures ANOVA was used to analyse the normally distributed data, followed by correction for multiple testing using Dunnett's multiple comparisons test. The Friedman test was used to analyse the not normally distributed data, followed by correction for multiple testing using Bonferroni correction. Regression analysis was performed of the outcome (MMP-8 and MMP-9 levels), the explorative factor (gingival index or plaque index) and the variation across time points. Statistical analyses were conducted using GraphPad Prism version 9.0 (GraphPad Software, USA). The difference was considered statistically significant if $p < 0.05$.

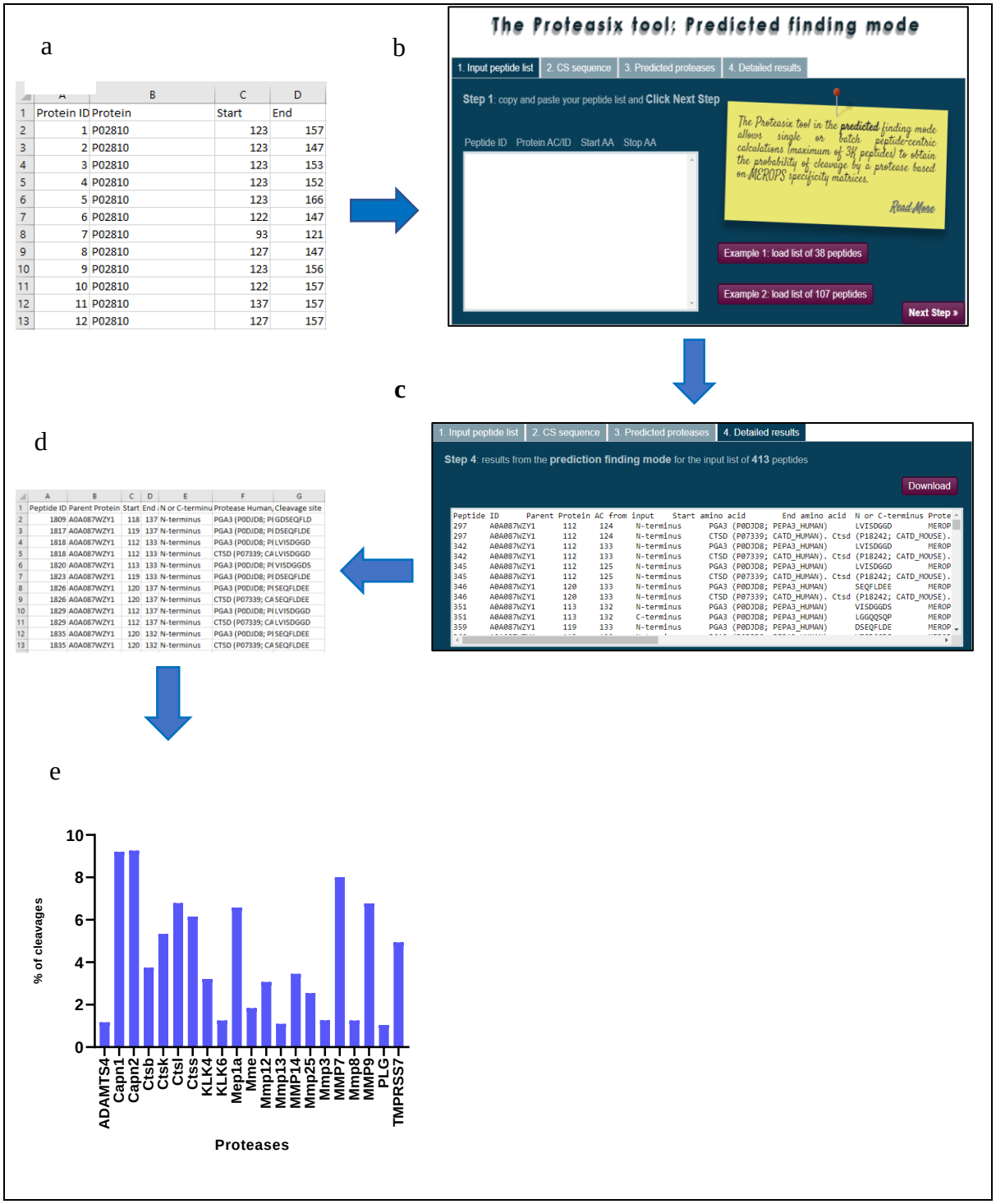

Figure 5.1 Representative example of the main steps of Proteasix analysis.

A spreadsheet was created with four columns from the list of detected peptides (peptide ID, protein accession number, position of the start amino acid, and position of the end amino acid) (a). On the Proteasix website (http://www.proteasix.org), the input list was pasted from the spreadsheet into the appropriate section (b). The analysis was then run until detailed results were produced (c); after that whole output list was selected and pasted into an Excel spreadsheet (d). The number of cleavage events was counted using the Excel function "COUNTIF", then the percentage of cleavage from the total cleavage events was calculated and presented in a bar chart using GraphPad Prism software (e).

5.3 Results

This study evaluated WMS from 16 participants (10 male, 6 female) with a mean (SD) age of 15.2 (1.6) years and a mean (SD) irregularity of 6.4 (2.2) mm. Mean (SD) plaque index was: 0.57 (0.3) at T1; 0.76 (0.32) at T3; and 1.09 (0.40) at T4. Mean (SD) gingival index was: 0.73 (0.31) at T1; 0.84 (0.22) at T3; and 1.25 (0.35) at T4. Plaque and gingival indices were increased significantly at T4 compared to T1 ($P<0.001$). Mean (SD) WMS flow rate (ml/min) was 0.67 (0.29) at T1; 0.85 (0.37) at T2; 1.03 (0.45) at T3; and 0.93 (0.37) at T4. Furthermore, mean (SD) protein content (mg/ml) was: 1.51 (0.48) at T1; 1.55 (0.34) at T2; 1.65 (0.68) at T3; and 1.14 (0.48) at T4. No significant differences were detected in either measurement between the time points.

5.3.1 Peptidome characteristics

Identifying which protein precursors the peptides belong to may reveal interesting aspects of the saliva proteome and proteolytic dynamics during OTM. Overall, 2852 naturally occurring peptides were identified by mass spectrometry, originating from 436 different proteins with 49 common to all time points, as depicted in the Venn diagram (Figure 5.2a). The percentage of peptides identified for each common protein was calculated, list of proteins and the percentages of peptides for the top 15 common protein precursors are presented in Figure 5.2b (and Appendix Table 5.1). The most abundant peptides belonged to the major salivary proteins, mainly proline-rich proteins, statherin, histatins, and P-B peptide. When the percentages of peptides identified for each common protein were compared over time, no significant changes were found for PIGR, PRP1, PRB2, PRB3, PRBC, SMR3B, and HIS1 at all time points of orthodontic alignment. Significant degradation of STAT, PROL4, CO1A1, and CO2A1 were observed at T2 compared with baseline levels (T1) (all $p <0.01$), and then this degradation

returned to the baseline levels at T4. Besides, peptides that belong to PRR27 were significantly increased at T2 and T3 (both $p<0.05$), returning to baseline levels at T4. On the contrary, the percentages of peptides that belong to PRB4 were significantly decreased at T2 ($p<0.01$.), returning to T1 levels by T4. The percentages of peptides that belong to HIS3 were significantly decreased at both T2 ($p<0.01$.) and T3 ($p<0.05$) (Figure 5.3).

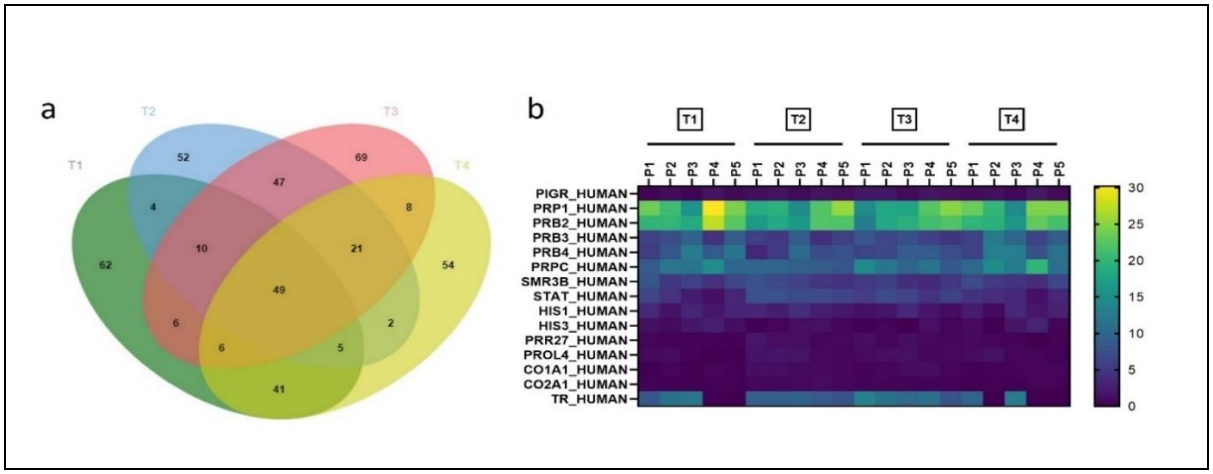

Figure 5.2 Venn diagram and heatmap of proteins of origin.

(a) Venn diagram showing the distribution of proteins of origin of the identified peptides by mass spectrometry at four time-points. T1, baseline (before placement of orthodontic appliance); T2, one hour after placement of orthodontic appliance; T3, one week after placement of orthodontic appliance; T4, end of the alignment. (b) Heatmap displaying the percentages of peptides for the most abundant common proteins among the participants (p1, p2, p3, p4, and p5) at four time-points. The scale refers to the percentages of peptides of each protein in the total number of peptides for each participant.

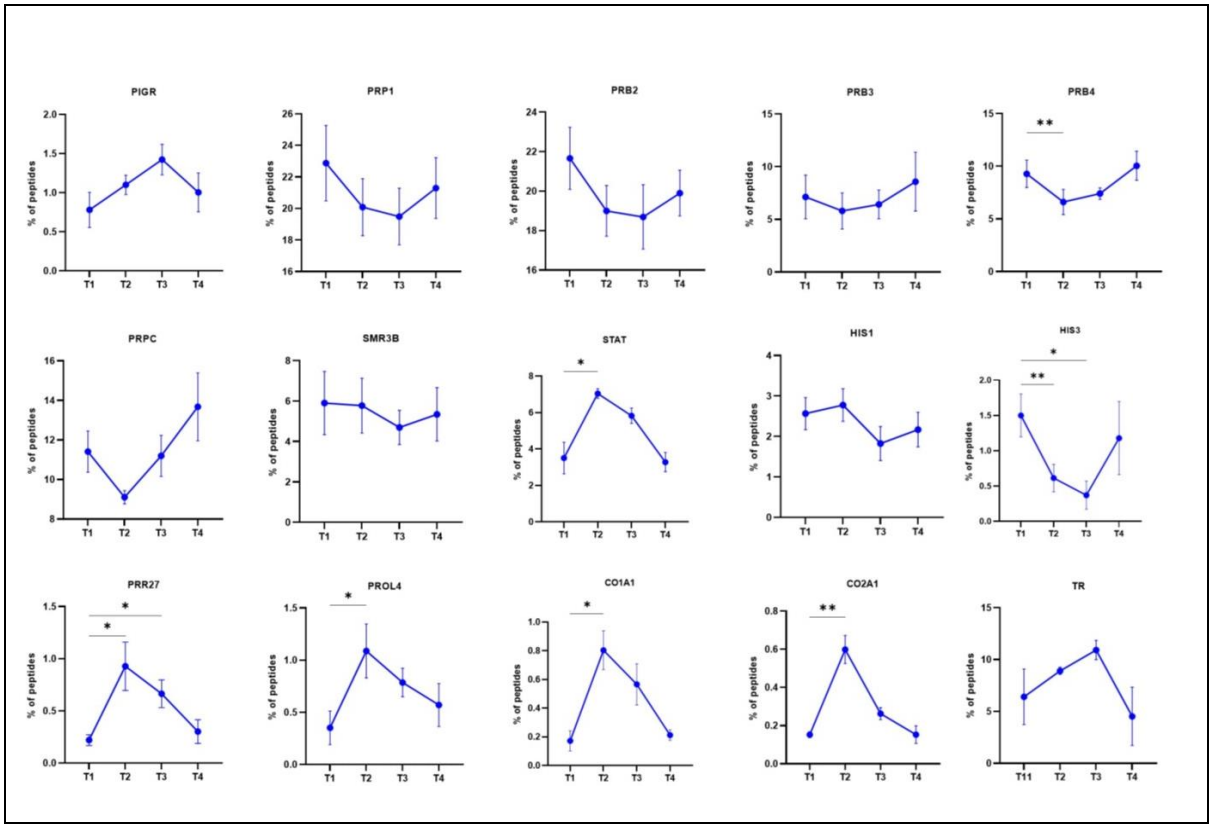

Figure 5.3 Graphs showing the percentage of peptides for each of the common proteins identified in unstimulated WMS at T1-T4.

T1, baseline (before placement of fixed appliances); T2, 1 hour after placement of fixed appliances; T3, 1 week after placement of fixed appliances; T4, completion of alignment. Data were analysed by repeated measures ANOVA; * = $p<0.05$; ** = $p<0.01$.

5.3.2 Prediction of protease activity

The profile of all proteases, as predicted by Proteasix, is shown in Figure 5.4a. In total, 73 proteases were predicted to be active in the WMS of the participants, and a list of the predicted proteases (symbols, accession numbers, and names) is shown in Table 5.1. For each protease, the percentage of cleavage from the total cleavages events for each participant was calculated, and a percentage threshold of cleavage was set at 1% to consider the activity of a particular protease, as shown in Figure 5.4 (Petra et al., 2021). Twenty-four proteases had a percentage threshold of cleavage above 1%; among these calpains, metalloproteinases and cathepsins were

the most prevalent groups of proteases potentially implicated in the generation of salivary peptides at all time points.

When the predicted activity of these proteases was compared over time, there was a significant increase in the predicted activity of CTSG (cathepsin G) ($p<0.05$, $p<0.05$), ELANE (neutrophil elastase) ($p<0.001$, $p<0.01$), MMP3 ($p<0.01$, $p<0.05$), MMP8 ($p<0.001$, $p<0.01$), MMP13 ($p<0.01$, $p<0.05$), PGA3 (pepsin) ($p<0.01$, $p<0.05$) at both T2 and T3 compared to T1. The predicted activity of MME (neprilysin) ($p<0.05$), MMP-9 ($p<0.05$), and MMP-25 ($p<0.05$) was significantly increased at T2, while MMP-12 ($p<0.05$) predicted activity was significantly increased at T4 compared to T1 (Figure 5.4b). Conversely, the predicted activity of CAPN1 ($p<0.001$, $p<0.001$), CAPN2 ($p<0.001$, $p<0.001$), CTSK (cathepsin K) ($p<0.01$, $p<0.05$), MEP1A ($p<0.01$, $p<0.05$), and TMPRSS7 ($p<0.01$, $p<0.05$) was significantly decreased at both T2 and T3 compared to the baseline levels. Additionally, the predicted activity of MMP-7 ($p<0.01$) and KLK4 (kallikrein 4) ($p<0.05$) was significantly decreased at T3 and T2. No changes in the predicted activity level of ADAMTS4, CTSB (cathepsin B), CTSL (cathepsin L), CTSS (cathepsin S), KLK6 (kallikrein 4), MMP14, PLG (plasminogen) were observed at any time points of orthodontic alignment.

To search for proteases identified by peptides in the LC-MS/MS data, "VLOOKUP" function in Excel and the UniProt accession numbers were used to find them. Only two of the 73 proteases predicted by Proteasix were identified in the LC-MS/MS data, which are Transmembrane protease serine 11D (TMPRSS11D; O60235) and Prothrombin (F2; P00734).

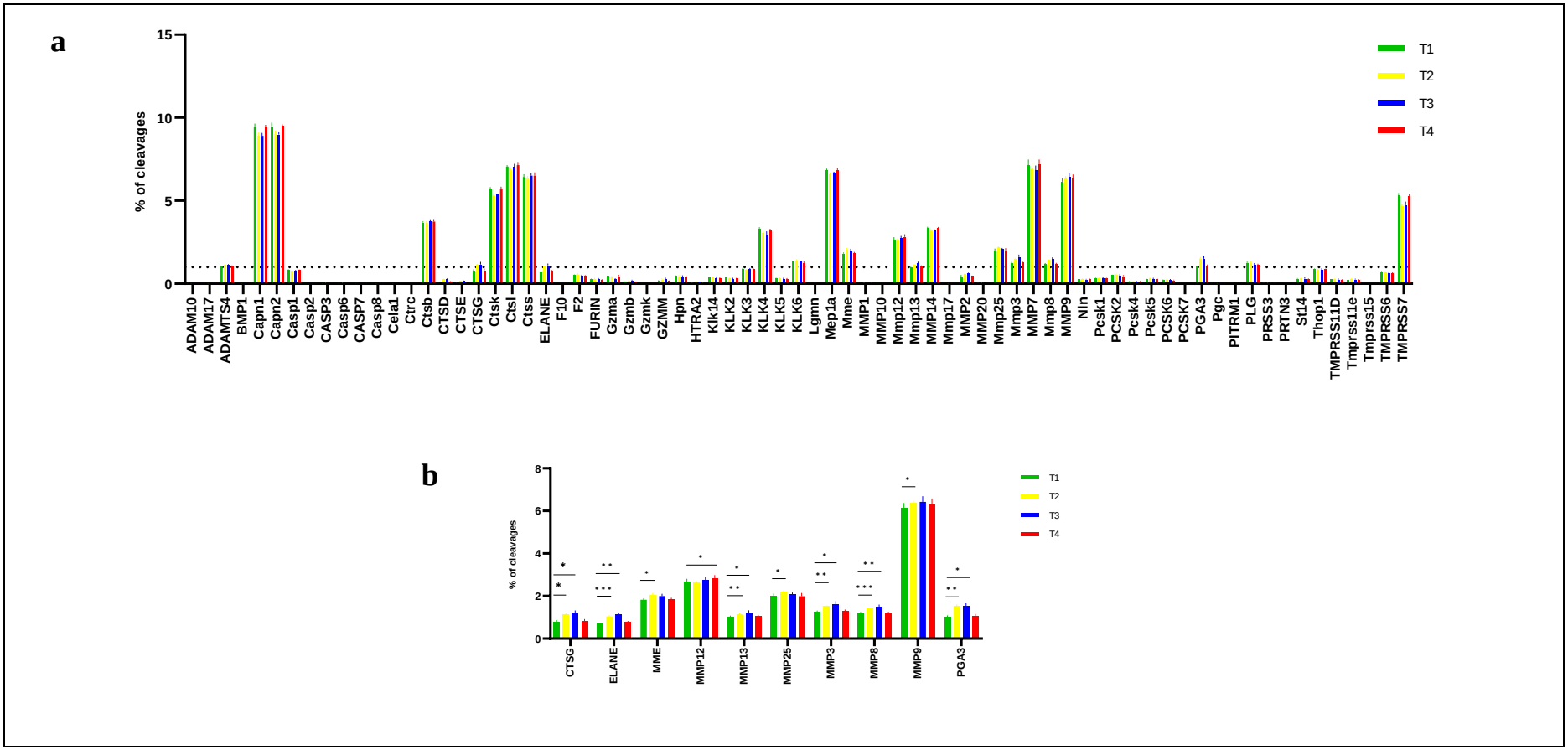

Figure 5.4 Graph showing the profile of proteases as predicted by Proteasix.

(a) Graph showing the profile of all proteases as predicted by Proteasix. The bars represent the percentage of cleavages for each predicted protease at four time-points. T1, baseline; T2, one hour after placement of orthodontic appliance; T3, one week after placement of orthodontic appliance; T4, end of the alignment. The interrupted line represents a percentage threshold (1%) to consider the activity of the proteases. **(b)** The predicted activity of ten proteases significantly increased during orthodontic treatment. Data are shown as mean ± SD. Data were analysed by repeated measures ANOVA; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

Table 5.1 List of all proteases (symbols, accession numbers, and names) predicted by Proteasix.

Protease symbol	Accession number	Protease name
ADAM10	O14672	Disintegrin and metalloproteinase domain-containing protein 10
ADAM17	P78536	Disintegrin and metalloproteinase domain-containing protein 17
ADAMTS4	O75173	A disintegrin and metalloproteinase with thrombospondin motifs 4
BMP1	P13497	Bone morphogenetic protein 1
CAPN1	P07384	Calpain-1 catalytic subunit
CAPN2	P17655	Calpain-2 catalytic subunit
CASP1	P29466	Caspase-1
CASP2	P42575	Caspase-2
CASP3	P42574	Caspase-3
CASP6	P55212	Caspase-6
CASP7	P55210	Caspase-7
CASP8	Q14790	Caspase-8
CELA1	Q9UNI1	Chymotrypsin-like elastase family member 1
CTRC	Q99895	Chymotrypsin-C
CTSB	P07858	Cathepsin B
CTSD	P07339	Cathepsin D
CTSE	P14091	Cathepsin E
CTSG	P08311	Cathepsin G
CTSK	P43235	Cathepsin K
CTSL	P07711	Cathepsin L1
CTSS	P25774	Cathepsin S
ELANE	P08246	Neutrophil elastase
F10	P00742	Coagulation factor X
F2	P00734	Prothrombin
FURIN	P09958	Furin
GZMA	P12544	Granzyme A
GZMB	P10144	Granzyme B
GZMK	P49863	Granzyme K
GZMM	P51124	Granzyme M
HPN	P05981	Serine protease hepsin

HTRA2	O43464	Serine protease HTRA2, mitochondrial
KLK14	Q9P0G3	Kallikrein-14
KLK2	P20151	Kallikrein-2
KLK3	P07288	Prostate-specific antigen
KLK4	Q9Y5K2	Kallikrein-4
KLK5	Q9Y337	Kallikrein-5
KLK6	Q92876	Kallikrein-6
LGMN	Q99538	Legumain
MEP1A	Q16819	Meprin A subunit alpha
MME	P08473	Neprilysin
MMP1	P03956	Interstitial collagenase
MMP10	P09238	Stromelysin-2
MMP12	P39900	Macrophage metalloelastase
MMP13	P45452	Collagenase 3
MMP14	P50281	Matrix metalloproteinase-14
MMP17	Q9ULZ9	Matrix metalloproteinase-17
MMP2	P08253	72 kDa type IV collagenase
MMP20	O60882	Matrix metalloproteinase-20
MMP25	Q9NPA2	Matrix metalloproteinase-25
MMP3	P08254	Stromelysin-1
MMP7	P09237	Matrilysins
MMP8	P22894	Neutrophil collagenase
MMP9	P14780	Matrix metalloproteinase 9
NLN	Q9BYT8	Neurolysin, mitochondrial
PCSK1	P29120	Neuroendocrine convertase 1
PCSK2	P16519	Neuroendocrine convertase 2
PCSK4	Q6UW60	Proprotein convertase subtilisin/kexin type 4
PCSK5	Q92824	Proprotein convertase subtilisin/kexin type 5
PCSK6	P29122	Proprotein convertase subtilisin/kexin type 6
PCSK7	Q16549	Proprotein convertase subtilisin/kexin type 7
PGA3	P0DJD8	Pepsin A-3
PGC	P20142	Gastricsin

PITRM1	Q5JRX3	Presequence protease, mitochondrial
PLG	P00747	Plasminogen
PRSS3	P35030	Trypsin-3
PRTN3	P24158	Myeloblastin
ST14	Q9Y5Y6	Suppressor of tumorigenicity 14 protein
THOP1	P52888	Thimet oligopeptidase
TMPRSS11D	O60235	Transmembrane protease serine 11D
TMPRSS11E	Q9UL52	Transmembrane protease serine 11E
TMPRSS15	P98073	Enteropeptidase
TMPRSS6	Q8IU80	Transmembrane protease serine 6
TMPRSS7	Q7RTY8	Transmembrane protease serine 7

5.3.3 Targeted approaches to validate proteases predictions

WMS from all 16 participants were used with ELISA to directly assess protease abundance and with gelatin zymography to assess gelatinolytic activity. Three distinct clear bands were identified in all samples at approximately 190 kDa (band 1), 72 kDa (band 2), and 62 kDa (band 3) (Figure 5.5a). Gelatinolytic activity increased over time and returned to the baseline levels at T4, being around 1.8 times more elevated at T3 than T1 for band 2 ($P<0.05$) and 1.4 times more elevated at T3 than T1 for band 3 ($p<0.05$) (Figure 5.5b-d).

Results of ELISA demonstrated that MMP-8 levels increased over time but were only significant at T3 and T4 compared with baseline levels (T1) ($P<0.001$; $P<0.05$, respectively) (Figure 5.6a). In addition, MMP-9 levels were significantly increased at T3 compared with baseline levels (T1) ($P<0.01$) (Figure 5.6b).

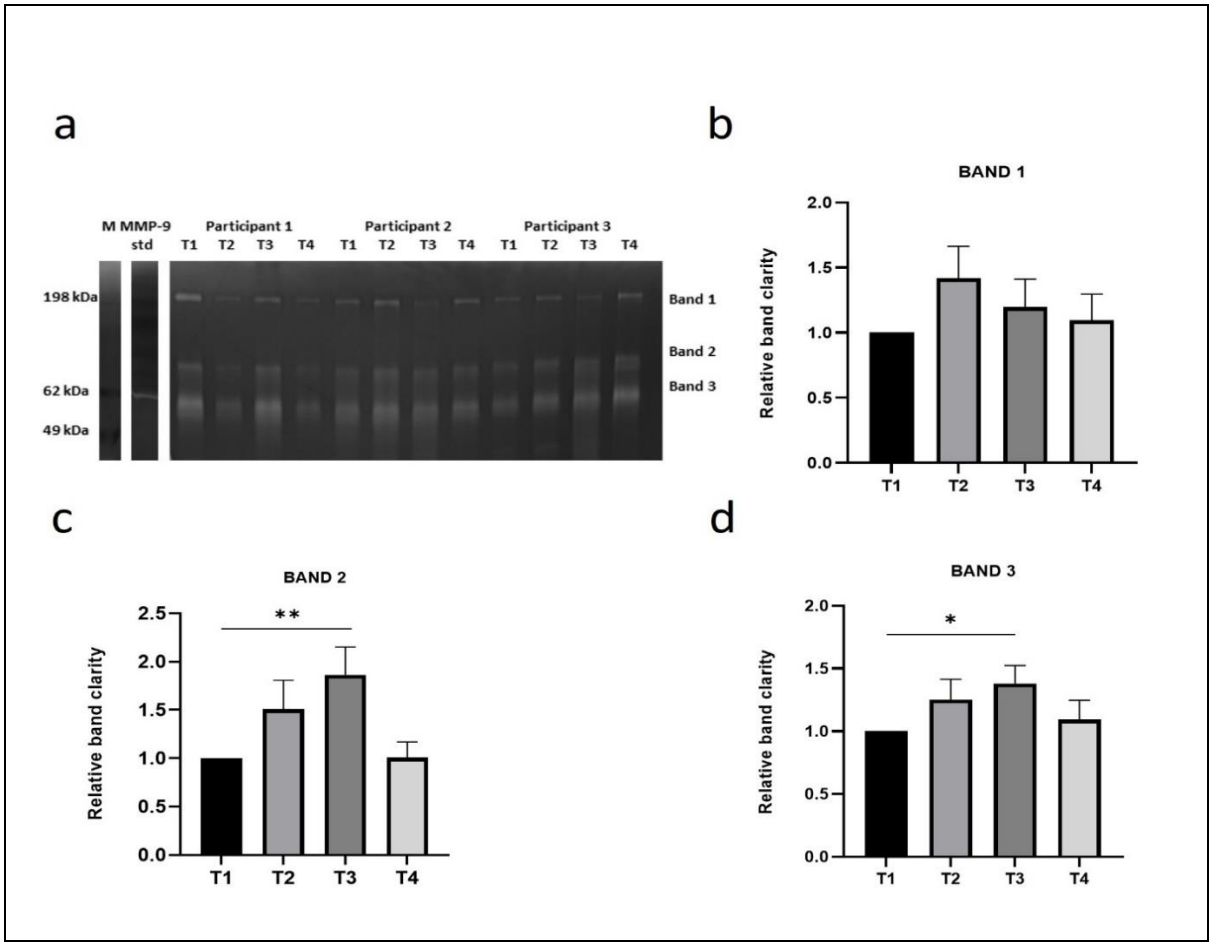

Figure 5.5 Gelatin zymography.

Gelatin zymography for the investigation of gelatinolytic activity in unstimulated whole mouth saliva of 16 participants at four time-points. **(a)** Representative example of Coomassie-stained zymogram gel demonstrating bands with gelatinolytic activity at three different molecular weights. **(b)**, **(c)**, and **(d)** Relative quantification of band intensity of bands 1, 2, and 3. The fold change of gelatinolytic activity at T2, T3, and T4 for each band was assessed relative to T1. T1, baseline (before placement of orthodontic appliance); T2, one hour after placement of orthodontic appliance; T3, one week after placement of orthodontic appliance; T4, end of the alignment; m, molecular weight markers; MMP-9 std, matrix metalloproteinase-9 standard; * = $p<0.05$; ** = $p<0.01$.

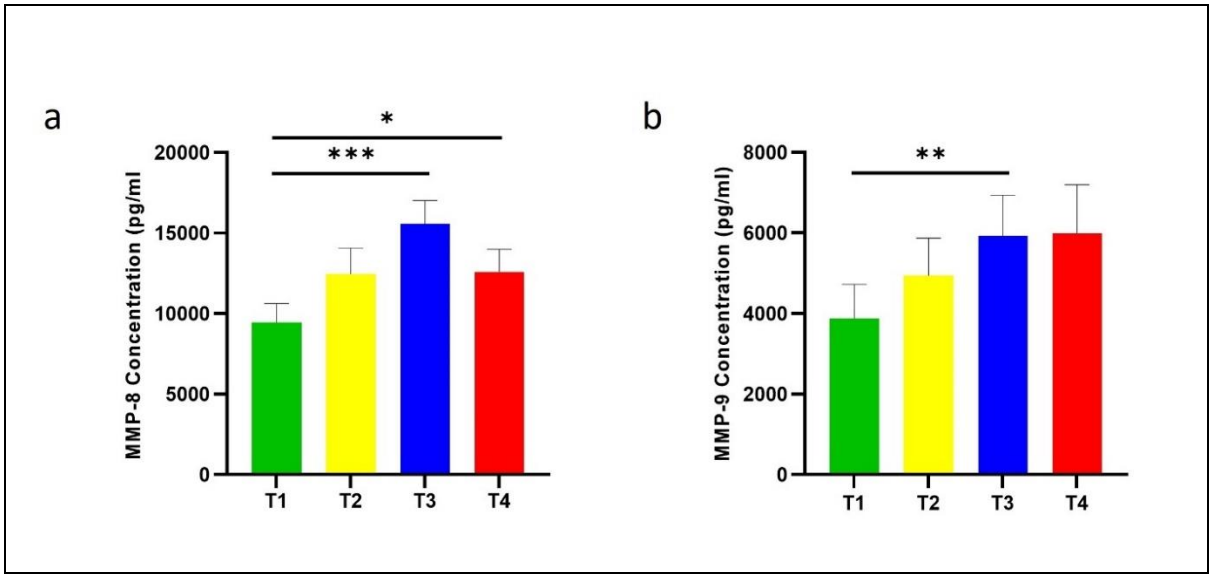

Figure 5.6 MMP-8 and MMP-9 levels measured by ELISA.

Graphs showing the levels of matrix metalloproteinase-8 (MMP-8) **(a)** and matrix metalloproteinase-9 (MMP-9) **(b)**, as measured by ELISA (Enzyme-linked immunosorbent assay), in unstimulated whole mouth saliva of 16 participants at four time-points. T1, baseline (before placement of orthodontic appliance); T2, one hour after placement of orthodontic appliance; T3, one week after placement of orthodontic appliance; T4, end of the alignment. Data were analysed by repeated measures ANOVA; * = $p<0.05$; ** = $p<0.01$; *** = $p<0.001$.

5.4 Discussion

This study describes the first characterisation of a WMS peptidome and protease profile during OTM using a peptidomic approach supplemented by mass spectrometry and bioinformatic analysis.

Proteolytic activity plays a crucial role in ECM remodelling, and numerous enzymes have been implicated, including serine, aspartate, cysteine, metallo, and threonine proteases (Kerrigan et al., 2000, Waddington and Embery, 2001, Krishnan and Davidovitch, 2006, Puente et al., 2003). In this study, proteases possibly implicated in the endogenous cleavage of the naturally occurring peptides were predicted by Proteasix software (Klein et al., 2013). This in silico analysis resulted in 73 predicted proteases responsible for producing peptides

identified by mass spectrometry, of which only 57 matched to a previous study of WMS using the same software (Trindade et al., 2015b). The additional 16 novel proteases predicted in our study could be attributed to changes in the Proteasix algorithm since the original publication (Mulkern et al., 2020), age-related differences in the WMS proteome and peptidome (as adults aged 33 years were included in their study, whereas adolescents aged 15.2 years were included in our study), or more likely due to the effect of OTM. Previous studies have reported age-related changes in the salivary proteome, specific salivary proteins, or total proteins in the WMS (Denny et al., 1991, Cabras et al., 2009, Messana et al., 2015, Schulz et al., 2013). However, the literature contains contradictory conclusions addressing the association between age and saliva composition (Cabras et al., 2009). Of these 73, 24 proteases had a percentage of cleavage greater than 1% (Petra et al., 2021); calpains, metalloproteinases, and cathepsins were the most prevalent groups of the proteases that seem to participate in the proteolysis of salivary proteins. Similar results were obtained by previous studies that predicted the activity of proteases in WMS using Proteasix software (Trindade et al., 2015b, Mulkern et al., 2020).

OTM occurs through remodelling of the ECM of the periodontium. The mechanical stimulus generates an aseptic acute inflammatory reaction characterized by vascular changes and leukocytes infiltration (Krishnan and Davidovitch, 2006, Li et al., 2018, Apajalahti et al., 2003, Marcaccini et al., 2010), with neutrophils being among the initially infiltrating cells (Marcaccini et al., 2010, Korkmaz et al., 2010) as early as an hour (Jayaprakash et al., 2019). Our results in this study showed a significant increase in the predicted activity of CTSG (cathepsin G), ELANE (neutrophil elastase), and PGA3 (pepsin) one hour and one week after orthodontic appliance placement. Azurophilic granules of neutrophils contain ELANE and CTSG, which are secreted from neutrophils during inflammation and infection (Pisano et al., 2005, Hartman et al., 2021, van der Plas et al., 2021). These proteases play a crucial role in regulation of the inflammation and modulation of the immune response due to retaining pro-

and anti-inflammatory activities (Korkmaz et al., 2010). Although PGA3 (pepsin) is found in the stomach, PGA3 was found to be upregulated in infected wound samples (Hartman et al., 2021). These proteases have not previously been investigated in relation to OTM; however, they seem to have a crucial role in the regulation of the acute inflammatory reaction during the initial phase of OTM.

MMPs play a vital role in ECM remodelling and regulating inflammation (Krishnan and Davidovitch, 2006, Kerrigan et al., 2000, Magalhães et al., 2018). Moreover, MMP-8, MMP-9, and MMP-13 are produced by polymorphonuclear leukocytes (Pisano et al., 2005, Amado et al., 2010, Apajalahti et al., 2003). In previous research, MMPs have been extensively investigated in GCF and, to a lower extent, in WMS during OTM. Our results revealed a significant increase in the predicted activity of MMP-3, MMP-8, and MMP-13 at 1 hour and 1 week, whilst MMP-9, MMP-25, and MME were significantly increased 1 hour after appliance placement and MMP-12 only at the end of the alignment. This prediction was validated by ELISA, which confirmed that MMP-8 levels were significantly increased at 1 week after orthodontic appliance placement and continued until the end of the alignment, whereas MMP-9 levels were significantly increased only at 1 week. In contrast to the predicted results, there was no significant difference in total MMP-8 and MMP-9 levels 1 hour after orthodontic appliance placement when assessed by ELISA; this could be explained because ELISA detects total MMPs (both pro- and active forms) (Cheng et al., 2008), and the actual activity might be masked by total protein measurement. The existing literature can mostly support these predictions on the correlation between OTM and different MMPs. Notably, MMP-8, MMP-9, and MMP-12 levels in WMS increased at 1 hour (Xu et al., 2020) and 1 week (Sioustis et al., 2021) during OTM. Furthermore, MMP-3, MMP-8, MMP-9, and MMP-13 levels in GCF were previously positively correlated with OTM (Garlet et al., 2007, Bildt et al., 2009, Zhang et al., 2020, Apajalahti et al., 2003, Behm et al., 2021b, Cantarella et al., 2006). It has also been

demonstrated that orthodontic force application significantly increased MMP-8 levels (Apajalahti et al., 2003), MMP-3, MMP-13, and MMP-9 levels (Capelli Junior et al., 2011) in the GCF 1 hour following orthodontic appliance activation, which is in agreement with our predicted results.

The role of MME in OTM has never been studied previously. However, one previous study found that MME mRNA levels were higher in periodontitis-affected gingival tissues than in healthy gingival tissues and that MME expression was seen in fibroblasts and neutrophils in those tissues. In addition, they reported that MME contributes to the regulation of inflammation by degrading IL-1β, an important cytokine in inflammation (Nezu et al., 2017). Thus, MME may have a role in the aseptic inflammatory reaction associated with OTM.

Additionally, our predicted results demonstrated that MMP-7 predicted activity was significantly decreased 1 week after orthodontic appliance placement. MMP-7 activity or presence has not been extensively studied; one previous study investigated MMP-7 levels in GCF of healthy teeth and found that MMP-7 levels were significantly decreased one hour after orthodontic force application (Patil et al., 2015). Whilst in another study, MMP-7 levels in GCF were not significantly changed over time (Canavarro et al., 2012).

The results of our study showed that CAPN1, CAPN2, MEP1A, TMPRSS7, and KLK4 predicted activity was significantly reduced during OTM. However, no information in the literature is available concerning the production of those proteases during this process. There are few studies in the literature on the role of cathepsins during OTM in humans, whilst OTM significantly increased CTSK gene expression in rats (Baloul et al., 2011, Kirschneck et al., 2020, Wang et al., 2021a). It has been reported that periodontal cells express CTSB but not CTSK (Sugiyama et al., 2003); therefore, previous studies focused on CTSB. Contradictory results were reported in the literature on CTSB levels in GCF; one study reported on an increase

in CTSB levels after 24 hours but no change after 1 hour and 1 week of orthodontic force application (Sugiyama et al., 2003), and the second study reported on a decrease CTSB levels after 24 hours (Rhee et al., 2009). The first study agrees with the present results that demonstrated no change in the predicted activity of CTSB one hour and one week after orthodontic appliance placement; however, the predicted activity of CTSB was not measured after 24 hours of force application in our study. Hence, further investigations are needed to establish the relationship between cathepsins and OTM in humans.

Proteolysis is the main source of peptides, and considerable efforts have been made to identify the resultant fragments, cleavage sites, and implicated proteases. In line with the literature, our results demonstrated that the most abundant peptides belonged to the major salivary proteins, mainly proline-rich proteins, histatins, statherin, and P-B peptide (Amado et al., 2010, Vitorino et al., 2009). The ECM of soft and hard periodontal tissues consists mainly of type I collagen, which is an essential element for the structural integrity of the supporting tissues and stability of the tooth in position. Collagen degradation is considered one of the critical factors in periodontal tissue and alveolar bone remodelling associated with OTM and periodontal disease (Ingman et al., 2012, Takahashi et al., 2003, Terajima et al., 2014). In addition, total type I collagenase activity in GCF has been reported to be increased ten times in GCF of orthodontic patients compared to control (Ingman et al., 2012). In the present study, levels of peptides derived from COL1A1 and COL1A2 were significantly increased 1-hour after orthodontic appliance placement, suggesting that these proteins display high susceptibility to proteolysis during OTM. This is supported by our protease activity prediction results which linked with the increased levels of detected collagen-derived peptides, as it is well-reported that MMPs play vital roles in collagen breakdown and ECM remodelling during OTM. MMP-8 and MMP-13 are members of the collagenase group, and MMP-9 is a member of the gelatinases group; these proteases degrade type I collagen and gelatin (Ingman et al., 2005,

Apajalahti et al., 2003, Ingman et al., 2012, Kerrigan et al., 2000), and our data showed increased predicted activity of these proteases. Furthermore, our gelatin zymography results demonstrated increased gelatinolytic activity for two bands identified at 72 and 62 kDa over time but was statistically significant only 1 week after orthodontic appliance placement. Differences in the methodology could justify the inability to detect the gelatinolytic activity 1 hour after orthodontic appliance placement but possibly suggests that the peptidomic approach may provide a better tool for detecting protease activity.

Our results also demonstrate that statherin, PROL4 and PRR27 show high susceptibility to proteolysis, while PRB4 and histatin-3 showed less susceptibility to proteolysis during OTM; but how they are related to OTM is unclear and has never been investigated before. However, it is worth noting that fixed orthodontic appliance placement involves acid etching of the teeth that leads to demineralization of teeth, and statherin is known to be involved in calcium homeostasis and remineralization of teeth (Vitorino et al., 2009, Amado et al., 2010) and it is known for its strong affinity for the tooth surface and was identified previously in the pellicle formed on metallic brackets (Siqueira et al., 2021). This might explain the increase in statherin-derived peptides 1 hour after orthodontic appliance placement. Further investigations will be necessary to establish the link between OTM, proteases, and protein substrates.

Bacterial plaque and inflamed gingiva can potentially influence the proteolytic activity of salivary proteases. However, there were no significant changes in plaque and gingival indices after 1 hour and 1 week of orthodontic force application compared to baseline levels, and linear regression results showed no significant association between MMP-8 and MMP-9 levels with the plaque and gingival indices over time. Consequently, since changes in the proteolytic activity were observed in this study 1 hour and 1 week following orthodontic appliance placement, we may assume that these changes were induced by orthodontic forces

rather than by gingival inflammation or bacterial plaque, proposing that orthodontic forces modulate the proteolytic activity in the periodontal tissues.

Limitations of this study include its retrospective design and the use of a small sample size with the mass spectrometry and bioinformatics approaches. Hence, prospective clinical trials with a larger sample size should be ideally performed. Furthermore, additional research focusing on the detailed characterization of the role of each identified protease and its substrates may improve our understanding of OTM biology and possibly reveal novel biomarkers linked with OTM.

5.5 Conclusions

The profile and activity pattern of proteases responsible for salivary peptide generation were mapped, and susceptible protein targets were identified during the alignment stage of orthodontic treatment with fixed appliances, setting the basis for further future validation. The proteases detected in WMS change over time, with the majority of MMPs and proteases associated with inflammation exhibiting increases as early as one hour after orthodontic force application, supported by elevated levels of collagen-derived peptides. Protease prediction using peptidome data demonstrates a potential tool for identifying and differentiating between the various phases of OTM.

Chapter 6 The influence of appointment interval on orthodontic tooth alignment: a prospective randomised controlled trial

6.1 Introduction

The first phase of orthodontic treatment with fixed appliances is concerned with tooth alignment. Clinically efficient alignment reflects a balance between maximising the speed of tooth movement and minimising potential damage to the teeth and supporting structures, and patient discomfort. Accelerating OTM could potentially reduce treatment duration and the risk of longer-term side effects associated with orthodontic treatment (Huang et al., 2014). The rate of OTM is a major determinant of treatment time, which is mostly controlled by the rate of alveolar bone remodelling. Therefore, continued efforts have been directed toward the search for a safe, predictable, and acceptable method to reduce orthodontic treatment time without compromising clinical results. The time interval between reactivation of the fixed appliance may influence tooth alignment rates, overall treatment duration and other variables, such as periodontal status.

The appropriate length of time between orthodontic appointments, called the appointment interval, has been the subject of debate for many years. Clinicians generally have their own preferences regarding the timing of appliance reactivation, based either on what they were taught in their orthodontic speciality programs, community norms or their own philosophy. Little evidence is present in the orthodontic literature to support these biases, and there is no evidence-based standard appointment interval (Keim, 2011). According to a survey of 59 randomly chosen orthodontists, the most common interval between reactivation

appointments was 5-6 weeks, followed by 7-8 weeks, with the availability of highly resilient wires, constant-force springs, self-ligating brackets, and non-compliance appliances, as well as time constraints in families being among the most frequently mentioned reasons for extending the period between appointments. It was demonstrated that longer appointment intervals minimised total chair time and overheads, reduced patients' absence from school and allowed clinicians to see more patients. However, disadvantages of longer appointment intervals included an increase in the number of out-of-control or overcorrected cases, a longer total treatment duration and issues with monitoring of compliance-dependent appliances and fee collection (Sheridan, 2005, Jerrold and Naghavi, 2011). Furthermore, orthodontic appliances are plaque traps that are in contact with or adjacent to the supporting periodontal structures for lengthy periods of time. Because oral hygiene is monitored less frequently when the appointment interval is extended, there is insufficient monitoring of patients with periodontal disease and poor oral hygiene and a greater risk of delay in diagnosing periodontal disease and decalcification (Jerrold and Naghavi, 2011).

There are no clinical studies in the literature regarding the effect of appointment interval on OTM. However, shorter intervals between appointments seem to contribute to keeping treatment under control and have been recommended for the following: adults; patients with periodontal diseases and poor oral hygiene; decalcification and white spot lesions; root resorption after a 2-3 month resting period; extraction cases; patients being treated with compliance-dependent appliances such as elastics or headgear (Jerrold and Naghavi, 2011, Moresca, 2018).

6.2 Aims and objectives

The main aim of this prospective randomised controlled trial was to investigate the effect of appointment interval on OTM in adolescent patients during routine treatment with fixed-appliances. The primary objective of this study was to measure the time taken to achieve orthodontic tooth alignment using fixed appliances in patients treated with either 2- or 8-week appointment intervals. The secondary objectives were to measure rate of OTM and assess variations in periodontal biochemistry.

The null hypothesis was that there is no difference in the time to orthodontic tooth alignment between 2-week and 8-week appointment interval groups.

6.3 Methods

6.3.1 Ethical approval

Ethical approval was obtained by the United Kingdom National Research Ethics Service, North of Scotland Research Ethics Committee (19/NS/0099) on 10th September 2019, and written informed consent was received from all parents, guardians, and children. Data was reported and presented according to CONSORT (Consolidated Standards of Reporting Trials) statement (Schulz et al., 2010). This trial was registered at ClinicalTrials.gov with identifier number NCT04050657.

6.3.2 Study design

This is a 2-arm parallel-group prospective randomized controlled trial comparing the effect of appointment interval on the duration and rate of orthodontic tooth alignment. Participants were

allocated randomly into 1 of 2 groups: the first was reviewed every 2 weeks (2-week group), and the second was reviewed every 8 weeks (8-week group).

6.3.3 Participants

The participants were recruited from patients attending the Department of Orthodontics at Guy's Hospital within the Faculty of Dentistry, Oral & Craniofacial Sciences, King's College London who satisfied the following criteria: undergoing routine fixed-appliance treatment with or without tooth extractions; 12-18 years old at the start of treatment; no medical contraindications or regular medication; non-smokers; in the permanent dentition; mandibular arch incisor irregularity index of 4-12 mm; and normal weight. Patients who had undergone any previous orthodontic treatment, growth modification or multidisciplinary treatment, those with systematic diseases and craniofacial abnormalities, and those unable to give consent were excluded.

Fixed appliances (Victory-APC 0.022-inch brackets, MBT prescription; 3M-Unitek) were placed for all participants. A particular archwire sequence was followed (0.014-inch nickel-titanium; 0.018-inch nickel-titanium; 0.017 x 0.025-inch nickel titanium and 0.019 x 0.025-inch stainless steel). Archwire progression occurred only if full bracket engagement was attainable, and the completion of alignment was indicated by placement of a 0.019 x 0.025-inch stainless steel archwire. All appliances were placed by consultant orthodontists. Participants in the first group were reviewed every 2-weeks, while the second group were reviewed every 8-weeks. Mandibular dental study casts were taken at taken at the start of treatment and each adjustment appointment.

For all participants, plaque levels and gingival health were measured at the start of treatment and each adjustment appointment using established validated plaque and gingival

indices (Löe and Silness, 1963, Silness and Loe, 1964) as described in section (5.2.2) in Chapter 5 of this thesis.

6.3.4 Rate of tooth alignment

Tooth alignment was calculated from serial scanned dental stone casts taken at the start of treatment and each adjustment appointment using Little's irregularity index. This index calculates the horizontal linear contact point displacement of each mandibular incisor from the next tooth and hence represents the total of the five individual displacements (Little, 1975) (Figure 6.1). The overall alignment rate for each group was calculated from the difference in the irregularity index of scanned casts taken at the start and end of the alignment, divided by the number of days between the two measurements (duration of alignment in days).

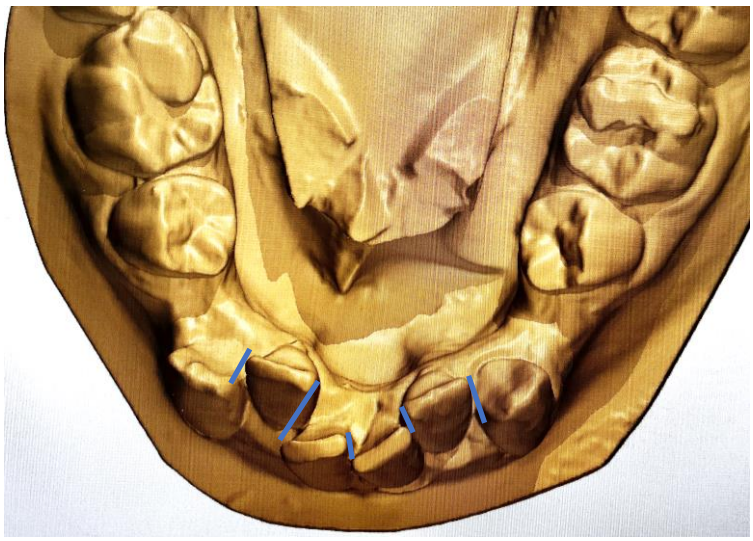

Figure 6.1 Little's Irregularity Index to calculate teeth irregularity by measuring the five liner distances between the contact points of lower anterior teeth.

6.3.5 Sample Size Calculation

Sample size calculation was based upon previous randomised prospective data on time to alignment completion with fixed appliances, which found a mean time to alignment of 200.7 days with standard deviation (SD) 73.6 days in the presence of 8.9 mm incisor irregularity (Woodhouse et al., 2015). A total of 50 participants were required to detect with an unpaired t-test a hypothesized 30% reduction (Schulz and Grimes, 2005) in alignment time with a common SD across groups to yield 80% power at 5% significance level.

6.3.6 Randomisation

The 1:1 randomisation sequence was generated using the Randbetween function in Microsoft Excel, with participants allocation undertaken centrally at King's College London independently from the clinical operators. The randomisation sequences were kept by an independent individual, who would allocate each participant to the proper group after they were recruited to the study and had signed the consent before the bond up (allowing allocation concealment). The patients and clinical operators were not blinded to their group allocation throughout treatment.

6.3.7 Statistical analysis

Descriptive statistics were used to summarise the data. Parametric and non-parametric analyses were conducted after checking for the normality distribution using the Shapiro-Wilk test of normality. Mann Whitney U test was used to compare the not normally distributed data, whereas the independent t-test was used to compare the normally distributed data in both groups. The chi-square test was used for categorical variables (gender and extraction involvement). Kaplan-Meier analysis was used to evaluate treatment effect in terms of time to

achieve alignment. Statistical analyses were done using GraphPad Prism version 9.0 (GraphPad Software, USA). Two-tailed P values were calculated, and the difference was considered statistically significant if $p < 0.05$.

6.4 Results

Due to the COVID-19 pandemic, the trial was halted for 18 months, so complete data is only currently available for 13 participants. The data for only these participants will be presented in this chapter as a pilot study.

6.4.1 Participants

A CONSORT diagram showing participant flow through the trial is presented in Figure 6.2 (Schulz et al., 2010). Thirteen participants (3 male, 10 female) were recruited, with n=7 and 6 participants allocated randomly to the 2-week and 8-week groups, respectively. Table 6.1 shows the demographics for the two groups at baseline (T1). The mean (SD) age of all participants at baseline before appliance placement (T1) was 17.46 (5.6) years; mean (SD) ages were 17.4 (6.3) and 17.5 (5.2) years for the 2-week and 8-week groups, respectively. The mean (SD) irregularity was 7.58 (3.8) mm in the 2-week group and 7.53(3.6) mm in the 8-week group. There were no statistically significant differences in demographics (age, gender, plaque index, gingival index, irregularity, and extraction involvement) between the two groups. All participants were followed up until alignment completion.

6.4.2 Primary outcome

The overall mean time to achieve alignment in the 2-week group was significantly lower than the 8-week group (122.7 (54.16) vs 291.7 (127.1) days; p=0.012). Overall, the 2-week group needed a mean of 168.5 days less than the 8-week group to achieve complete alignment (Table

6.2 and Figure 6.4a). Figure 6.3 shows Kaplan-Meier curves comparing alignment patterns for the 2-week and 8-week groups. There is a clear separation between the curves occurring as early as 80 days, which is continued throughout the duration of the trial, reflecting group alignment patterns significantly different from one another (P = 0.013; log-rank test).

Based on these results, we reject the null hypothesis that there would be no difference in the time to alignment completion between the 2-week and 8-week appointment interval groups.

6.4.3 Secondary outcomes

Overall mean alignment rate from baseline to completion of alignment was also significantly increased in the 2-week group than in the 8-week group (0.066 (0.03) vs 0.028 (0.014) mm/day; p=0.011) (Table 6.2 and Figure 6.4b). Moreover, the mean alignment rate from baseline to week 8 was higher in the 2-week group compared with the 8-week group but without any significant difference (0.096 (0.047) vs 0.071 (0.026) mm/day; p>0.05) (Table 6.2).

There were no significant differences in the number of appointments between the two groups (p>0.05). The 2-week group needed a mean number of 8.29 (3.64) visits compared to 6 (2.1) visits for the 8-week group.

Plaque and gingival indices deteriorated significantly for the 8-week group at the fifth and eighth adjustment appointments (T5 and T8) compared to baseline (T1) (both p<0.05). However, no significant changes in plaque and gingival indices were observed in the 2-week group over time (Figure 6.5).

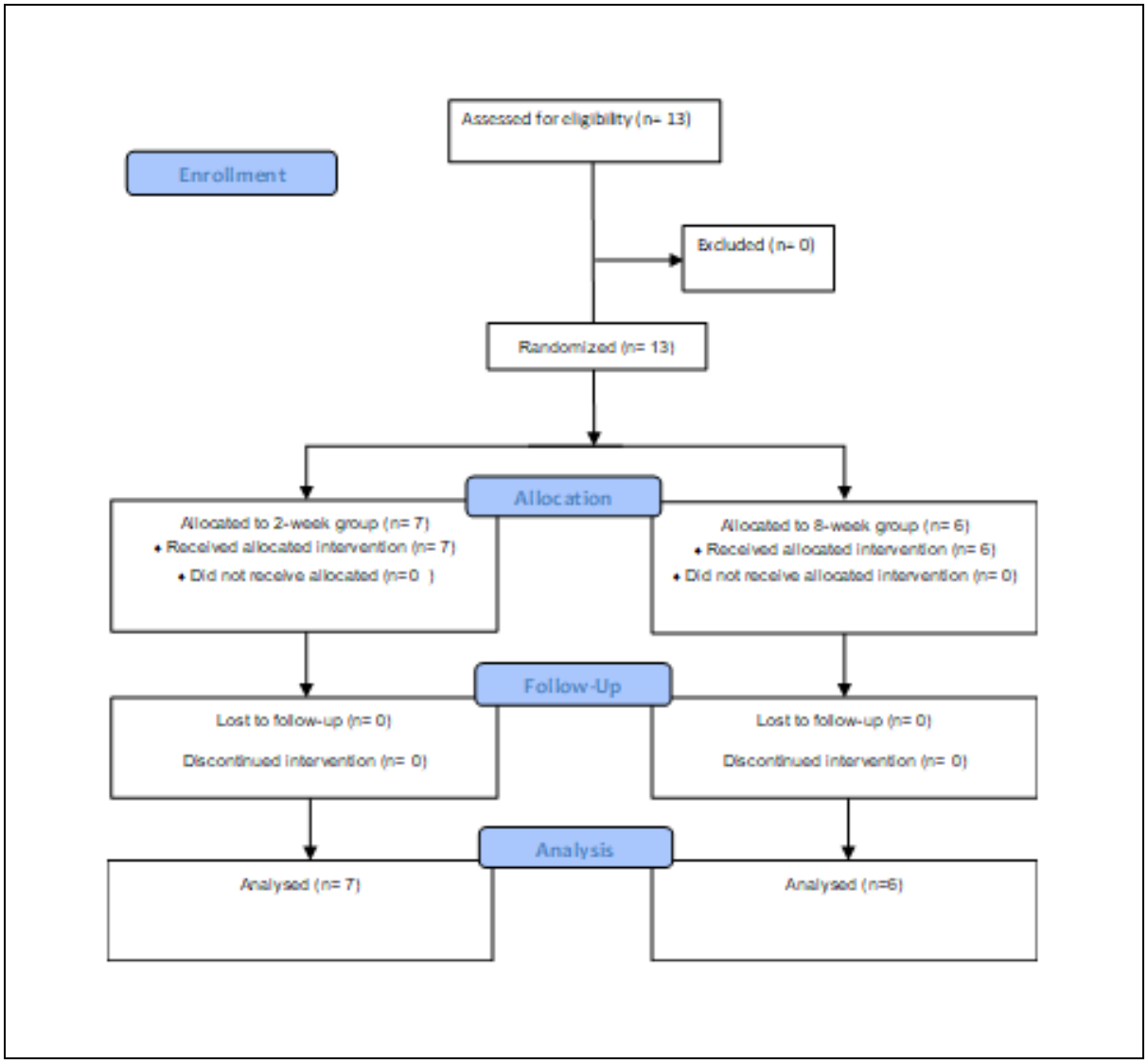

Figure 6.2 CONSORT diagram depicting the flow of participants in the trial.

Table 6.1 Demographics of participants at baseline.

	Overall	**2-week group**	**8-week group**	**P value**
Patients (n)	13	7	6	
Female / male (n)	10/13	05/07	05/06	0.612¥
Age - mean (SD)	17.46 (5.6)	17.4 (6.3)	17.5 (5.2)	0.703 #
Plaque index (SD)	0.15 (0.1)	0.14 (0.14)	0.16 (0.16)	0.99 #
Gingival index (SD)	0.15(0.1)	0.14 (0.14)	0.16 (0.16)	0.99 #
Irregularity - mean (SD)	7.6 (2.4)	7.58 (3.8)	7.53(3.6)	0.98*
Tooth extraction – n (%)	2 (15%)	2 (29%)	0	0.155¥

SD, standard deviation; ¥ from chi-square test; * from independent t-test; # from Mann-Whitney test.

Table 6.2 Rate of tooth movement (mm/day) during the study period.

Outcome	Overall (n=13)	2-week group (n=7)	8-week group (n=6)	P value
Time to completion of alignment (d) – mean (SD)	200.7 (126.0)	122.7 (54.16)	291.7 (127.1)	0.012#
Tooth alignment rate: baseline to completion of alignment (mm/d) – mean (SD)	0.048 (0.029)	0.066 (0.03)	0.028 (0.014)	0.011*
Tooth alignment rate: baseline to week 8 (mm/d) – mean (SD)	0.084 (0.039)	0.096 (0.047)	0.071 (0.026)	0.304*

d, days; SD, standard deviation; * from independent t-test; # from Mann-Whitney test.

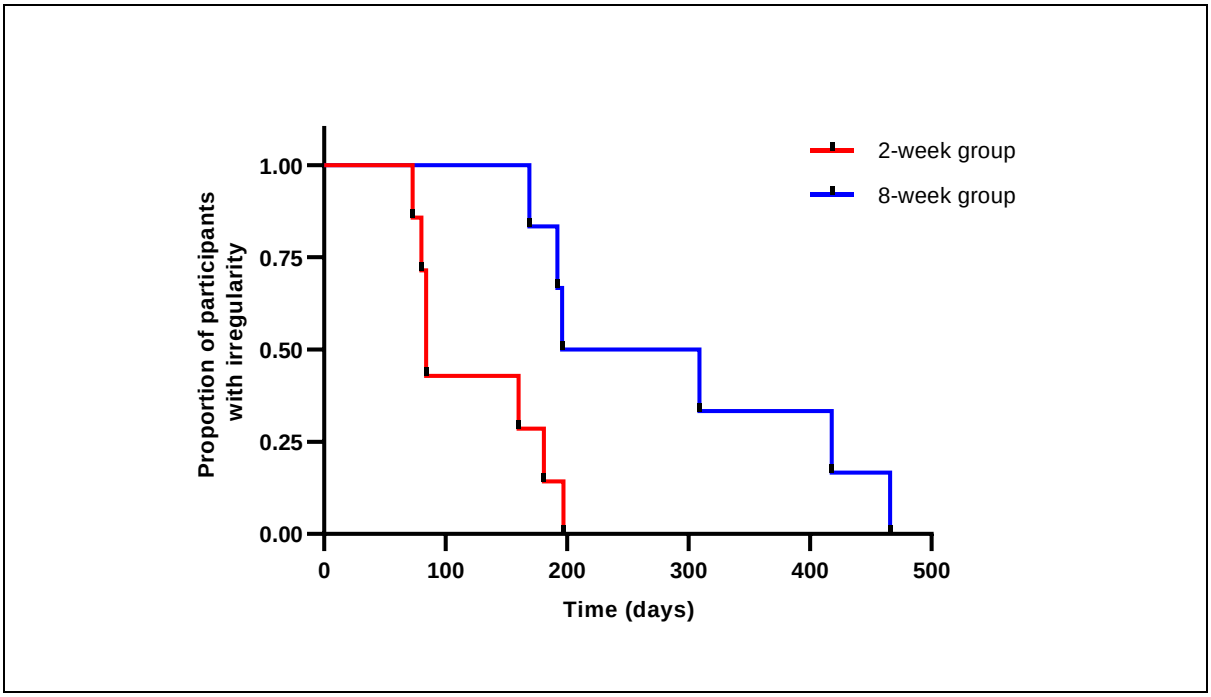

Figure 6.3 Kaplan-Meier survival curves for the 2-week and 8- week groups used in the study.

The y-axis gives the proportion of participants still in treatment (not aligned) over time (days on x-axis). By drawing a line perpendicular to the x-axis at a given time value, the proportions of participants not completed for each group is determined from the corresponding y-axis. There is clear separation occurring as early as 80 days. This separation is maintained throughout the duration of the trial.

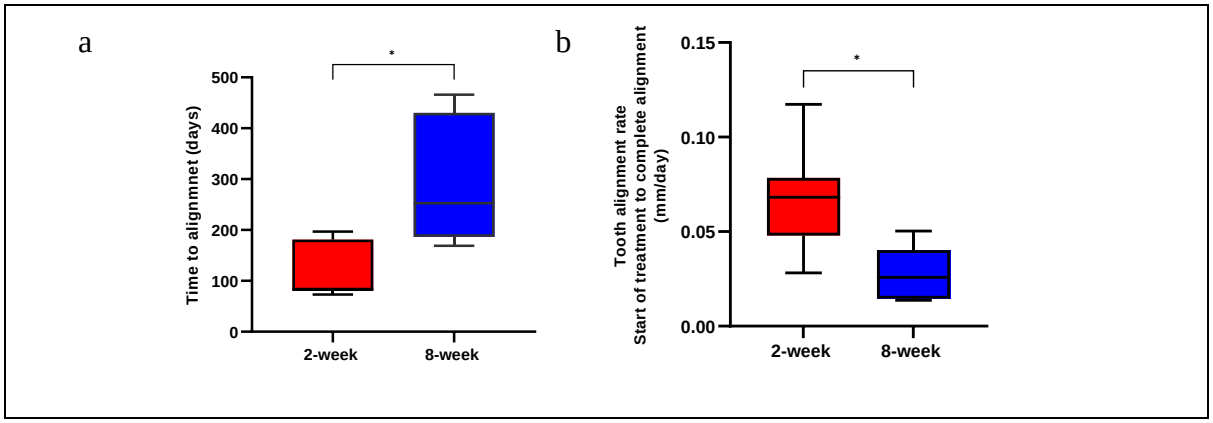

Figure 6.4 Box plots of measured values in 2-week and 8-week groups for study outcomes.

(a) primary (time to completion of alignment in days) and (b) secondary (tooth alignment rate from start of treatment to completion of alignment in mm/day) outcomes. Plotted boxes with horizontal lines indicate interquartile ranges with medians. Vertical whiskers represent upper and lower values.

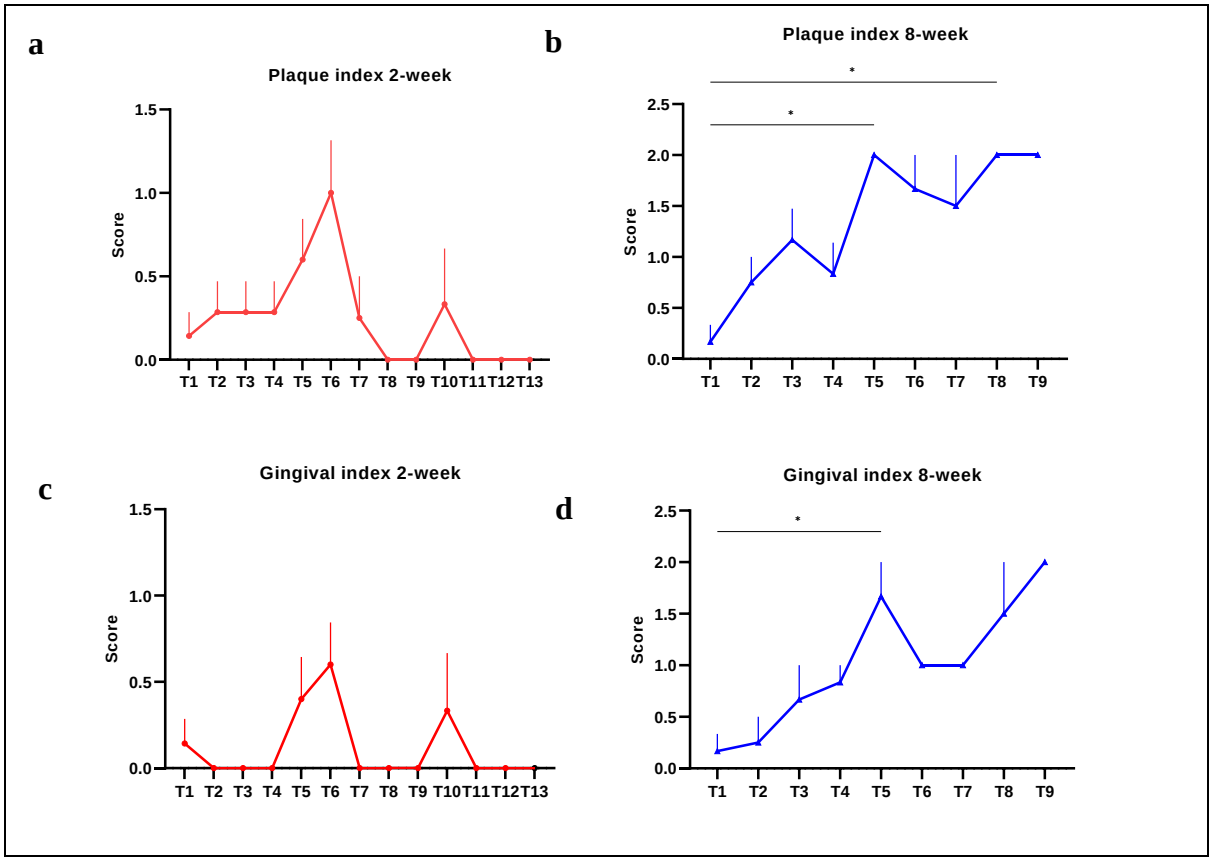

Figure 6.5 Graph showing the plaque index (a & b) and the gingival index (c & d) of the 2-week and 8-week groups.

Plaque and gingival indices were measured at the start of treatment and each adjustment appointment for the 2-week (T1-T13) and the 8-week (T1-T9) groups. * = $p<0.05$: significant difference with T1 ($p<0.05$) for the 8-week group. Data were analysed by the Kruskal-Wallis test.

6.5 Discussion

This is the first RCT to assess the effect of appointment interval on treatment duration to achieve alignment of the mandibular dentition during the initial phase of orthodontic treatment using fixed orthodontic appliances. Interestingly, we found that patients reviewed every 2 weeks had significantly shorter alignment duration and increased the rate of OTM. Specifically, patients reviewed every 2 weeks needed a mean of 168.5 days less than the 8-week group to achieve complete alignment, equivalent to a 58% reduction in duration to alignment completion. Moreover, our results demonstrated a faster rate of OTM within the 2-week group with an overall rate of 0.07 mm per day compared to 0.03 mm per day in the 8-week group.

Orthodontic treatment duration is affected by the rate of OTM, which is mediated by PDL and alveolar bone remodelling. The orthodontic force causes tooth displacement within its socket, initiating an immediate aseptic acute inflammatory response characterised by the release of multiple inflammatory cytokines. This acute inflammatory reaction lasts for one or two days, then it subsides and is replaced by a chronic aseptic inflammation that persists until the orthodontic appliance is re-activated in the next orthodontic treatment appointment and thus inducing another acute inflammatory reaction (Krishnan and Davidovitch, 2006). It is well reported in the literature the vital role of acute inflammation in OTM, confirmed by the early release of inflammatory mediators after orthodontic force application as early as 1 minute (Dudic et al., 2006) and 1 hour (Karacay et al., 2007, Hamamcı et al., 2012), then peaks at 24 hours (Sarı and Uçar, 2007, Alikhani et al., 2013, Ren et al., 2002). However, these inflammatory mediators returned to baseline levels at 48 hours, 7 days, 14 days, and 21 days (Sarı and Uçar, 2007, Karacay et al., 2007, Grieve et al., 1994). Therefore, by reviewing the patients every 2 weeks, the acute inflammatory reaction is induced at each adjustment appointment, leading to the release of inflammatory cytokines, which mediate PDL and alveolar bone resorption and hence increase OTM rate. This might explain the faster rate of

OTM and shorter alignment duration in patients reviewed every 2 weeks than those reviewed every 8 weeks. However, this should be confirmed by measuring the levels of the inflammatory mediators in the saliva and GCF of those patients in future studies.

Our results demonstrated a faster OTM rate from baseline to week 8 in the 2-week group compared with the 8-week group but without any significant difference (0.096 vs 0.071 mm/day). However, it is worth noting that the 8-week time point represented the fourth visit for the 2-week group when most of the lower anterior irregularity was alleviated, whilst it was the second visit for the 8-week group. Furthermore, no significant differences in the number of visits were found between the two groups. Therefore, the alignment rate was measured for each group at the second visit, with the 2-week group exhibiting a significantly faster rate of OTM (0.18 mm per day) compared to the 8-week group (0.07 mm per day). This indicates that reviewing patients every 2 weeks causes a faster OTM rate.

Orthodontic treatment with fixed appliances can cause external root resorption and loss of periodontal attachment. However, no clinical evidence exists that accelerated OTM is associated with adverse changes in any of these parameters over the short or long term (Dab et al., 2019). There is little data relating to human studies of appointment interval periods, but a recent animal study found no correlation between interval periods and external root resorption in rats exposed to a retraction force (Nagata et al., 2016). Furthermore, there is no association between root resorption and the choice of archwire sequence used (Weltman et al., 2010). It is crucial not to use excessive force during treatment (Weltman et al., 2010), which is why in the present study, archwire progression was undertaken in both randomised groups when clinically indicated.

Orthodontic treatment with fixed appliances has a negative effect on oral health (Cantekin et al., 2011, Atack et al., 1996). It is associated with increased plaque retention and bleeding on probing (Ristic et al., 2007, Levin et al., 2008, Fornell et al., 2002), which can

jeopardize oral health, causing several adverse effects such as enamel demineralization (white spot lesions) and gingival inflammation (Fornell et al., 2002, Weyland et al., 2022), with white spot lesions being seen within 4 weeks of starting orthodontic treatment (Ogaard et al., 1988). Most orthodontic patients are adolescents who are reported to be more likely to miss orthodontic appointments and be poorly compliant with plaque prevention and control (Fornell et al., 2002, Weyland et al., 2022). Therefore, shorter intervals between appointments have been recommended to keep treatment under control for patients with periodontal diseases and poor oral hygiene, decalcification, and white spot lesions (Jerrold and Naghavi, 2011, Moresca, 2018). This is consistent with our findings which demonstrated a significant deterioration in plaque and gingival indices over time in patients reviewed every 8 weeks, whilst patients reviewed every 2 weeks exhibited no significant changes in those indices, indicating better treatment control when shorter appointment intervals are employed. This can be explained by the effects of motivation and oral hygiene instruction given at each adjustment appointment which can keep treatment under control.

Other factors that might affect the appointment interval period and should be taken into consideration include treatment financing, scheduling convenience, and monitoring of compliance-dependent appliances like elastics or headgear. In addition, the number of adults seeking orthodontic treatment is increasing. However, prolonged treatment time might lead to adults discontinuing or resorting to alternative treatments such as implants or veneers. As a result, approaches for reducing treatment duration are desired by adults (Ong and Wang, 2002). Also, with the slower cellular response and a higher risk for periodontal disease, adult patients might benefit from being seen at shorter appointment intervals.

The limitations of this study include the inability to: recruit the required sample, to measure in vivo levels of salivary and GCF biomarkers during OTM and the reported pain and discomfort, and to measure root resorption. These limitations were the results of halting the

study for an extended period due to the COVID-19 pandemic. The trial is still going at present, and the targeted sample size will be reached as well as these outcomes will be measured. On the other hand, the methods used in the study are robust, and the results of the pilot study showed a significant effect of appointment interval on tooth alignment.

6.6 Conclusions

This prospective randomized clinical trial found evidence that shorter intervals between appointments can significantly reduce the time required to achieve complete alignment and increase the rate of tooth alignment. Furthermore, shorter appointment intervals seem to contribute to keeping periodontal health under control during orthodontic treatment.

Chapter 7 General discussion

7.1 Duration and rate of OTM

People seeking orthodontic treatment have made shortening the duration of treatment a high priority. In the last few decades, patients and orthodontists have been interested in the factors that influence the speed of OTM; hence, the modulation of OTM rate has become the dominant focus of orthodontic trials, with several approaches and devices being reported to speed OTM. However, any adjunct treatment or new approaches to speed orthodontic OTM requires more robust clinical evidence of its efficiency before being marketed and introduced into orthodontic practices. Therefore, it was essential to systematically review the literature to assess the current evidence on the duration and rate of OTM during orthodontic treatment with fixed appliances and to evaluate factors associated with those variables.

Orthodontic treatment with fixed appliances encompasses several stages: alignment and levelling, overbite and overjet reduction, space closure, and finishing. According to a recent systematic review, the average duration of treatment with fixed appliances is 19.9 months; however, with a wide variation between studies (with mean values ranging from 14 to 33 months) (Tsichlaki et al., 2016). The alignment of dentition is the first phase and key goal of orthodontic treatment; consequently, the duration of this phase influences the overall duration and burden of a course of orthodontic treatment. In addition, it is a commonly selected outcome in clinical studies investigating orthodontic treatment interventions and the speed of OTM. Therefore, a systematic review of the literature was conducted to evaluate treatment duration to achieve alignment of the mandibular dentition using fixed appliances. Thirty-five RCTs were included, giving a pooled duration to achieve whole-arch alignment of the mandibular dentition of 263.0 days (8.8 months) and 110.7 days (3.4 months) to achieve mandibular incisor alignment.

In this systematic review, high levels of heterogeneity were observed across the studies. As a result, confidence intervals may be more informative than pooled averages. However, the overall strength of evidence using the GRADE approach suggested high-quality evidence about the results, which enhances our confidence in the findings. Specifically, the quality of evidence was high for the surgically-assisted orthodontics (reducing the time to initial alignment of the anterior teeth by 44.3 days) and for the lack of benefit for both thermal NiTi archwires and self-ligating brackets. This is consistent with previous reviews demonstrating the little effect of appliance design on alignment rates (Papageorgiou et al., 2014b, Papageorgiou et al., 2014a, Wang et al., 2018, Fleming and Johal, 2010). On the other hand, surgically assisted orthodontics reduced incisor alignment time, and this treatment adjunct seems to be associated with increased OTM rates, although on a relatively short-term basis (Fleming et al., 2015). This intervention's transient nature may be overcome if performed surgical procedure is repeated several times. However, most surgical procedures are invasive and might not be readily acceptable to most patients (Uribe et al., 2014).

Space closure is the most time-consuming phase of orthodontic treatment and shortening this time might lead to a shorter overall treatment duration. Space closure can be achieved by either retracting the maxillary canine teeth as a separate stage followed by retracting the four incisors as a second stage of treatment or by a single stage of en-masse retraction of all six anterior teeth simultaneously. Although the former approach is more time-consuming (Rizk et al., 2018), extensive investigations have used single canine retraction as an experimental model to assess the effectiveness of different treatment modalities during orthodontic treatment. Therefore, a second systematic review was performed to assess evidence from RCTs on duration and rate of single canine retraction following maxillary first premolar extraction using full-arch fixed appliances. Fifty RCTs were included, with the estimated average pooled duration to achieve complete canine retraction being 4.98 months. Similar to

the alignment duration systematic review, this review found that surgically-assisted orthodontics resulted in a shorter retraction duration than control groups (1.11 months less) and a greater canine retraction rate consistent with other reviews (Gil et al., 2018, Apalimova et al., 2020) while there was no effect of adjunct vibration, self-ligation, photobiomodulation, and platelet-rich plasma or fibrin.

Both reviews found limited research assessing the primary outcome of the complete duration of each phase. Out of the 35 included RCTs, 13 assessed alignment duration, with only 4 trials assessing complete alignment duration, while the rest of the studies assessed incisor alignment. However, out of the 50 included RCTs, 4 trials assessed complete canine retraction, with only 2 studies being used in the data synthesis. Most clinical trials have focused on OTM rate as a primary outcome of their interventions. However, useful clinical data on complete duration of each phase or overall treatment, which is the most clinically relevant outcome for both patient and orthodontist, is lacking. There is a recognition that outcomes of research should be of relevance and benefit to patients instead of focusing on technical features of interventions. However, it has been indicated that there is an overemphasis on technical and clinician-centred outcomes in dental research across all specialities (Fleming et al., 2016, Tsichlaki and Fleming, 2019). In addition, there was inconsistency in the definition of alignment duration among the included studies and in outcome assessment methods and tools of tooth alignment and canine retraction. There is a plethora of evidence showing that outcome heterogeneity is widespread in healthcare research. In orthodontic research, systematic reviews frequently conclude that there is a lack of quality evidence, an inability to do data synthesis from diverse studies, and a need for further research. This inability to undertake meaningful syntheses is one of the challenges associated with using inconsistent outcomes in clinical research investigations (Tsichlaki et al., 2018).

Overall treatment duration depends on many factors, which can be patient-related, such as age, gender, the severity of the underlying malocclusion, and compliance, or treatment-related, including factors such as teeth extraction, the need for overbite reduction, and space closure (Beckwith et al., 1999, Skidmore et al., 2006, Fisher et al., 2010). Subgroup and meta-regression analyses in both systematic reviews indicated that patient and treatment-related characteristics could significantly affect tooth alignment and canine retraction duration. Premolar extraction and 0.022-inch bracket slot size were associated with greater tooth alignment, consistent with previous findings (Little and Spary, 2017, Cobb 3rd et al., 1998), while age and baseline irregularity were associated with longer alignment duration. On the other hand, anchorage reinforcement method and patient gender are associated with canine retraction rate. Furthermore, differences in applied treatment methods impacted alignment and canine retraction duration. Therefore, these factors should be taken into account both clinically and when designing trial outcomes.

The efficiency of most approaches for accelerating OTM proposed in recent years is limited, in addition to their high prices and orthodontists' and patients' opposition to more invasive surgical procedures (Moresca, 2018). Therefore, new non-invasive and cost-effective approaches should be pursued. Existing information about the effect of the interval between appointments on OTM is lacking. Hence, in this thesis, a prospective randomised controlled trial was conducted for the first time to investigate the effect of appointment interval on OTM in adolescent patients during routine orthodontic treatment with fixed appliances. Notably, the groups in this RCT were not different regarding baseline demographics, including plaque or gingival indices and baseline irregularity. In this RCT, we were able to reject our null hypothesis since we found that the duration to complete alignment was significantly shorter in patients reviewed every 2 weeks than those reviewed every 8 weeks (122.7 vs 291.7 days; p=0.012). In addition, the overall alignment rate was significantly higher in the 2-week group

compared to the 8-week group (0.066 vs 0.028 mm/day; p=0.011). Our first systematic review results demonstrated that the pooled duration to achieve complete alignment of mandibular dentition was nearly double that of the 2-week group (263 vs 122.7 days) whilst almost similar to that of the 8-week group (263 vs 291.7 days).

Orthodontic forces create an acute aseptic inflammation in the surrounding supporting structure that lasts 1-2 days and is characterised by the dilatation of blood vessels, leukocyte adhesion and migration, and the release of inflammatory mediators. This leads to ECM in the PDL, gingiva, and alveolar bone. This acute phase is then replaced by an aseptic and transitory chronic inflammation which continues until the next orthodontic treatment appointment, after which acute inflammation returns due to activation by orthodontic appliances (Andrade et al., 2012, Krishnan and Davidovitch, 2006, Meikle, 2006); this could be one explanation for the faster alignment and hence duration in the 2- week group. In addition, the presence of acute inflammation is confirmed by the results of chapter 5 of this thesis, which demonstrated increased levels of many proteases as early as 1 hour after orthodontic force application, with many of those proteases having essential roles in acute inflammation. Specifically, our results revealed a significant increase in the predicted activity of CTSG, ELANE, PGA3, MMP-3, MMP-8, and MMP-13 at 1 hour and 1 week, whilst MMP-9, MMP-25, and MME were significantly increased 1 hour after orthodontic force application.

Orthodontic treatment in patients with periodontal disease is particularly risky, as the combination of aseptic and periodontal-associated inflammation triggers accelerated attachment loss and disease progression (Li et al., 2018). The data of our RCT showed that plaque and gingival indices deteriorated significantly over time for the 8-week group confirming the results of the retrospective study in chapter 5 of this thesis, which demonstrated that plaque and gingival indices were increased significantly at the end of alignment compared to baseline in orthodontic patients who were reviewed every 6 weeks. Whilst no significant

changes in plaque and gingival indices were observed in the 2-week group over time, indicating that reviewing patients with shorter appointment intervals would be beneficial to patients providing control over periodontal health during orthodontic treatment.

7.2 Adipokines and OTM

Obesity is defined as an abnormal increase of body fat produced by adipocyte cells which are responsible for the dysregulation of immunological responses by producing bioactive molecules known as adipocytokines (Ouchi et al., 2011). Adipocytes produce a variety of adipokines, mainly increased amounts of pro-inflammatory adipokines like leptin and decreased amounts of anti-inflammatory adipokines like adiponectin. This imbalance between pro- and anti-inflammatory adipokines causes a subclinical chronic systemic inflammation status of obese individuals (Ruiz-Heiland et al., 2021, Deschner et al., 2014). Although there are very scarce studies in the literature on the association between obesity and OTM (Consolaro, 2017), our research group previously found that levels of leptin differed significantly between obese and normal-weight patients before and during orthodontic treatment, with higher rates of OTM in obese patients (Saloom et al., 2017).

Because OTM mainly depends on PDL and alveolar bone remodelling, obesity can potentially affect orthodontic treatment, specifically the inflammatory and ECM mediators secreted in the periodontal sulcus. Therefore, understanding the effect of leptin and adiponectin on OTM could make an important contribution to the long-term success of orthodontic treatment, especially in view of the increasing obesity rates in children and adolescents worldwide. Our in vitro study in chapter 4 of this thesis investigated the effect of compressive force, leptin, and AdipoRon on the expression of inflammatory and ECM remodelling biomarkers in hPDLFs and hGFs, as well as their effect on IL-1α induced expression of

inflammatory and ECM remodelling biomarkers in the presence or absence of compressive force. The results demonstrated that leptin showed pro-inflammatory properties by selectively enhancing IL-1α-induced expression of IL-6 and IL-8 as well as several MMPs in compressed hPDLFs and hGFs. Whereas AdipoRon exhibited anti-inflammatory properties by attenuating these biomarkers under similar conditions. Because obese patients have higher levels of leptin and lower levels of adiponectin than normal weight patients, this might explain the increased rates of OTM observed in obese individuals (Saloom et al., 2017).

Elevated leptin concentrations during orthodontic treatment of obese individuals may result in higher bone resorption and thus increased orthodontic tooth movement, as well as increased inflammation, periodontal bone loss and dental root resorption due to increased osteoclast activity. Although enhanced tooth movement velocity in these patients would be beneficial clinically, the expected risk of increased related periodontal bone loss and dental root resorptions would advise caution and vigilance in the orthodontic treatment of obese individuals. Therefore, understanding the complicated molecular mechanisms induced by adipokines will hopefully assist in better estimating the likelihood of unwanted side effects during orthodontic treatment for obese individuals and hence ensure effective treatment (Schröder et al., 2021, Ruiz-Heiland et al., 2021).

Adiponectin warrants attention due to its numerous functions in metabolic processes. Based on the anti-inflammatory properties of adiponectin and continuous inflammation in periodontitis, it has been postulated that adiponectin might play a protective role in periodontitis. However, the administration of adiponectin is challenging because of the requirement for a megadose given via intravenous injection and the possible induction of adverse effects. Therefore, AdipoRon, an orally active synthetic analogue of endogenous adiponectin, has been introduced. It possesses pharmacological properties like those of adiponectin and can bind and activate both AdipoR1 and AdipoR2 receptors (Wang et al.,

2021b, Bhat et al., 2020), making it a suitable candidate to treat several disorders. Hence, AdipoRon might have potential therapeutic value in treating periodontitis by inhibiting the inflammatory lesions, promoting wound healing and tissue regeneration contributing to bone tissue regeneration (Wu et al., 2021, Iwayama et al., 2012).

The work in this thesis demonstrated that in the absence of induced inflammation, compressive forces, AdipoRon, and leptin have little effect on the production of inflammatory and ECM remodelling biomarkers. The acute inflammation induced by orthodontic forces, which is characterized by blood vessel dilatations in the surrounding periodontal tissues, elicits a typical innate immune response, with neutrophils being among the first cells to infiltrate (Chaushu et al., 2022, Marcaccini et al., 2010, Korkmaz et al., 2010). Additionally, as demonstrated by the results in chapter 5 of this thesis, increased levels of several proteases, including CTSG, ELANE, MMP-8, MMP-9, and MMP-13, were observed 1 hour after orthodontic force application. These proteases are secreted mainly from neutrophils during inflammation and infection (Apajalahti et al., 2003, Pisano et al., 2005, Amado et al., 2010, van der Plas et al., 2021, Hartman et al., 2021). This might indicate that periodontal fibroblasts are not the first cells to respond to orthodontic forces. However, other cells, like neutrophils, are the first to respond and release inflammatory mediators initiating acute inflammation. After that, in the presence of acute inflammation, periodontal fibroblasts respond to compression and adipokines, enhancing the acute inflammation and promoting ECM remodelling.

Our findings suggest an impact of compressive force on the mTOR signalling pathway manifested by downregulating the expression of phosphorylated AKT and 4EPB1 proteins. Activation of the mTOR signalling pathway generally induces an anabolic response, such as protein synthesis (Jiang et al., 2022), whereas compressive forces induce catabolic changes on the compression side during OTM. Moreover, it has been suggested previously that the in vitro weight loading approach might induce hypoxia (Liu et al., 2019, Ullrich et al., 2019), which

in turn affects the mTOR signalling pathway by inhibiting mTORC1 signal transduction (Brugarolas et al., 2004). Therefore, mTOR integrates several inputs, and it is unclear if specific inputs are dominant over others and if regulatory mechanisms depend on cell type (Laplante and Sabatini, 2012).

7.3 Proteolytic activity during OTM

Proteases are responsible for shaping entire proteomes and peptidomes of tissues and body fluids. Peptides originating from large proteins are likely due to the net result of protease activity and the counterbalancing regulation from their inhibitors. Therefore, studying the peptidome could help decipher the coordinated action of proteases (Magalhães et al., 2018). Advances in proteomic and peptidomic fields have opened new possibilities and are expected to change how diseases are diagnosed. Screening human body fluids for disease biomarkers is challenging, but it is hoped that alterations in the proteome or peptidome can be detected even before clinical symptoms appear (Loo et al., 2010).

Aside from bone remodelling, constant remodelling and reorganization of the ECM of the PDL occurs during OTM (Tantilertanant et al., 2019, Behm et al., 2021a), involving secreting both matrix proteins (ECM deposition) and proteases (Behm et al., 2021b, Chen et al., 2013). The significance of proteases in orthodontics has drawn much interest. Their primary physiological function is the modulation and regulation of ECM turnover by direct proteolytic degradation of the ECM proteins, including collagen, proteoglycans and fibronectin (Klein and Bischoff, 2011).

Our findings demonstrated the first characterisation of the salivary peptidome and protease profile during OTM. Mass spectrometry and bioinformatics were used to identify 73 active proteases, of which around 25 % changed during OTM, suggesting an active role. This

far exceeds the usual targeted approach and opens up new areas regarding the cells involved and biological processes. Using this novel approach, the role of MMPs was confirmed, and new proteases possibly derived from inflammatory cells were identified, which may also be necessary during the alignment stage of fixed appliance orthodontic treatment.

Typically, MMP levels are very low in normal conditions; however, their expression is elevated in inflamed tissues or those undergoing remodelling in both physiological and pathological conditions (Birkedal-Hansen, 1993, Ye, 2015). This is confirmed by our results showing that levels of several MMPs increased 1 hour and 1 week after orthodontic force application. Furthermore, since no visible plaque and gingival inflammation was detected at these time points indicating healthy infection and inflammation-free periodontium, it was assumed that changes observed in MMPs, and other proteases were due to orthodontic treatment rather than periodontal infection, demonstrating the crucial role of those proteases in PDL remodelling during OTM.

7.4 Future work

The findings of this thesis point to the necessity for further clinical and biochemical research.

Data in each systematic review provides a basis for appropriate trial design for future RCTs investigating the duration and rate of OTM with fixed appliances. Substantial heterogeneity was observed across studies. Therefore, future research studies with adequate sample sizes (based on power calculation), appropriate trial design, and a more consistent methodology in outcome assessment are needed.

One noteworthy limitation of the literature assessed in both systematic reviews was that most included trials only report over the short-term, often failing to follow up patients beyond one or a few months. Orthodontic treatment is a time-consuming process comprising several phases and various occlusal goals. As a result, it is essential to assess the effectiveness of any adjuncts or interventions during the overall duration of treatment because any potential benefit of the adjuncts may decrease with time. Future prospective randomised parallel-group trials should be conducted to investigate the relative influence of single versus repeated applications assessing the complete duration of each phase or overall treatment. Overall treatment duration would enable a reasonable calculation of the adjunct efficiency and cost-benefit to justify its clinical relevance.

Additionally, the thesis showed that the overall duration and rate of tooth movement significantly increased in the 2-week group compared to the 8-week group during the alignment phase of orthodontic treatment. Therefore, it would be interesting also to measure the duration and rate of tooth movement of the canine retraction phase and complete course of treatment, to assess the differences in pain and discomfort between the two groups, and to closely assess the difference in the root resorption between the two groups.

Orthodontic forces initiate local inflammation and greater capillary permeability in paradental tissues during orthodontic treatment. GCF occurs near the sites of these activities and is unlikely to be diluted, so it has a better diagnostic potential than saliva for the biomarkers of these activities (Rody et al., 2011). It would be valuable to use mass spectrometry and bioinformatics to investigate the profile of naturally occurring peptides in the GCF of the 2-week and 8-week groups and to predict proteases potentially implicated in the generation of these peptides using Proteasix software. In addition, it would be essential to use targeted approaches to assess the differences between the 2-week and 8-week groups from the biochemical aspect in the saliva and GCF. This was not possible in this thesis because of limited time and funding.

The weight method used in this thesis to simulate orthodontic forces in vitro might induce hypoxia, especially in the central area of the glasses. This is an inherent limitation of this method. Additionally, it is worth noting that an in vitro model can never attain the complexity of the in vivo conditions. Therefore, future studies should aim to apply this knowledge and investigate these findings using an in vivo model.

AdipoRon could be a potential promising therapy to treat periodontitis associated with diabetes via regulating hyperglycaemia, repressing inflammation, improving bone regeneration, and inhibiting bone loss (Wu et al., 2019). Data of this thesis highlight potential implication for orthodontic treatment by using AdipoRon to enhance retention and reduce relapse after orthodontic treatment by attenuating the inflammation induced by orthodontic forces.

Analysis of MMPs and TIMPs in orthodontics may contribute to more predictable treatment regimens in the future. It may serve as a diagnostic aid to predict the rate of OTM, root resorption severity, and the relapse (Bildt et al., 2009). Chairside MMP tests have already

been developed to monitor the treatment of periodontitis (Mäntylä et al., 2003). It would be valuable to develop MMP tests to monitor OTM during orthodontic treatment with different interventions.

Further studies should be performed to investigate the extra- and intracellular mechanisms by which leptin and AdipoRon exert their pro- and anti-inflammatory properties in compressed hPDLFs and hGFs. In addition, our results suggest that the mTOR signalling pathway might have an influence on autophagy regulation; hence, it is crucial to explore the role of autophagy in OTM, as aberrant autophagy is associated with uncontrolled degradation of the periodontium. Future in vitro studies should be conducted to measure autophagy activity and markers in compressed hPDLFs and hGFs. In addition, further research on the relationship between the mTOR signalling pathway with other regulatory pathways and how to target this signalling pathway will be necessary from the orthodontic perspective.

In addition, inhibitors of the mTOR signalling pathway may help to prevent protein synthesis and cell growth which would be beneficial for tooth stabilization after orthodontic treatment. Therefore, it would be interesting to use non-invasive localized drug delivery to accelerate OTM and hence shorten treatment duration or reversibly stop OTM for anchorage reinforcement and retention enhancement. Moreover, modulating autophagy and inflammation may present novel targets for treating periodontitis and orthodontic-induced root resorption.

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ZHANG, L., FARRELL, J. J., ZHOU, H., ELASHOFF, D., AKIN, D., PARK, N. H., CHIA, D. & WONG, D. T. 2010. Salivary transcriptomic biomarkers for detection of resectable pancreatic cancer. *Gastroenterology,* 138**,** 949-57.e1-7.

ZHANG, Y., PROENCA, R., MAFFEI, M., BARONE, M., LEOPOLD, L. & FRIEDMAN, J. M. 1994. Positional cloning of the mouse obese gene and its human homologue. *Nature,* 372**,** 425-32.

ZHAO, Y., WANG, C., LI, S., SONG, H., WEI, F., PAN, K., ZHU, K., YANG, P., TU, Q. & CHEN, J. 2008. Expression of Osterix in mechanical stress-induced osteogenic differentiation of periodontal ligament cells in vitro. *European Journal of Oral Sciences,* 116**,** 199-206.

ZHENG, J. & YANG, K. 2021. Clinical research: low-level laser therapy in accelerating orthodontic tooth movement. *BMC Oral Health,* 21**,** 324.

ZHONG, W., XU, C., ZHANG, F., JIANG, X., ZHANG, X. & YE, D. 2008. Cyclic stretching force-induced early apoptosis in human periodontal ligament cells. *Oral Diseases,* 14**,** 270-276.

ZHOU, J. & WINDSOR, L. J. 2006. Porphyromonas gingivalis affects host collagen degradation by affecting expression, activation, and inhibition of matrix metalloproteinases. *J Periodontal Res,* 41**,** 47-54.

ZHU, J., GUO, B., GAN, X., ZHANG, L., HE, Y., LIU, B., CHEN, X., ZHANG, S. & YU, H. 2017. Association of circulating leptin and adiponectin with periodontitis: a systematic review and meta-analysis. *BMC Oral Health,* 17**,** 104.

ZHU, J., HOOP, C. L., CASE, D. A. & BAUM, J. 2018. Cryptic binding sites become accessible through surface reconstruction of the type I collagen fibril. *Scientific Reports,* 8**,** 16646.

ZIEGLER, N., ALONSO, A., STEINBERG, T., WOODNUTT, D., KOHL, A., MÜSSIG, E., SCHULZ, S. & TOMAKIDI, P. 2010. Mechano-transduction in periodontal ligament cells identifies activated states of MAP-kinases p42/44 and p38-stress kinase as a mechanism for MMP-13 expression. *BMC Cell Biology,* 11**,** 10.

ZIMMERMANN, G. S., BASTOS, M. F., DIAS GONÇALVES, T. E., CHAMBRONE, L. & DUARTE, P. M. 2013. Local and circulating levels of adipocytokines in obese and normal weight individuals with chronic periodontitis. *J Periodontol,* 84**,** 624-33.

Appendices

Chapter 2

Appendix 2.1. Search strategies applied for each database

MEDLINE (Ovid) Search Strategy (searched from 1946 to 07/01/2021)

1 orthodon$.mp.
2 orthodontic.ti,ab.
3 ((tooth or teeth) adj5 move$).ti,ab.
4 1 or 2 or 3
5 exp Orthodontic Wires/
6 "orthodontic wire$".mp.
7 (wire$ or "arch-wire" or archwire$ or "arch wire").mp.
8 5 or 6 or 7
9 (initial$ or first or aligning or sequence$ or order or crowd$ or decrowd$ or "de-crowd$" or levelling$ or alleviat$).mp.
10 4 and 8 and 9

This subject search was linked to the Cochrane Highly Sensitive Search Strategy (CHSSS) for identifying randomized trials in MEDLINE: sensitivity maximising version (2008 revision) as referenced in Chapter 6.4.11.1 and detailed in box 6.4.c of The Cochrane Handbook for Systematic Reviews of Interventions, Version 5.1.0 [updated March 2011] (Higgins 2011).

1. randomized controlled trial.pt.
2. controlled clinical trial.pt.
3. randomized.ab.
4. placebo.ab.
5. drug therapy.fs.
6. randomly.ab.
7. trial.ab.
8. groups.ab.
9. or/1-8
10. exp animals/ not humans.sh.
11. 9 not 10

Embase (Ovid) search strategy (searched from 1947 to 07/01/2021)

1 orthodontic.ti,ab.
2 orthodon*.mp.
3 ((tooth or teeth) adj5 move$).ti,ab.
4 1 or 2 or 3 (60718)
5 exp orthodontic wire/ or exp stainless steel/ or exp nickel/ or exp titanium/
6 exp orthodontic wire/
7 orthodontic wire$.mp.
8 (wire$ or "arch-wire" or archwire$ or "arch wire").mp
9 5 or 6 or 7 or 8 (203903)
10 (initial$ or first or aligning or sequence$ or order or crowd$ or decrowd$ or "de-crowd$" or levelling$ or alleviat$).mp.
11 4 and 9 and 10

This subject search was linked to an adapted version of the Cochrane Centralised Search Project filter for identifying RCTs in Embase Ovid

1. Randomized controlled trial/
2. Controlled clinical study/
3. Random$.ti,ab.
4. randomization/
5. intermethod comparison/
6. placebo.ti,ab.
7. (compare or compared or comparison).ti.
8. ((evaluated or evaluate or evaluating or assessed or assess) and (compare or compared or comparing or comparison)).ab.
9. (open adj label).ti,ab.
10. ((double or single or doubly or singly) adj (blind or blinded or blindly)).ti,ab.
11. double blind procedure/
12. parallel group$1.ti,ab.
13. (crossover or cross over).ti,ab.
14. ((assign$ or match or matched or allocation) adj5 (alternate or group$1 or intervention$1 or patient$1 or subject$1 or participant$1)).ti,ab.
15. (assigned or allocated).ti,ab.
16. (controlled adj7 (study or design or trial)).ti,ab.
17. (volunteer or volunteers).ti,ab.
18. trial.ti.

19. or/1-18
20. (exp animal/ or animal.hw. or nonhuman/) not (exp human/ or human cell/ or (human or humans).ti.)
21. 19 not 20

Cochrane database of systematic reviews (CDSR) search via Cochrane Library

orthodon* AND (wire* OR "arch-wire" OR archwire* OR "archwire")

Cochrane Central Register of controlled trials (CENTRAL) search strategy via Cochrane Library

orthodon* AND (wire* OR "arch-wire" OR archwire* OR "archwire")

Scopus search strategy

Orthodont* AND random* AND (wire* OR "arch-wire*" OR archwire*)

Web of Science search strategy

(orthodont* AND random* AND (wire* OR "arch-wire*" OR archwire*))

LILACS search strategy

http://pesquisa.bvsalud.org/portal/?lang=en

(orthodon* AND random* AND (wire* OR “arch-wire” OR archwire*) AND (initial* OR first OR aligning OR sequence* OR order OR crowd* OR decrowd* OR “de-crowd*))

The Database of Abstracts of Reviews of Effects (DARE)

https://www.crd.york.ac.uk/CRDWeb

Appendix Table 2.1 List of excluded studies by full text with reasons.

Paper	Status
Excluded by full text with reasons	
Alam MK. Laser assisted orthodontic tooth movement in saudi population: A randomized clinical trial. Bangladesh Journal of Medical Science. 2019;18(2):385-90.	Excluded; movement of upper canines
Andreasen G. A clinical trial of alignment of teeth using a 0.019 inch thermal nitinol wire with a transition temperature range between 31 degrees C. and 45 degrees C. American journal of orthodontics. 1980;78(5):528-37.	Excluded, case report
Alzahawi K, Færøvig E, Brudvik P, Bøe OE, Mavragani M. Root resorption after leveling with super-elastic and conventional steel arch wires: a prospective study. Progress in Orthodontics. 2014;15(1).	Excluded; no lower teeth alignment rate or time
Aragón MLC, Bichara LM, Flores-Mir C, Almeida G, Normando D. Efficiency of compensatory orthodontic treatment of mild class III malocclusion with two different bracket systems. Dental Press Journal of Orthodontics. 2017;22(6):49-55.	Excluded; total treatment duration
Atik E, Akarsu-Guven B, Kocadereli I. Mandibular dental arch changes with active self-ligating brackets combined with different archwires. Nigerian journal of clinical practice. 2018;21(5):566-72.	Excluded; no lower teeth alignment rate or time
Bloom KL, Bhatia SN. Comparison of copper NiTi and Nitinol archwires in initial alignment. European journal of orthodontics. 1998;20(5):614.	Excluded; no full-text found
DiBiase AT, Nasr IH, Scott P, Cobourne MT. Duration of treatment and occlusal outcome using Damon3 self-ligated and conventional orthodontic bracket systems in extraction patients: A prospective randomized clinical trial. Am J Orthod Dentofacial Orthop 2011;139(2).	Excluded; no lower teeth alignment rate or time
DiBiase AT, Woodhouse NR, Papageorgiou SN, Johnson N, Slipper C, Grant J, et al. Effect of supplemental vibrational force on orthodontically induced inflammatory root resorption: A multicenter randomized clinical trial. Am J Orthod Dentofacial Orthop 2016;150(6):918-27.	Excluded; no lower teeth alignment rate or time
Dywer L, Littlewood SJ, Rahman S, Spencer RJ, Barber SK, Russell JS. A multi-center randomized controlled trial to compare a self-ligating bracket with a conventional bracket in a UK population: Part 1: Treatment efficiency. Angle Orthod. 2016;86(1):142-8.	Excluded; no lower teeth alignment rate or time
Gravina MA, Brunharo IH, Fraga MR, Artese F, Campos MJ, Vitral RW, et al. Clinical evaluation of dental alignment and leveling with three different types of orthodontic wires. Dental Press J Orthod. 2013;18(6):31-7.	Excluded; no lower anterior teeth alignment rate or time
Johansson K, Lundstrom F. Orthodontic treatment efficiency with self-ligating and conventional edgewise twin brackets A prospective randomized clinical trial. Angle Orthodontist. 2012;82(5):929-34.	Excluded; no lower anterior teeth alignment rate or time
Jones ML, Staniford H, Chan C. Comparison of superelastic NiTi and multistranded stainless steel wires in initial alignment. Journal of clinical orthodontics : JCO. 1990;24(10):611-3.	Excluded; no full-text found
Kaklamanos EG, Mavreas D, Tsalikis L, Karagiannis V, Athanasiou AE. Treatment duration and gingival inflammation in Angle's Class I malocclusion patients treated with the conventional straight-wire method and the Damon technique: a single-centre, randomised clinical trial. Journal of Orthodontics. 2017;44(2):75-81.	Excluded; no lower anterior teeth alignment rate or time
Lew K. A comparison of archwires used for initial alignment in Begg treatment. Australian orthodontic journal. 1988;10(3):180-2.	Excluded; no full-text found
Lo Giudice A, Nucera R, Perillo L, Paiusco A, Caccianiga G. IS LOW-LEVEL LASER THERAPY AN EFFECTIVE METHOD TO ALLEVIATE PAIN INDUCED BY ACTIVE ORTHODONTIC ALIGNMENT ARCHWIRE? A RANDOMIZED CLINICAL TRIAL. Journal of Evidence-Based Dental Practice. 2019;19(1):71-8.	Excluded; no lower anterior teeth alignment rate or time
Markovic E, Fercec J, Scepan I, Glisic B, Nedeljkovic N, Juloski J. The correlation between pain perception among patients with six different orthodontic archwires and the degree of dental crowding. Srpski arhiv za celokupno lekarstvo. 2015;143(3-4):134-40.	Excluded; no lower anterior teeth alignment rate or time
Megat Abdul Wahab R, Idris H, Yacob H, Zainal Ariffin SH. Comparison of self- and conventional-ligating brackets in the alignment stage. European Journal of Orthodontics. 2012;34(2):176-81.	Excluded; alignment of upper anterior teeth
Nagalakshmi S, Sriram G, Balachandar K, Dhayanithi D. A comparative evaluation of mandibular incisor decrowding with coaxial and optiflex arch wires and their load-deflection rates. Journal of Pharmacy and Bioallied Sciences. 2014;6(SUPPL. 1):S118-S21.	Excluded; not RCT
O'Brien K, Lewis D, Shaw W, Combe E. A clinical trial of aligning archwires. European Journal of Orthodontics. 1990;12(4):380-4.	Excluded; alignment of upper anterior teeth
O'Dywer L, Littlewood SJ, Rahman S, Spencer RJ, Barber SK, Russell JS. A multi-center randomized controlled trial to compare a self-ligating bracket with a conventional bracket in a UK population: Part 1: Treatment efficiency. Angle Orthodontist. 2016;86(1):142-8.	Excluded; no lower anterior teeth alignment rate or time
Ong E, McCallum H, Griffin MP, Ho C. Efficiency of self-ligating vs conventionally ligated brackets during initial alignment. Am J Orthod Dentofacial Orthop. 2010;138(2):138.e1-7; discussion -9.	Excluded; retrospective study
Sandhu SS, Sandhu J. Arandomized clinical trial investigating pain associated with superelastic nickel-titanium and multistranded stainless steel archwires during the initial leveling and aligning phase of orthodontic treatment. Journal of Orthodontics. 2013;40(4):276-85.	Excluded; no lower anterior teeth alignment rate or time
Pandis N, Polychronopoulou A, Makou M, Eliades T. Mandibular dental arch changes associated with treatment of crowding using self-ligating and conventional brackets. Eur J Orthod. 2010;32(3):248-53.	Excluded; no lower anterior teeth alignment rate or time

Pandis N, Polychronopoulou A, Sifakakis I, Makou M, Eliades T. Effects of levelling of the curve of Spee on the proclination of mandibular incisors and expansion of dental arches: a prospective clinical trial. Aust Orthod J. 2010;26(1):61-5.	Excluded; no lower anterior teeth alignment rate or time
Quintao CCA, Jones ML, Menezes LM, Koo D, Elias CN. A prospective clinical trial to compare the performance of four initial orthodontic archwires. Korean journal of orthodontics. 2005;35(5):381-7.	Excluded; no lower anterior teeth alignment rate or time
Koroluk LD. A randomized clinical trial investigating pain associated with superelastic nickel-titanium and multistranded stainless steel archwires during the initial leveling and aligning phase of orthodontic treatment. Journal of Orthodontics. 2013;40(4):273.	Excluded; no lower anterior teeth alignment rate or time
Joseph J, Ninan VS, Abraham ME, John J, Cherian KK, Thomas RM. Arch Expansion Efficiency of Coaxial Tubular Superelastic Nickel-Titanium in Comparison to Single-Stranded Superelastic Nickel-Titanium While Relieving Mandibular Anterior Crowding: A Randomized Controlled Study. Journal of International Society of Preventive & Community Dentistry. 2019;9(1):60-4.	Excluded; no lower anterior teeth alignment rate or time
Yildirim K, Saglam-Aydinatay B. Comparative assessment of treatment efficacy and adverse effects during nonextraction orthodontic treatment of Class I malocclusion patients with direct and indirect bonding: A parallel randomized clinical trial. Am J Orthod Dentofacial Orthop 2018;154(1):26-+.	Excluded; no lower anterior teeth alignment rate or time
Evans TJ, Jones ML, Newcombe RG. Clinical comparison and performance perspective of three aligning arch wires. Am J Orthod Dentofacial Orthop 1998;114(1):32-9.	Excluded; no lower anterior teeth alignment rate or time
West AE, Jones ML, Newcombe RG. MULTIFLEX VERSUS SUPERELASTIC - A RANDOMIZED CLINICAL-TRIAL OF THE TOOTH ALIGNMENT ABILITY OF INITIAL ARCH WIRES. Am J Orthod Dentofacial Orthop 1995;108(5):464-71.	Excluded; no lower anterior teeth alignment rate or time
Kau CH, Kantarci A, Shaughnessy T, Vachiramon A, Santiwong P, de la Fuente A, et al. Photobiomodulation accelerates orthodontic alignment in the early phase of treatment. Prog Orthod. 2013;14:30.	Excluded; not RCT
Shaughnessy T, Kantarci A, Kau CH, Skrenes D, Skrenes S, Ma D. Intraoral photobiomodulation-induced orthodontic tooth alignment: a preliminary study. Bmc Oral Health. 2016;16.	Excluded; not RCT
Ren X, Li J, Zhao Y, Li H, Lei L. Torque expression by active and passive self-ligating brackets in patients with four premolar extractions: A retrospective study. Orthodontics & Craniofacial Research.23(4):509-16.	Excluded; retrospective study
Wichelhaus A, Dulla M, Sabbagh H, Baumert U, Stocker T. Stainless steel and NiTi torque archwires and apical root resorption. Journal of Orofacial Orthopedics-Fortschritte Der Kieferorthopadie. 2020.	Excluded; retrospective study
Aksoy A, Cesur MG, Dagdeviren BH, Ozkaynak YA, Karacin G, Gultekin F. Assessment of Pain, Anxiety, and Cortisol Levels During the Initial Aligning Phase of Fixed Orthodontic Treatment. Turkish Journal of Orthodontics.32(1):34-40.	Excluded; not RCT
Alexander L, Kommi PB, Arani N, Mathew A, Yashwant A, Senkutvan RS. Evaluation and comparison of surface characteristics of commercially available TMA wires using scanning electron microscopy and optical profilometer. Indian journal of dental research. 2019;30(4):548-52.	Excluded; not RCT
Alsabti N, Bourauel C, Talic N. Comparison of force loss during sliding of low friction and conventional TMA orthodontic archwires : An in vitro study. Journal of Orofacial Orthopedics. 2020;02:02.	Excluded; not RCT
Alsabti N, Talic N. Comparison of static friction and surface topography of low friction and conventional TMA orthodontic arch wires: An in-vitro study. Saudi Dental Journal. 2020.	Excluded; not RCT
Amaya S, Perez A, Guzman H, Espinosa A, Motta G, Mojica J, et al. Changes in the mechanical properties of two nickel-titanium archwires after 3 months of clinical usage. Journal of the World Federation of Orthodontists. 2020;9(4):175-80.	Excluded; not RCT
Amm EW, Antoszewska-Smith J, Boley J. Canine substitution of congenitally missing maxillary lateral incisors in Class I and Class III malocclusions by using skeletal anchorage. American journal of orthodontics and dentofacial orthopedics : official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics. 2019;156(4):512-21.	Excluded; not RCT
Ata-Ali F, Ata-Ali J, Lanuza-Garcia A, Ferrer-Molina M, Melo M, Plasencia E. Clinical outcomes of lingual fully customized vs labial straight wire systems : Assessment based on American Board of Orthodontics criteria. Journal of Orofacial Orthopedics. 2020;08:08.	Excluded; not RCT
Deana NF, Alves N, Sandoval P. Effectiveness of photobiomodulation therapy in accelerating orthodontic tooth movement: A meta-analysis of randomized clinical trials. Journal of Oral Research. 2019;8(5):416-32.	Excluded; not RCT
Jung M-H. Factors influencing treatment efficiency. The Angle orthodontist. 2020.	Excluded; not RCT
Kawaguchi K, Iijima M, Muguruma T, Endo K, Mizoguchi I. Effects of bioactive glass coating by electrophoretic deposition on esthetical, bending, and frictional performance of orthodontic stainless steel wire. Dental materials journal. 2020.	Excluded; not RCT
Ledra IM, Gandini LG, Martins RP. Expansion with transpalatal arch or continuous arch mechanics. American journal of orthodontics and dentofacial orthopedics : official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics. 2020;157(5):611-8.	Excluded; not RCT
Manikandan S, Chandra D, Gnanashanmugam K, Sivakumar S. Evaluation of intrusive forces created by 'v' bend, intrusion arches at various deflections: An invitro study. Indian Journal of Public Health Research and Development. 2020;11(2):523-6.	Excluded; not RCT
Moyano J, Montagut D, Perera R, Fernandez-Bozal J, Puigdollers A. Comparison of changes in the dental transverse and sagittal planes between patients treated with self-ligating and with conventional brackets. Dental press journal of orthodontics. 2020;25(1):47-55.	Excluded; not RCT
Nahas-Scocate ACR, Neves MB, de Souza LT, de Cerqueira Kasaz A, Listik E, da Silva HDP, et al. An in vitro assessment of the influences of different wire materials and bracket systems when correcting dental crowding. Journal of Materials Science: Materials in Medicine. 2020;31(11).	Excluded; not RCT
Palone M, Spedicato GA, Lombardo L. Analysis of tooth anatomy in adults with ideal occlusion: A preliminary study. American Journal of Orthodontics & Dentofacial Orthopedics.157(2):218-27.	Excluded; not RCT

Pradal A, Nucci L, Derton N, De Felice ME, Turco G, Grassia V, et al. Mechanical evaluation of the stability of one or two miniscrews under loading on synthetic bone. Journal of functional biomaterials. 2020;11(4).	Excluded; not RCT
Sar SK, Shetty D, Kumar P, Juneja S, Sharma P. Leptin levels in gingival crevicular fluid during canine retraction: in vivo comparative study. Journal of orthodontics. 2019;46(1):27-33.	Excluded; not RCT
Soboku T, Motegi E, Sueishi K. Effect of Different Bracket Prescriptions on Orthodontic Treatment Outcomes Measured by Three-dimensional Scanning. Bulletin of Tokyo Dental College.60(2):69-80.	Excluded; not RCT
Yazicioglu S, Oz AA, Oz AZ, Arici N, Ozer M, Arici S. Buccolingual Inclination Effects of Self-Ligating and Conventional Premolar Brackets: A Cone Beam Computed Tomography Study. Turkish Journal of Orthodontics.33(2):110-4.	Excluded; not RCT
Abu Alhaija ES, Taha NA. A comparative study of initial changes in pulpal blood flow between conventional and self-ligating fixed orthodontic brackets during leveling and alignment stage. Clinical oral investigations. 2020.	Excluded; no lower anterior teeth alignment rate or time
Abu Alhaija ESJ, Al-Abdallah SY, Taha NA. A comparative study of initial changes in pulpal blood flow between clear aligners and fixed orthodontic appliances. American journal of orthodontics and dentofacial orthopedics. 2019;156(5):603-10.	Excluded; no lower anterior teeth alignment rate or time
Afshar MK, Safarian F, Torabi M, Farsinejad A, Mohammadzadeh I. Comparison of TNF-alpha and IL-1beta concentrations in gingival crevicular fluid during early alignment stage of orthodontic treatment in adults and adolescents. Pesquisa Brasileira em Odontopediatria e Clinica Integrada. 2020;20:1-8.	Excluded; no lower anterior teeth alignment rate or time
Al Shayea EI. Comparative Assessment between Ibuprofen, Chewing Gum, and Bite Wafers in Pain Control Following First Archwire Placement in Orthodontic Patients. The journal of contemporary dental practice. 2020;21(4):416-20.	Excluded; no lower anterior teeth alignment rate or time
Alqareer A, Alyahya A, Al-Anezi SA, AlAwadhi A, Al Qabandi S, Alyaseen M. Efficacy of Chewing Gum to Reduce Orthodontic Pain Compared to Placebo: A Blinded, Parallel-Group, Preliminary Clinical Trial. Journal of Oral & Facial Pain and Headache. 2019;33(3):301-7.	Excluded; no lower anterior teeth alignment rate or time
AlSayed Hasan MMA, Sultan K, Ajaj M, Voborná I, Hamadah O. Low-level laser therapy effectiveness in reducing initial orthodontic archwire placement pain in premolars extraction cases: a single-blind, placebo-controlled, randomized clinical trial. BMC oral health. 2020;20(1):1-9.	Excluded; no lower anterior teeth alignment rate or time
Alyassiri HAA, Kamaruddin AF, Ismail K, Shafiai NAA, Rahman NA, Ahmad WMAW. Preliminary result of randomised controlled trial of three different coated archwires Part 1: Tooth alignment and coating loss. Malaysian Journal of Medicine and Health Sciences. 2020;16:1-8.	Excluded; no lower anterior teeth alignment rate or time
Arash V, Teimoorian M, Farajzadeh Jalali Y, Sheikhzadeh S. Clinical comparison between Multi-Stranded Wires and Single strand Ribbon wires used for lingual fixed retainers. Progress in orthodontics. 2020;21(1):22.	Excluded; no lower anterior teeth alignment rate or time
Babanouri N, Ajami S, Salehi P. Effect of mini-screw-facilitated micro-osteoperforation on the rate of orthodontic tooth movement: a single-center, split-mouth, randomized, controlled trial. Progress in Orthodontics. 2020;21(1).	Excluded; no lower anterior teeth alignment rate or time
Bashir R, Sonar S, Batra P, Srivastava A, Singla A. Comparison of transverse maxillary dental arch width changes with self-ligating and conventional brackets in patients requiring premolar extraction - A randomised clinical trial. International orthodontics. 2019;17(4):687-92.	Excluded; no lower anterior teeth alignment rate or time
Bidwai P, Kamble RH. "Comparative evaluation of rate of canine retraction in corticotomy facilitated orthodontics and piezocision technique in adult population" - Protocol for a in vivo study. European Journal of Molecular and Clinical Medicine. 2020;7(2):2144-50.	Excluded; no lower anterior teeth alignment rate or time
Charavet C. Localized Piezoelectric Alveolar Decortication for Orthodontic Treatment in Adults: a Randomized Controlled Trial. Journal of oral and maxillofacial surgery. 2019;77(9):e56-e7.	Excluded; no lower anterior teeth alignment rate or time
Chen H, Han B, Jiang R, Su H, Feng T, Teng F, et al. PASS versus MBTTM for evaluation of anchorage control in three-dimensional measurements: a randomized controlled trial. European journal of orthodontics. 2020.	Excluded; no lower anterior teeth alignment rate or time
Costa Lima KC, Benini Paschoal MA, Gurgel JdA, Salvatore Freitas KM, Maio Pinzan-Vercelino CR. Comparative analysis of microorganism adhesion on coated, partially coated, and uncoated orthodontic archwires: A prospective clinical study. American Journal of Orthodontics and Dentofacial Orthopedics. 2019;156(5):611-6.	Excluded; no lower anterior teeth alignment rate or time
Delavarian M, Imani MM, Delavarian F, Bayani S. Comparison of chewing gum and ibuprofen in alleviating orthodontic pain: a single centre, randomised clinical trial. Australasian Orthodontic Journal. 2020;36(1):38-44.	Excluded; no lower anterior teeth alignment rate or time
Diddige R, Negi G, Kiran KVS, Chitra P. Comparison of pain levels in patients treated with 3 different orthodontic appliances - a randomized trial. Medicine and Pharmacy Reports. 2020;93(1):81-8.	Excluded; no lower anterior teeth alignment rate or time
El-Timamy A, El Sharaby F, Eid F, El Dakroury A, Mostafa Y, Shaker O. Effect of platelet-rich plasma on the rate of orthodontic tooth movement: A split-mouth randomized trial. Angle Orthodontist. 2020;90(3):354-61.	Excluded; no lower anterior teeth alignment rate or time
Farid KA, Eid AA, Kaddah MA, Elsharaby FA. The effect of combined corticotomy and low level laser therapy on the rate of orthodontic tooth movement: Split mouth randomized clinical trial. Laser Therapy. 2019;28(4):275-83.	Excluded; no lower anterior teeth alignment rate or time
Gnaneswar SM, Sridhar P. Comparison of dual-dimensional and rectangular wires in terms of space closure and anchorage loss during retraction with miniimplants: A prospective clinical study. Journal of dental research, dental clinics, dental prospects. 2020;14(1):54-60.	Excluded; no lower anterior teeth alignment rate or time
Goymen M, Gulec A. Effect of photobiomodulation therapies on the root resorption associated with orthodontic forces: a pilot study using micro computed tomography. Clinical oral investigations. 2020;24(4):1431-8.	Excluded; no lower anterior teeth alignment rate or time

Hasan MMAA, Sultan K, Ajaj M, Voborna I, Hamadah O. Low-level laser therapy effectiveness in reducing initial orthodontic archwire placement pain in premolars extraction cases: a single-blind, placebo-controlled, randomized clinical trial. Bmc Oral Health. 2020;20(1).	Excluded; no lower anterior teeth alignment rate or time
Lai T-T, Chiou J-Y, Lai T-C, Chen T, Wang H-Y, Li C-H, et al. Perceived pain for orthodontic patients with conventional brackets or self-ligating brackets over 1 month period: A single-center, randomized controlled clinical trial. Journal of the Formosan Medical Association. 2020;119(1):282-9.	Excluded; no lower anterior teeth alignment rate or time
Maan AS, Patil AK. Assessment of salivary interleukin-1beta (IL-1beta), prostaglandin E₂ (PGE₂) levels and pain intensity in children and adults during initial orthodontic treatment. Journal of Orthodontic Science.8:16.	Excluded; no lower anterior teeth alignment rate or time
Mollabashi V, Farmany A, Alikhani MY, Sattari M, Soltanian AR, Kahvand P, et al. Effects of tio2-coated stainless steel orthodontic wires on streptococcus mutans bacteria: a clinical study. International journal of nanomedicine. 2020;15:8759-66.	Excluded; no lower anterior teeth alignment rate or time
Monini AdC, Gandini LG, Jr., Vianna AP, Martins RP, Jacob HB. Tooth movement rate and anchorage lost during canine retraction: A maxillary and mandibular comparison. Angle Orthodontist. 2019;89(4):559-65.	Excluded; no lower anterior teeth alignment rate or time
Pacheco AAR, Collins JR, Contreras N, Lantigua A, Pithon MM, Tanaka OM. Distalization rate of maxillary canines in an alveolus filled with leukocyte-platelet-rich fibrin in adults: A randomized controlled clinical split-mouth trial. American Journal of Orthodontics and Dentofacial Orthopedics. 2020;158(2):182-91.	Excluded; no lower anterior teeth alignment rate or time
Ramaiah PT, Thomas T, Hanumanthaiah S, Dakshina CK, Sabu JK, Subramonia S. Use of single-dose low-level laser therapy for pain control on initial archwire activation of orthodontic appliance: A randomized control clinical trial. World Journal of Dentistry. 2019;10(3):214-8.	Excluded; no lower anterior teeth alignment rate or time
Ren C, McGrath C, Gu M, Jin L, Zhang C, Sum F, et al. Low-level laser-aided orthodontic treatment of periodontally compromised patients: a randomised controlled trial. Lasers in medical science. 2020;35(3):729-39.	Excluded; no lower anterior teeth alignment rate or time
Reyes Pacheco AA, Collins JR, Contreras N, Lantigua A, Pithon MM, Tanaka OM. Distalization rate of maxillary canines in an alveolus filled with leukocyte-platelet-rich fibrin in adults: a randomized controlled clinical split-mouth trial. American journal of orthodontics and dentofacial orthopedics. 2020;158(2):182-91.	Excluded; no lower anterior teeth alignment rate or time
Sahu A, Bhosale V, Ghunawat D, Madanshetty P, Rathi G, Khan MY. An In Vivo Randomized Clinical Evaluation of the Surface Morphology of Nickel-Titanium, Beta-titanium, and Copper-Nickel-Titanium. The journal of contemporary dental practice. 2020;21(6):636-9.	Excluded; no lower anterior teeth alignment rate or time
Shahrin AA, Ghani SHA, Norman NH. Pain experience with micro-osteoperforations during initial orthodontic alignment: A randomized clinical trial. Journal of Oral Research. 2020;9(4):309-18.	Excluded; no lower anterior teeth alignment rate or time
Shetty M, Sangamesh B, Shetty A, Patil A. Evaluation of the efficacy of bite wafer chewing in pain reduction in fixed orthodontic appliance treatment. Gulhane Medical Journal. 2020;62:28-32.	Excluded; no lower anterior teeth alignment rate or time
Wang G, Li M, Han Y, Xu X, Xu J, Huang S. Comparison of the impacts of two kinds of brackets on external apical root resorption in orthodontic treatment of bimaxillary protrusion patients. Chinese journal of tissue engineering research. 2021;25(10):1539-44.	Excluded; no lower anterior teeth alignment rate or time
Xiao L, Chen R-X, Luo N. Effect of single low level laser therapy on initial pain during fixed orthodontic treatment. Shanghai kou qiang yi xue = Shanghai journal of stomatology. 2019;28(5):549-52.	Excluded; no lower anterior teeth alignment rate or time
Yilmaz HN, Alakus E, Erdem B, Kucukkeles N. Effect of piezocision on molar intrusion in open-bite treatment using a modified MEAW technique. Journal of Orofacial Orthopedics. 2020;25:25.	Excluded; no lower anterior teeth alignment rate or time
Zhao CY, Wang YY, Wang MY, Wang J, Huo F, Wang P. Effect of double chain method on the closure of orthodontic teeth. [Chinese]. Acta Anatomica Sinica. 2020;51(1):66-71.	Excluded; no lower anterior teeth alignment rate or time
Atik E, Gorucu-Coskuner H, Akarsu-Guven B, Taner T. A comparative assessment of clinical efficiency between premium heat-activated copper nickel-titanium and superelastic nickel-titanium archwires during initial orthodontic alignment in adolescents: a randomized clinical trial. Progress in orthodontics. 2019;20(1):46.	Excluded; alignment of upper anterior teeth

RCT, randomised clinical trial.

Appendix Table 2.2 Detailed assessment of included randomized trials with the Risk-of-Bias 2.0 tool.

Domain	Reference	Abdelrahman 2015	Aydın 2018	Bansal 2019	Celikoglu 2014	Charavet 2019	Cobb 1998	de Araujo Gurgel 2020	El Shehawya 2020	Fleming 2009	Gibreal 2019	Huang 2010	Irvine 2004
1. Randomization process	**1.1**	NI	Y	Y	Y	Y	Y	Y	Y	Y	Y	NI	Y
	1.2	NI	NI	Y	NI	Y	NI	Y	PY	PY	Y	NI	NI
	1.3	N	PN	N	N	N	N	PN	PN	N	N	NI	N
	Assessor's Judgement	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns	Low	Low	Low	Low	Some concerns	Some concerns
2. Deviations from intended interventions	**2.1**	N	N	Y	Y	NI	NI	N	NI	NI	Y	NI	NI
	2.2	N	Y	Y	NI	NI	NI	Y	NI	NI	Y	NI	NI
	2.3	NA	N	N	N	N	PN	N	PN	N	N	PN	PN
	2.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2.6	PY	PY	Y	PY	Y	PY	PY	PY	Y	Y	PY	Y
	2.7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low
3. Mising outcome data	**3.1**	Y	PY	Y	PY	Y	PY	PY	PN	Y	Y	Y	PY
	3.2	NA	NA	NA	NA	NA	NA	NA	PN	NA	NA	NA	NA
	3.3	NA	NA	NA	NA	NA	NA	NA	PN	NA	NA	NA	NA
	3.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low
4. Measurement of the outcome	**4.1**	N	N	N	N	N	N	N	N	N	N	N	N
	4.2	N	N	N	N	N	N	N	N	N	N	PN	N
	4.3	N	NI	N	N	NI	NI	N	N	NI	N	NI	NI
	4.4	NA	PN	NA	NA	N	N	NA	NA	N	NA	PN	N
	4.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low
5. Selection of the reported result	**5.1**	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	PY	Y
	5.2	N	N	N	N	N	N	N	N	N	N	PN	N
	5.3	N	N	N	N	N	N	N	N	N	N	PN	N
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low
Overall	**Assessor's Judgement**	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns	Low	Low	Low	Low	Some concerns	Some concerns
	General Note	-	-	-	-	Incomplete reporting of alignment duration (no means & SDs)	Incomplete reporting of alignment duration and rate (no means & SDs)	-	-	-	-	Incomplete reporting for alignment duration (no SDs)	-

Y=yes; PY=probably yes; PN=probably no; N=no; NA=not applicable; NI=no information

Appendix Table 2.2 (continued) **Detailed assessment of included randomized trials with the Risk-of-Bias 2.0 tool.**

Domain	Reference	Little 2017	Jahanbin 2019	Miles 2005	Miles 2006	Miles 2012	Mahmoudzadeh 2018	Mandall 2006	Nabbat 2020	Nahas 2017	Nordstrom 2018	Ong 2011	Pandis 2007
1. Randomization process	**1.1**	Y	Y	N	N	Y	Y	Y	Y	PY	Y	Y	NI
	1.2	Y	Y	N	N	NI	Y	Y	Y	PY	NI	Y	NI
	1.3	N	N	N	N	N	N	N	N	N	N	N	N
	Assessor's Judgement	Low	Low	High	High	Some concerns	Low	Low	Low	Low	Some concerns	Low	Some concerns
2. Deviations from intended interventions	**2.1**	Y	NI	NI	NI	NI	N	NI	Y	NI	N	N	NI
	2.2	Y	NI	NI	NI	N	Y	Y	Y	NI	N	Y	NI
	2.3	N	PN	PN	PN	N	N	N	N	N	NA	N	N
	2.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2.6	Y	Y	Y	Y	PY	Y	Y	PN	Y	NI	Y	Y
	2.7	NA	NA	NA	NA	NA	NA	NA	N	NA	NI	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Some concerns	Low	High	Low	Low
3. Mising outcome data	**3.1**	Y	Y	Y	Y	Y	Y	Y	PY	PY	NI	Y	Y
	3.2	NA	NA	NA	NA	NA	NA	NA	NA	NA	PN	NA	NA
	3.3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NI	NA	NA
	3.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NI	NA	NA
	Assessor's judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low	High	Low	Low
4. Measurement of the outcome	**4.1**	N	N	N	N	N	N	N	N	N	N	N	N
	4.2	N	N	N	N	N	N	N	N	N	N	N	N
	4.3	N	NI	NI	NI	N	N	Y	N	N	N	N	NI
	4.4	NA	N	NA	NA	NA	NA	PN	NA	NA	NA	NA	N
	4.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low
5. Selection of the reported result	**5.1**	Y	PY	PY	PY	Y	Y	Y	Y	Y	Y	Y	Y
	5.2	N	N	N	N	N	N	N	N	PN	N	N	N
	5.3	N	N	N	N	N	N	N	N	N	N	N	N
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low
Overall	**Assessor's Judgement**	Low	Low	High	High	Some concerns	Low	Low	Some concerns	Low	High	Low	Some concerns
	General Note	-	-	Incomplete reporting for alignment rate (noSDs)	Incomplete reporting for alignment rate (no SDs)	-	-	-	-	-	Incomplete reporting for alignment rate (no means and SDs)	-	-

Y=yes; PY=probably yes; PN=probably no; N=no; NA=not applicable; NI=no information

Appendix Table 2.2 (continued) **Detailed assessment of included randomized trials with the Risk-of-Bias 2.0 tool.**

Domain	Reference	Pandis 2009	Sandhu 2012	Scott 2008	Sebastian 2012	Sebastian 2019	Serafim 2015	Sirri 2020	Songra 2014	Ulhaq 2017	Uribe 2017	Woodhouse 2015
1. Randomization process	**1.1**	Y	NI	Y	Y	Y	PY	Y	Y	Y	Y	Y
	1.2	Y	NI	NI	Y	Y	PY	Y	Y	Y	Y	Y
	1.3	N	N	N	N	N	PN	N	PN	N	N	N
	Assessor's Judgement	Low	Some concerns	Low	Low	Low	Low	Low	Low	Low	Low	Low
2. Deviations from intended interventions	**2.1**	N	NI	NI	N	N	NI	Y	Y	Y	NI	NI
	2.2	N	NI	NI	PN	Y	NI	N	Y	Y	NI	NI
	2.3	NA	N	N	NA	N	PN	N	N	N	N	N
	2.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2.6	Y	Y	Y	Y	Y	PN	Y	Y	Y	PN	Y
	2.7	NA	NA	NA	NA	NA	NI	NA	NA	NA	NI	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	High	Low	Low	Low	High	Low
3. Mising outcome data	**3.1**	Y	Y	Y	Y	Y	PN	Y	Y	Y	PN	Y
	3.2	NA	NA	NA	NA	NA	PN	NA	NA	NA	PN	NA
	3.3	NA	NA	NA	NA	NA	NI	NA	NA	NA	PY	NA
	3.4	NA	NA	NA	NA	NA	NI	NA	NA	NA	PN	NA
	Assessor's judgement	Low	Low	Low	Low	Low	High	Low	Low	Low	Some concerns	Low
4. Measurement of the outcome	**4.1**	N	N	N	N	PN	N	N	N	N	N	N
	4.2	N	N	N	N	PN	PN	N	N	N	N	N
	4.3	PN	N	N	N	N	N	N	N	N	N	N
	4.4	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	4.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low	Low
5. Selection of the reported result	**5.1**	Y	Y	Y	Y	PY	PY	Y	Y	Y	Y	Y
	5.2	N	N	N	N	N	PN	N	N	N	N	N
	5.3	N	N	N	N	N	PN	N	N	N	N	N
	Assessor's Judgement	Low	Low	Low	Low	Low		Low	Low	Low	Low	Low
Overall	**Assessor's Judgement**	Low	Some concerns	Low	Low	Low	High	Low	Low	Low	High	Low
	General Note	Incomplete reporting for alignment duration (no SDs)	-	-	-	-	Incomplete reporting for alignment duration (no means & SDs)	-	-	-	-	

Y=yes; PY=probably yes; PN=probably no; N=no; NA=not applicable; NI=no information

Chapter 3

Appendix 3.1. Search strategies applied for each database

MEDLINE (Ovid) Search Strategy (searched from 1946 to 09/07/2021)

1 orthodon$.mp.
2 orthodontic.ti,ab.
3 orthodontic wire$.mp..
4 orthodontic tooth movement.mp. or exp Tooth Movement Techniques/
5 tooth displacement.mp.
6 "orthodontic wire$".mp.
7 orthodontic treatment.mp..
8 orthodontic Therapy.mp.
9 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8
10 retract$.mp.
11 canine retraction.mp.
12 space closure.mp.
13 10 or 11 or 12
14 9 and 13

This subject search was linked to the Cochrane Highly Sensitive Search Strategy (CHSSS) for identifying randomized trials in MEDLINE: sensitivity maximising version (2008 revision) as referenced in Chapter 6.4.11.1 and detailed in box 6.4.c of The Cochrane Handbook for Systematic Reviews of Interventions, Version 5.1.0 [updated March 2011](Higgins 2011).

1. randomized controlled trial.pt.
2. controlled clinical trial.pt.
3. randomized.ab.
4. placebo.ab.
5. drug therapy.fs.
6. randomly.ab.
7. trial.ab.
8. groups.ab.
9. or/1-8
10. exp animals/ not humans.sh.
11. 9 not 10

Embase (Ovid) search strategy (searched from 1947 to 09/07/2021)

1 orthodontic.ti,ab.
2 orthodon*.mp.
3 orthodontic wire$.mp.
4 orthodontic tooth movement.mp. or exp orthodontic tooth movement/
5 tooth movement.mp.
6 tooth displacement.mp.
7 orthodontic treatment.mp.
8 orthodontic Therapy.mp.
9 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8
10 retract$.mp.
11 canine retraction$.mp.
12 space closure.mp.
13 10 or 11 or 12
14 9 and 13

This subject search was linked to an adapted version of the Cochrane Centralised Search Project filter for identifying RCTs in Embase Ovid
1. Randomized controlled trial/
2. Controlled clinical study/
3. Random$.ti,ab.
4. randomization/
5. intermethod comparison/
6. placebo.ti,ab.
7. (compare or compared or comparison).ti.
8. ((evaluated or evaluate or evaluating or assessed or assess) and (compare or compared or comparing or comparison)).ab.
9. (open adj label).ti,ab.
10. ((double or single or doubly or singly) adj (blind or blinded or blindly)).ti,ab.
11. double blind procedure/
12. parallel group$1.ti,ab.
13. (crossover or cross over).ti,ab.
14. ((assign$ or match or matched or allocation) adj5 (alternate or group$1 or intervention$1 or patient$1 or subject$1 or participant$1)).ti,ab.
15. (assigned or allocated).ti,ab.
16. (controlled adj7 (study or design or trial)).ti,ab.
17. (volunteer or volunteers).ti,ab.

18. trial.ti.

19. or/1-18

20. (exp animal/ or animal.hw. or nonhuman/) not (exp human/ or human cell/ or (human or humans).ti.)

21. 19 not 20

Cochrane database of systematic reviews (CDSR) search strategy (searched on 09/07/2021) via Cochrane Library

1 orthodon*

2 [Orthodontic Space Closure] MeSH

3 retraction

4 canine retraction

5 retract*

6 2 OR 3 OR 4 OR 5

7 1 AND 6

Cochrane Central Register of controlled trials (CENTRAL) search strategy (searched on 09/07/2021) via Cochrane Library

1 orthodon*

2 [Orthodontic Space Closure] MeSH

3 retraction

4 canine retraction

5 retract*

6 2 OR 3 OR 4 OR 5

7 1 AND 6

Scopus search strategy (searched from 1960- 09/07/2021)

orthodont* AND random* AND (retract* OR "canine retraction*" OR "space closure*")

Web of Science search strategy (searched from 1900- 09/07/2021)

orthodont* AND random* AND (retract* OR "canine retraction*" OR "space closure*")

LILACS search strategy (searched on 09/07/2021)

http://pesquisa.bvsalud.org/portal/?lang=en

orthodont* AND random* AND (retract* OR "canine retraction*" OR "space closure*")

The Database of Abstracts of Reviews of Effects (DARE)

https://www.crd.york.ac.uk/CRDWeb

Appendix Table 3.1. List of excluded studies by full text with reasons.

Paper	Status
Excluded by full text with reasons	
Abohabib, A. M., et al. (2018). "Effects of low-intensity laser therapy on the stability of orthodontic mini-implants: a randomised controlled clinical trial." Journal of Orthodontics 45(3): 149-156.	Excluded; no canine retraction duration or rate
Abu-Shahba, R. and A. Alassiry (2019). "Comparative evaluation of the maxillary canine retraction rate and anchorage loss between two types of self-ligating brackets using sliding mechanics." Journal of orthodontic science **8**: 3.	Excluded; CBCT measurements
Addanki, P., et al. (2017). "Clinical and Radiographic Comparative Evaluation of Buccal and Palatal Corticotomy with Buccal Corticotomy in Periodontally Accelerated Osteogenic Orthodontics with Surgical Bur." Contemporary Clinical Dentistry **8**(2): 321-326.	Excluded; unclear inclusion criteria (enmasse retraction)
Al-Hasani, N. R., et al. (2011). CLINICAL EFFICACY OF LOCALLY INJECTED CALCITRIOL IN ORTHODONTIC TOOTH MOVEMENT Research Article	Excluded; the split mouth design was not randomized
Al-Ainawi, K. I., et al. (2016). "The Effect of Using a Modified Dentoalveolar Distractor on Canine Angulation following Rapid Canine Retraction: A Split-mouth Design Randomized Controlled Trial." J Contemp Dent Pract **17**(1): 49-57.	Excluded; segmented arch mechanics
Alfawal, A. M. H., et al. (2020). "Evaluation of patient-centered outcomes associated with the acceleration of canine retraction by using minimally invasive surgical procedures: a randomized clinical controlled trial." Dental and Medical Problems 57(3): 285-293.	Excluded; no canine retraction duration or rate
Alhadlaq, A., et al. (2016). "Anchorage condition during canine retraction using transpalatal arch with continuous and segmented arch mechanics." Angle Orthodontist **86**(3): 380-385.	Excluded; segmented arch mechanics
Al-Sibaie, S. and M. Y. Hajeer (2014). "Assessment of changes following en-masse retraction with mini-implants anchorage compared to two-step retraction with conventional anchorage in patients with class II division 1 malocclusion: a randomized controlled trial." European Journal of Orthodontics **36**(3): 275-283.	Excluded; en-masse retraction
Arantes, G. M., et al. (2009). "Tenoxicam controls pain without altering orthodontic movement of maxillary canines." Orthodontics & Craniofacial Research 12(1): 14-19.	Excluded; cross-over trial
Attri, S., et al. (2018). "Comparison of rate of tooth movement and pain perception during accelerated tooth movement associated with conventional fixed appliances with micro-osteoperforations–a randomised controlled trial." Journal of Orthodontics **45**(4): 225-233.	Excluded; en-masse retraction
Baeshen, H. A. (2020). "The Effect of Partial Corticotomy on the Rate of Maxillary Canine Retraction: Clinical and Radiographic Study." Molecules (Basel, Switzerland) 25(20).	Excluded; non-randomized clinical trial
Barsoum, H. A., et al. (2021). "Comprehensive comparison of canine retraction using NiTi closed coil springs vs elastomeric chains: A split-mouth randomized controlled trial." Angle Orthodontist 91(4): 441-448.	Excluded; segmented arch mechanics
Bidwai, P. and R. H. Kamble (2020). "Comparative evaluation of rate of canine retraction in corticotomy facilitated orthodontics and piezocision technique in adult population - protocol for a in vivo study." European Journal of Molecular and Clinical Medicine 7(2): 2144-2150	Excluded; no canine retraction duration or rate (protocol)
Boester, C. H. and L. E. Johnston (1974). "A clinical investigation of the concepts of differential and optimal force in canine retraction." Angle Orthodontist **44**(2): 113-119.	Excluded; segmented arch mechanics
Bokas, J. and M. Woods (2006). "A clinical comparison between nickel titanium springs and elastomeric chains." Australian orthodontic journal 22(1): 39-46.	Excluded; non-randomized clinical trial
Borsos, G., et al. (2011). "Comparison of bone-borne and tooth tissue-borne anchorage during the maxillary canine retraction in growing patients: a randomised clinical trial." Timisoara medical journal **61**(1-2): 98-101.	Excluded; Cephalometric measurements
Borsos, G., et al. (2012). "Tooth movement using palatal implant supported anchorage compared to conventional dental anchorage." Anatomischer Anzeiger [Annals of anatomy] **194**(6): 556-560.	Excluded; Cephalometric measurements
Burrow, S. J. (2010). "Canine retraction rate with self-ligating brackets vs conventional edgewise brackets." Angle Orthodontist 80(4): 438-445.	Excluded; non-randomized clinical trial
Cetinsahin, A., et al. (2010). "Effects of the zygoma anchorage system on canine retraction." European Journal of Orthodontics **32**(5): 505-513.	Excluded; segmented arch mechanics
Chen, H., et al. (2020). "PASS versus MBTTM for evaluation of anchorage control in three-dimensional measurements: a randomized controlled trial." European Journal of Orthodontics.	Excluded; no canine retraction duration or rate
Chopra, S. S., et al. (2017). "Comparative evaluation of anchorage reinforcement between orthodontic implants and conventional anchorage in orthodontic management of bimaxillary dentoalveolar protrusion." Medical journal, Armed Forces India **73**(2): 159-166.	Excluded; Cephalometric measurements
Chung, S. E., et al. (2015). "The effect of light emitting diode phototherapy on rate of orthodontic tooth movement: a split mouth, controlled clinical trial." J Orthod **42**(4): 274-283.	Excluded; extraction of 5s
da C Monini, A., et al. (2019). "Tooth movement rate and anchorage lost during canine retraction: A maxillary and mandibular comparison." The Angle orthodontist **89**(4): 559-565.	Excluded; Cephalometric measurements
Da Costa Monini, A., et al. (2014). "Canine retraction and anchorage loss: Self-ligating versus conventional brackets in a randomized split-mouth study." Angle Orthodontist **84**(5): 846-852.	Excluded; Cephalometric measurements
da Costa Monini, A., et al. (2017). "A comparison of lower canine retraction and loss of anchorage between conventional and self-ligating brackets: a single-center randomized split-mouth controlled trial." Clinical Oral Investigations **21**(4): 1047-1053.	Excluded; Cephalometric measurements
Darendeliler, M. A., et al. (1997). "The drum spring (DS) retractor: constant and continuous force for canine retraction." European Journal of Orthodontics **19**(2): 115-130.	Excluded; segmented arch mechanics
Daskalogiannakis, J. and K. R. McLachlan (1996). "Canine retraction with rare earth magnets: an investigation into the validity of the constant force hypothesis." American Journal of Orthodontics and Dentofacial Orthopedics **109**(5).	Excluded; segmented arch mechanics
Davis, S., et al. (2019). "Comparative evaluation of the efficiency of canine retraction using modified Marcotte and T-loop retraction springs - A split-mouth, randomized clinical trial." J Orthod Sci **8**: 9.	Excluded; segmented arch mechanics
De Almeida, M. R., et al. (2013). "A comparative anchorage control study between conventional and self-ligating bracket systems using differential moments." Angle Orthodontist **83**(6): 937-942.	Excluded; Cephalometric measurements
Deepak, C. (2019). "Corticotomy assisted fixed orthodontic treatment vs non corticotomy assisted fixed orthodontic treatment: a randomised clinical trial study." Indian journal of public health research and development 10(11): 2873-2875.	Excluded; no canine retraction duration or rate

Deforest, W. N., et al. (2014). "Human tooth movement by continuous high and low stresses." Angle Orthodontist **84**(1): 102-108.	Excluded; segmented arch mechanics
Dholakia, K. D. and S. R. Bhat (2012). "Clinical efficiency of nonconventional elastomeric ligatures in the canine retraction phase of preadjusted edgewise appliance therapy: an in-vivo study." American journal of orthodontics and dentofacial orthopedics : official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics 141(6): 715-722.	Excluded; non-randomized clinical trial
DiBiase, A. T., et al. (2018). "Effects of supplemental vibrational force on space closure, treatment duration, and occlusal outcome: A multicenter randomized clinical trial." American journal of orthodontics and dentofacial orthopedics : official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics **153**(4): 469-480.	Excluded; en-masse retraction
Dincer, M. and H. N. Iscan (1994). "The effects of different sectional arches in canine retraction." European Journal of Orthodontics **16**(4): 317-323.	Excluded; segmented arch mechanics
Dixon, V., et al. (2002). "A randomized clinical trial to compare three methods of orthodontic space closure." Journal of Orthodontics **29**(1): 31-36.	Excluded; en-masse retraction
El-Bialy, T., et al. (2020). "Effect of Low Intensity Pulsed Ultrasound (LIPUS) on Tooth Movement and Root Resorption: A Prospective Multi-Center Randomized Controlled Trial." Journal of Clinical Medicine 9(3).	Excluded; segmented arch mechanics
Erdur, E. A., et al. (2021). "Effect of injectable platelet-rich fibrin (i-PRF) on the rate of tooth movement: A randomized clinical trial." Angle Orthodontist 91(3): 285-292.	Excluded; en-masse retraction
Eroglu, T., et al. (2010). "Success of zygomatic plate-screw anchorage system." Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons **68**(3): 602-605.	Excluded; no canine retraction duration or rate
Fang, S., et al. (2017). "Comparing two methods of orthodontics space closure: a randomized clinical trial." International Journal of Clinical and Experimental Medicine **10**(10): 14667-14672.	Excluded; en-masse retraction
Fattori, L., et al. (2020). "Micro-osteoperforation effectiveness on tooth movement rate and impact on oral health related quality of life." Angle Orthodontist 90(5): 640-647.	Excluded; en-masse retraction
Ganzer, N., et al. (2018). "Anchorage reinforcement with miniscrews and molar blocks in adolescents: A randomized controlled trial." American journal of orthodontics and dentofacial orthopedics : official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics **154**(6): 758-767.	Excluded; en-masse retraction
Gnaneswar, S. M. and P. Sridhar (2020). "Comparison of dual-dimensional and rectangular wires in terms of space closure and anchorage loss during retraction with miniimplants: A prospective clinical study." Journal of dental research, dental clinics, dental prospects 14(1): 54-60.	Excluded; en-masse retraction
Guram, G., et al. (2018). "Evaluation of Low-Level Laser Therapy on Orthodontic Tooth Movement: A Randomized Control Study." Contemp Clin Dent 9(1): 105-109.	Excluded; segmented arch mechanics
Gurgan, C. A., et al. (2005). "Alterations in gingival dimensions following rapid canine retraction using dentoalveolar distraction osteogenesis." European Journal of Orthodontics **27**(4): 324-332.	Excluded; segmented arch mechanics
Hasler, R., et al. (1997). "A clinical comparison of the rate of maxillary canine retraction into healed and recent extraction sites--a pilot study." European Journal of Orthodontics **19**(6).	Excluded; segmented arch mechanics
Hayashi, K., et al. (2004). "Comparison of maxillary canine retraction with sliding mechanics and a retraction spring: a three-dimensional analysis based on a midpalatal orthodontic implant." European Journal of Orthodontics 26(6): 585-589.	Excluded; segmented arch mechanics
Huffman, D. J. and D. C. Way (1983). "A clinical evaluation of tooth movement along arch wires of two different sizes." American journal of orthodontics **83**(6): 453-459.	Excluded; segmented arch mechanics
Fa, A., et al. (2020). "Photobiomodulation Therapy on Orthodontic Movement: analysis of Preliminary Studies with a New Protocol." International Journal of Environmental Research and Public Health 17(10).	Excluded; both upper and lower canine retraction rate together
Insee, K., et al. (2014). "Comparisons of the chondroitin sulphate levels in orthodontically moved canines and the clinical outcomes between two different force magnitudes." European Journal of Orthodontics 36(1): 39-46.	Excluded; non-randomized clinical trial
Ireland, A. J., et al. (2016). "Effect of gender and Frankfort mandibular plane angle on orthodontic space closure: a randomized controlled trial." Orthodontics & Craniofacial Research **19**(2): 74-82.	Excluded; en-masse retraction
Iseri, H., et al. (2005). "Rapid canine retraction and orthodontic treatment with dentoalveolar distraction osteogenesis." American Journal of Orthodontics and Dentofacial Orthopedics **127**(5): 533-541.	Excluded; segmented arch mechanics
Isola, G., et al. (2019). "Effectiveness of Low-Level Laser Therapy during Tooth Movement: A Randomized Clinical Trial." Materials (Basel, Switzerland) **12**(13).	Excluded; segmented arch mechanics
Iwasaki, L. R., et al. (2000). "Human tooth movement in response to continuous stress of low magnitude." American Journal of Orthodontics and Dentofacial Orthopedics **117**(2): 175-183.	Excluded; segmented arch mechanics
Jahanbakhshi, M. R., et al. (2016). "The effect of buccal corticotomy on accelerating orthodontic tooth movement of maxillary canine." Dent Res J (Isfahan) **13**(4): 303-308.	Excluded; segmented arch mechanics
Jayachandran, B., et al. (2016). "Comparative evaluation of efficacy of self-ligating interactive bracket with conventional preadjusted bracket: A clinical study." Contemp Clin Dent **7**(2): 158-162.	Excluded; en-masse retraction
Joshi, H. N., et al. (2021). "Evaluation of Angular Changes of Canine in En Masse Retraction of Maxillary Anterior Teeth Using Power Arm and Titanium Mini-Implant - A Split Mouth Randomised Control Study." Journal of Evolution of Medical and Dental Sciences-Jemds 10(8): 522-526.	Excluded; en-masse retraction
Joshi, H. N., et al. (2021). "Comparison of anchor loss and rate of retraction between modified conventional anchorage with a power arm and titanium mini-implant in En-masse retraction-A randomized control split mouth study." International Journal of Pharmaceutical Research 13(2): 4140-4151.	Excluded; en-masse retraction
Kalha, A. S., et al. (2010). "Redefining orthodontic space closure: sequential repetitive loading of the periodontal ligament--a clinical study." World journal of orthodontics **11**(3): 221-229.	Excluded; en-masse retraction
Kalra, A., et al. (2013). "Comparison of rate of canine retraction into recent extraction site with and without gingival fiberotomy: A clinical study." Journal of Contemporary Dental Practice **14**(3): 419-426.	Excluded; segmented arch mechanics
Karakasli, K. and E. A. Erdur (2021). "The effect of platelet-rich fibrin (PRF) on maxillary incisor retraction rate." Angle Orthodontist 91(2): 213-219.	Excluded; en-masse retraction
Kecik, D. (2016). "Comparison of temporary anchorage devices and transpalatal arch-mediated anchorage reinforcement during canine retraction." Eur J Dent **10**(4): 512-516.	Excluded; no canine retraction duration or rate

Keng FY, Quick AN, Swain MV, Herbison P. A comparison of space closure rates between preactivated nickel-titanium and titanium-molybdenum alloy T-loops: a randomized controlled clinical trial. European journal of orthodontics. 2012;34(1):33-8.	Excluded; Extraction of either first or second premolars
Khanemasjedi, M., et al. (2017). "Efficacy of elastic memory chains versus nickel-titanium coil springs in canine retraction: a two-center split-mouth randomized clinical trial." International Orthodontics 15(4): 561-574.	Excluded; both upper and lower canine retraction rate together
Shreya Kishore, Saravana Dinesh SP, Srirengalakshmi, & Arvind Sivakumar. (2019). Comparing the rate of retraction in canines in males and females. International Journal of Research in Pharmaceutical Sciences, 10(2), 1332-1339.	Excluded; non-randomized clinical trial
Krishnan, P., et al. (2013). "An adjunctive minor surgical procedure for increased rate of retraction." Journal of Pharmacy and Bioallied Sciences **5**(SUPPL.1): S39-S42.	Excluded; en-masse retraction
Kula, K., et al. (1998). "Effect of ion implantation of TMA archwires on the rate of orthodontic sliding space closure." American Journal of Orthodontics and Dentofacial Orthopedics **114**(5): 577-580.	Excluded; en-masse retraction
Hatrom, A. A., et al. (2020). "Effect of piezocision corticotomy on en-masse retraction." Angle Orthodontist 90(5): 648-654.	Excluded; en-masse retraction
Hatrom, A. A., et al. (2021). "Pulp volume changes after piezocision-assisted tooth movement: a randomized clinical trial." BMC Oral Health 21(1): 28.	Excluded; en-masse retraction
Hayashi, K., et al. (2004). "Comparison of maxillary canine retraction with sliding mechanics and a retraction spring: a three-dimensional analysis based on a midpalatal orthodontic implant." European Journal of Orthodontics **26**(6): 585-589.	Excluded; segmented arch mechanics
Kishore, S., et al. (2019). "Comparing the rate of retraction in canines in males and females." International journal of research in pharmaceutical sciences 10(2): 1332-1339.	Excluded; non-randomized clinical trial
Lalnunpuii, H., et al. (2020). "Comparison of rate of orthodontic tooth movement in adolescent patients undergoing treatment by first bicuspid extraction and en-mass retraction, associated with low level laser therapy in passive self-ligating and conventional brackets: A randomized controlled trial." Int Orthod 18(3): 412-423.	Excluded; en-masse retraction
Leethanakul, C., et al. (2014). "Interseptal bone reduction on the rate of maxillary canine retraction." Angle Orthodontist 84(5): 839-845.	Excluded; segmented arch mechanics
Li, S. N., et al. (2019). "Comparison of movement rate with different initial moment-to-force ratios." American Journal of Orthodontics and Dentofacial Orthopedics **156**(2): 203-209.	Excluded; segmented arch mechanics
Lotzof, L. P., et al. (1996). "Canine retraction: a comparison of two preadjusted bracket systems." American Journal of Orthodontics and Dentofacial Orthopedics 110(2): 191-196.	Excluded; both upper and lower canine retraction rate together
Lucchese, A., et al. (2012). "Orthodontic tooth movement and distraction osteogenesis." European Journal of Inflammation **10**(1 Supplement 3): 49-54.	Excluded; segmented arch mechanics
Luppanapornlarp, S., et al. (2010). "Interleukin-1beta levels, pain intensity, and tooth movement using two different magnitudes of continuous orthodontic force." European Journal of Orthodontics **32**(5): 596-601.	Excluded; segmented arch mechanics
Makhlouf, M., et al. (2018). "Evaluating the amount of tooth movement and root resorption during canine retraction with friction versus frictionless mechanics using cone beam computed tomography." Open Access Macedonian Journal of Medical Sciences 6(2): 384-388.	Excluded; segmented arch mechanics
Malhotra, A., et al. (2021). "A clinical comparative study using anchorage from mini-implants and conventional anchorage methods to retract anterior teeth." Journal of Family Medicine & Primary Care 10(1): 468-474.	Excluded; en-masse retraction
Mani, P. T., et al. (2020). "Comparison of the rate of orthodontic space closure, with and without probiotic supplements: a randomised control trial." International journal of research in pharmaceutical sciences 11(Special ue 4): 1042-1050.	Excluded; en-masse retraction
Miles, P., et al. (2018). "Assessment of the rate of premolar extraction space closure in the maxillary arch with the AcceleDent Aura appliance vs no appliance in adolescents: a single-blind randomized clinical trial." American Journal of Orthodontics and Dentofacial Orthopedics **153**(1): 8-14.	Excluded; en-masse retraction
Miles, P. and N. Pandis (2020). "AcceleDent Aura does not influence treatment duration or number of visits." Australasian Orthodontic Journal 36(1): 2-8.	Excluded; no canine retraction duration or rate
Mittal, R., et al. (2020). "Comparison of orthodontic space closure using micro-osteoperforation and passive self-ligating appliances or conventional fixed appliances:A randomized controlled trial." The Angle orthodontist.	Excluded; en-masse retraction
Monini, A. d. C. (2012). "Estudo clínico do tempo de fechamento de espaço e do movimento dentário durante a retratação de caninos entre dois tipos de braquetes." 78-78.	Excluded; Cephalometric measurements
Moqadam, A. S., et al. (2016). "Effect of alveolar ridge preservation with PDFDBA on orthodontic tooth movement rate, formation of gingival invagination and root resorption: A randomized, controlled pilot study." Biomedical and Pharmacology Journal 9(3): 1229-1235.	Excluded; extraction of mandibular first premolars
Mowafy, M. I. and A. R. Zaher (2012). "Anchorage loss during canine retraction using intermittent versus continuous force distractions; a split mouth randomized clinical trial." Progress in Orthodontics **13**(2): 117-125.	Excluded; segmented arch mechanics
Nakamura, Y., et al. (2015). "Velocity of Canine Retraction in Angle Class I Treated with First Premolar Extraction: Effect of Facial Pattern." The Bulletin of Tokyo Dental College **56**(3): 145-151.	Excluded; Cephalometric measurements
Norman, N. H., et al. (2016). "Nickel titanium springs versus stainless steel springs: a randomized clinical trial of two methods of space closure." Journal of Orthodontics **43**(3): 176-185.	Excluded; en-masse retraction
Ozkan, S. and M. Bayram (2016). "Comparison of direct and indirect skeletal anchorage systems combined with 2 canine retraction techniques." American Journal of Orthodontics and Dentofacial Orthopedics **150**(5): 763-770.	Excluded; segmented arch mechanics
Pavlin, D., et al. (2015). "Cyclic loading (vibration) accelerates tooth movement in orthodontic patients: A double-blind, randomized controlled trial." Seminars in Orthodontics 21(3): 187-194.	Excluded; en-masse retraction
Patel, P., et al. (2016). "Comparative evaluation of pentraxin 3 levels in GCF during canine retraction with active tieback and NiTi coil spring: An in vivo study." Journal of orthodontic science **5**(2): 52-56.	Excluded; no canine retraction duration or rate
Pereira, S. C. d. C., et al. (2020). "Low Intensity Laser Influence on Orthodontic Movement: A Randomized Clinical and Radiographic Trial." Journal of Indian Orthodontic Society **54**(2): 127-134.	Excluded; segmented arch mechanics
Prasad, A. S., et al. (2020). "Comparison of mesial molar migration associated with different depths of micro-osteoperforation assisted canine retraction." European Journal of Molecular and Clinical Medicine 7(2): 242-250.	Excluded; no canine retraction duration or rate
Qamruddin, I., et al. (2020). "Pain perception and rate of canine retraction through self-ligating brackets and conventional elastomeric ligation system: A split mouth study." Pesquisa Brasileira em Odontopediatria e Clinica Integrada 20: e5147.	Excluded; both upper and lower canine retraction rate together

Raj, S. C., et al. (2020). "Retraction With and Without Piezocision-Facilitated Orthodontics: a Randomized Controlled Trial." International Journal of Periodontics & Restorative Dentistry 40(1): e19-e26.	Excluded; CBCT measurements
Rajasekaran, U. B. and U. S. Krishna Nayak (2014). "Effect of prostaglandin E1 versus corticotomy on orthodontic tooth movement: an in vivo study." Indian journal of dental research : official publication of Indian Society for Dental Research 25(6): 717-721.	Excluded; segmented arch mechanics and not randomised clinical trial
Sabrina, et al. (2016). "The comparison of space closure rate between conventional and passive self-ligating system using elastomeric chain in maxilla." Journal of International Dental and Medical Research **9**(Specialissue): 356-361.	Excluded; en-masse retraction
Samuels, R. H., et al. (1993). "A comparison of the rate of space closure using a nickel-titanium spring and an elastic module: a clinical study." American Journal of Orthodontics and Dentofacial Orthopedics **103**(5): 464-467.	Excluded; en-masse retraction
Sandler, J., et al. (2014). "Effectiveness of 3 methods of anchorage reinforcement for maximum anchorage in adolescents: A 3-arm multicenter randomized clinical trial." American Journal of Orthodontics and Dentofacial Orthopedics **146**(1): 10-20.	Excluded; no canine retraction duration or rate
Sandoval, P.; Bizcar, B.; Navarro, P. & Knösel, M. Efficacy of diode lasertherapy in acceleration of orthodontic space closure: a split-mouthrandomized clinical trial. Int. J. Dent. Oral Health, 3(2), 2017	Excluded; en-masse retraction
Sar, S. K., et al. (2019). "Leptin levels in gingival crevicular fluid during canine retraction: in vivo comparative study." Journal of Orthodontics 46(1): 27-33.	Excluded; non-randomized clinical trial
Shaik, J. A. and G. Guram (2018). "A Comparative Evaluation of Canine Retraction Using Ceramic Bracket and Ceramic Bracket with Metal Slot with Conventional Preadjusted Edgewise Appliance Bracket Systems: A Clinical Study." Journal of International Society of Preventive & Community Dentistry 8(4): 296-303.	Excluded; non-randomized clinical trial
Sharma, M., et al. (2012). "Mini-screw implant or transpalatal arch-mediated anchorage reinforcement during canine retraction: a randomized clinical trial." Journal of Orthodontics **39**(2): 102-110.	Excluded; no canine retraction duration or rate
Showkatbakhsh, R., et al. (2010). "The effect of pulsed electromagnetic fields on the acceleration of tooth movement." World journal of orthodontics 11(4): e52-56.	Excluded; non-randomized clinical trial
Shpack N, Davidovitch M, Sarne O, Panayi N, Vardimon AD. Duration and anchorage management of canine retraction with bodily versus tipping mechanics. Angle Orthodontist. 2008;78(1):95-100.	Excluded; non-randomized clinical trial
Simre, S. S. and K. Rajanikanth (2020). "Evaluation of conventional corticotomy with novel piezosurgery in orthodontic treatment - study protocol for a comparative study." European Journal of Molecular and Clinical Medicine 7(2): 2128-2131.	Excluded; protocol
Sivarajan S, Doss JG, Papageorgiou SN, Cobourne MT, Wey MC. Mini-implant supported canine retraction with micro-osteoperforation: a split-mouth randomized clinical trial. Angle orthodontist. 2019;89(2):183-9.	Excluded; both upper and lower canine retraction rate together
Sobouti, F., et al. (2015). "Effect of single-dose low-level helium-neon laser irradiation on orthodontic pain: a split-mouth single-blind placebo-controlled randomized clinical trial." Progress in Orthodontics **16**: 32.	Excluded; no canine retraction duration or rate
Songra, G., et al. (2014). "Comparative assessment of alignment efficiency and space closure of active and passive self-ligating vs conventional appliances in adolescents: a single-center randomized controlled trial." American Journal of Orthodontics and Dentofacial Orthopedics **145**(5): 569-578.	Excluded; en-masse retraction
Sonis AL, Van der Plas E, Gianelly A. A comparison of elastomeric auxiliaries versus elastic thread on premolar extraction site closure: an in vivo study. American journal of orthodontics. 1986;89(1).	Excluded; both upper and lower canine retraction rate together
Sousa, M. V., et al. (2011). "Influence of low-level laser on the speed of orthodontic movement." Photomedicine and laser surgery **29**(3): 191-196.	Excluded; segmented arch mechanics
Storniolo-Souza, J., et al. (2020). "INFLUENCE OF LOW-LEVEL LASER IRRADIATION ON ORTHODONTIC MOVEMENT AND PAIN LEVEL - A RANDOMIZED CLINICAL TRIAL." Orthodontic Waves 79(2-3): 105-112.	Excluded; segmented arch mechanics
Talwar A, Bhat S, editors. Comparative evaluation of Nickel-Titanium closed coil spring and Elastomeric chain for canine retraction . A Randomized Clinical Trial2018.	Excluded; mandibular canine retraction rate
Sueri, M. Y. and T. Turk (2006). "Effectiveness of laceback ligatures on maxillary canine retraction." Angle Orthodontist 76(6): 1010-1014.	Excluded; Cephalometric measurements
Teh, N. H. K., et al. (2020). "Distribution of mandibular trabeculae bone volume fraction in relation to different MOP intervals for accelerating orthodontic tooth movement." Angle Orthodontist 90(6): 774-782.	Excluded; mandibular canine retraction rate
Thiruvenkatachari, B., et al. (2008). "Comparison of rate of canine retraction with conventional molar anchorage and titanium implant anchorage." American Journal of Orthodontics and Dentofacial Orthopedics **134**(1): 30-35.	Excluded; Cephalometric measurements
Thiruvenkatachari, B., et al. (2006). "Comparison and measurement of the amount of anchorage loss of the molars with and without the use of implant anchorage during canine retraction." American Journal of Orthodontics and Dentofacial Orthopedics **129**(4): 551-554.	Excluded; no canine retraction duration or rate
Turker, G., et al. (2020). "Which method is more effective for accelerating canine distalization short term, low-level laser therapy or piezocision? A split-mouth study." Welche Methode ist fur die kurzfristige Beschleunigung der Eckzahndistalisierung effektiver, die Low-Level-Lasertherapie oder die Piezozision? Eine Split-mouth-Studie.	Excluded; non-randomized clinical trial
Üretürk, S. E., et al. (2017). "The effect of low-level laser therapy on tooth movement during canine distalization." Lasers Med Sci 32(4): 757-764.	Excluded; the split mouth design was not randomized
Wahab, R. M. A., et al. (2011). "Crevicular tartrate resistant acid phosphatase activity and rate of tooth movement under different continuous force applications." African Journal of Pharmacy and Pharmacology 5(20): 2213-2219.	Excluded; en-masse retraction
Wong, H., et al. (2013). "Does the bracket-ligature combination affect the amount of orthodontic space closure over three months? A randomized controlled trial." Journal of Orthodontics **40**(2): 155-162.	Excluded; en-masse retraction
Youssef, M., et al. (2008). "The effect of low-level laser therapy during orthodontic movement: A preliminary study." Lasers in Medical Science **23**(1): 27-33.	Excluded; segmented arch mechanics
Alikhani, M., et al. (2018). "Age-dependent biologic response to orthodontic forces." American journal of orthodontics and dentofacial orthopedics: official publication of the American Association of Orthodontists, its constituent societies, and the American Board of Orthodontics 153(5): 632-644.	Excluded; non-randomized clinical trial
Ziegler P, Ingervall B. A clinical study of maxillary canine retraction with a retraction spring and with sliding mechanics. American journal of orthodontics and dentofacial orthopedics. 1989;95(2):99-106.	Excluded; segmented arch mechanics

Supplementary Table 3.2. Detailed assessment of included randomized trials with the Risk-of-Bias 2.0 tool.

Domain	Reference	Abbas 2016	Ahmad 2020	Abdelhameed 2018	Aboalnaga 2019	Aboul-Ela 2011	Alfawal 2018	Alikhani 2013	Alkebsi 2018	Alqadasi 2019
1. Randomization process	**1.1**	Y	NI	Y	Y	Y	Y	NI	Y	NI
	1.2	NI	NI	NI	Y	NI	Y	NI	Y	Y
	1.3	PN	PN	PN	PN	PN	N	N	N	PN
	Assessor's Judgement	Some concerns	Some concerns	Some concerns	Low	Some concerns	Low	Some concerns	Low	Low
2. Deviations from intended interventions	**2.1**	PY	PY	PY	NI	PY	Y	Y	Y	PY
	2.2	PY	PY	PY	NI	PY	Y	Y	Y	PY
	2.3	PN	PN	PN	PN	PN	N	N	N	PN
	2.4	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2.5	NA	NA	NA	NA	NA	NA	NA	NA	NA
	2.6	NI	NI	PY	PY	PY	Y	Y	Y	PY
	2.7	NI	NI	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	High	High	Low	Low	Low	Low	Low	Low	Low
3. Mising outcome data	**3.1**	NI	NI	PY	PY	PY	Y	Y	Y	PY
	3.2	PN	N	NA	NA	NA	NA	NA	NA	NA
	3.3	NI	NI	NA	NA	NA	NA	NA	NA	NA
	3.4	PN	PN	NA	NA	NA	NA	NA	NA	NA
	Assessor's judgement	Some concerns	Some concerns	Low	Low	Low	Low	Low	Low	Low
4. Measurement of the outcome	**4.1**	PN	PN	PN	PN	PN	N	N	N	PN
	4.2	PN	PN	PN	PN	PN	N	N	N	PN
	4.3	NI	NI	NI	N	NI	N	N	N	N
	4.4	PN	PN	PN	NA	PN	NA	NA	NA	NA
	4.5	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low
5. Selection of the reported result	**5.1**	PY	PY	PY	PY	PY	Y	PY	Y	PY
	5.2	PN	PN	PN	PN	PN	N	N	N	PN
	5.3	PN	PN	PN	PN	PN	N	N	N	PN
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low
Overall	**Assessor's Judgement**	High	High	Some concerns	Low	Some concerns	Low	Some concerns	Low	Low
	General Note	-	Incomplete reporting for retraction duration and rate (no SDs)	-	-	Incomplete reporting for retraction rate (no SDs)	Incomplete reporting for retraction duration (no means & SDs)	Incomplete reporting for retraction rate (no means & SDs	-	-

PN, probably no; N, no; NA, not applicable; NI, no information; PY, probably yes; Y, yes.

Supplementary Table 3.2 (continued). Detailed assessment of included randomized trials with the Risk-of-Bias 2.0 tool.

Domain	Reference	Alqadasi 2020	Al-Naoum 2014	Al-Shafi 2021	Araghbidikashani 2017	Babanouri 2020	Cruz 2004	Deguchi 2007	Doshi-Mehta 2012
1. Randomization process	**1.1**	Y	PN	Y	NI	Y	NI	PY	PY
	1.2	Y	Y	Y	NI	Y	NI	NI	NI
	1.3	PN	PN	N	PN	PN	PN	PN	PN
	Assessor's Judgement	Low	Some concerns	Low	Some concerns	Low	Some concerns	Some concerns	Some concerns
2. Deviations from intended interventions	**2.1**	Y	PY	Y	NI	N	PY	NI	PN
	2.2	Y	PY	Y	NI	N	PY	NI	PY
	2.3	N	PN	N	PN	NA	PN	PN	PN
	2.4	NA	NA	NA	NA	NA	NA	NA	NA
	2.5	NA	NA	NA	NA	NA	NA	NA	NA
	2.6	PY	PY	Y	NI	PY	NI	NI	PY
	2.7	NA	NA	NA	PN	NA	PN	PN	NA
	Assessor's Judgement	Low	Low	Low	Some concerns	Low	Some concerns	Some concerns	Low
3. Mising outcome data	**3.1**	PY	PY	Y	NI	PY	NI	NI	PY
	3.2	NA	NA	NA	PN	NA	N	N	NA
	3.3	NA	NA	NA	NI	NA	NI	NI	NA
	3.4	NA	NA	NA	PN	NA	PN	PN	NA
	Assessor's judgement	Low	Low	Low	Some concerns	Low	Some concerns	Some concerns	Low
4. Measurement of the outcome	**4.1**	PN	PN	N	PN	PN	PN	PN	PN
	4.2	PN	PN	N	PN	PN	PN	PN	PN
	4.3	N	NI	N	NI	N	NI	NI	N
	4.4	NA	PN	NA	PN	NA	PN	PN	NA
	4.5	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low
5. Selection of the reported result	**5.1**	PY	PY	PY	PY	PY	PY	PY	PY
	5.2	PN	PN	PN	PN	PN	PN	PN	PN
	5.3	PN	PN	PN	PN	PN	PN	PN	PN
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low
Overall	**Assessor's Judgement**	Low	Some concerns	Low	High	Low	High	High	Some concerns
	General Note	-	-	-	-	-	-	-	-

PN, probably no; N, no; NA, not applicable; NI, no information; PY, probably yes; Y, yes.

Supplementary Table 3.2 (continued). Detailed assessment of included randomized trials with the Risk-of-Bias 2.0 tool.

Domain	Reference	El-Timamy 2020	Farid 2019	Feizbakhsh 2018	Haliloglu-Ozkan 2018	Haliloglu-Ozkan 2021	Hassan 2016	Heravi 2014	Jaber 2021
1. Randomization process	**1.1**	Y	Y	NI	PN	Y	PY	NI	Y
	1.2	Y	Y	NI	PN	PN	NI	NI	NI
	1.3	PN	PN	PN	PN	N	PN	PN	N
	Assessor's Judgement	Low	Low	Some concerns	High	Low	Some concerns	Some concerns	Some concerns
2. Deviations from intended interventions	**2.1**	PY	PY	Y	Y	N	NI	N	Y
	2.2	PY	PY	Y	Y	Y	PY	Y	Y
	2.3	PN	PN	PN	PN	N	PN	PN	N
	2.4	NA	NA	NA	NA	NA	NA	NA	NA
	2.5	NA	NA	NA	NA	NA	NA	NA	NA
	2.6	PY	PY	PY	PY	Y	PY	NI	Y
	2.7	NA	NA	NA	NA	NA	NA	PN	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Some concerns	Low
3. Mising outcome data	**3.1**	PY	Y	PY	PY	Y	PY	NI	Y
	3.2	NA	NA	NA	NA	NA	NA	PN	NA
	3.3	NA	NA	NA	NA	NA	NA	PN	NA
	3.4	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's judgement	Low	Low	Low	Low	Low	Low	Low	Low
4. Measurement of the outcome	**4.1**	PN	PN	PN	PN	N	PN	PN	N
	4.2	PN	PN	PN	PN	N	PN	PN	N
	4.3	N	N	N	N	N	N	NI	N
	4.4	NA	NA	NA	NA	NA	NA	PN	NA
	4.5	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low
5. Selection of the reported result	**5.1**	PY	PY	PY	PY	PY	PY	PY	PY
	5.2	PN	PN	PN	PN	PN	N	PN	PN
	5.3	PN	PN	PN	PN	PN	N	PN	PN
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low
Overall	**Assessor's Judgement**	Low	Low	Some concerns	High	Low	Some concerns	High	Some concerns
	General Note	-	-	-	-	-	-	-	-

PN, probably no; N, no; NA, not applicable; NI, no information; PY, probably yes; Y, yes.

Supplementary Table 3.2 (continued). Detailed assessment of included randomized trials with the Risk-of-Bias 2.0 tool.

Domain	Reference	Jivrajani 2020	Kansal 2014	Karci 2021	Kundi 2020	Liao 2017	Limpanichkul 2006	Mahmoudzadeh 2020	Mezomo 2011
1. Randomization process	**1.1**	PN	PY	Y	Y	NI	PY	PY	NI
	1.2	PN	NI	NI	Y	NI	NI	PY	NI
	1.3	PN	PN	N	PN	PN	PN	PN	PN
	Assessor's Judgement	High	Some concerns	Some concerns	Low	Some concerns	Some concerns	Low	Some concerns
2. Deviations from intended interventions	**2.1**	N	PN	PY	Y	NI	N	Y	NI
	2.2	PY	PN	PY	Y	NI	N	Y	NI
	2.3	PN	NA	N	PN	NI	NA	PN	PN
	2.4	NA	NA	NA	NA	NI	NA	NA	NA
	2.5	NA	NA	NA	NA	NI	NA	NA	NA
	2.6	NI	PY	Y	Y	NI	NI	PY	PY
	2.7	PN	NA	NA	NA	PN	PN	NA	NA
	Assessor's Judgement	Some concerns	Low	Low	Low	High	Some concerns	Low	Low
3. Mising outcome data	**3.1**	PN	PY	Y	Y	NI	NI	PY	PY
	3.2	PN	NA	NA	NA	N	N	NA	NA
	3.3	N	NA	NA	NA	NI	NI	NA	NA
	3.4	NA	NA	NA	NA	PN	N	NA	NA
	Assessor's judgement	Some concerns	Low	Low	Low	Some concerns	Some concerns	Low	Low
4. Measurement of the outcome	**4.1**	PN	PN	N	PN	PN	PN	PN	PN
	4.2	N	PN	N	PN	PN	PN	PN	PN
	4.3	N	N	NI	N	NI	NI	N	NI
	4.4	NA	NA	PN	NA	PN	PN	NA	PN
	4.5	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low
5. Selection of the reported result	**5.1**	PY	PY	PY	PY	PY	PY	PY	PY
	5.2	PN	PN	PN	PN	PN	PN	PN	PN
	5.3	PN	PN	PN	PN	PN	PN	PN	PN
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low
Overall	**Assessor's Judgement**	High	Some concerns	Some concerns	Low	High	High	Low	Some concerns
	General Note	-	-	-	-	-	-	-	-

PN, probably no; N, no; NA, not applicable; NI, no information; PY, probably yes; Y, yes.

Supplementary Table 3.2 (continued). Detailed assessment of included randomized trials with the Risk-of-Bias 2.0 tool.

Domain	Reference	Mistry 2020	Pacheco 2020	Qamruddin 2017	Qamruddin 2021	Dakshina 2019	Sharma 2020	Siriphan 2019	Ekizer 2016
1. Randomization process	**1.1**	Y	Y	Y	Y	PY	NI	PY	Y
	1.2	Y	NI	NI	NI	NI	NI	NI	Y
	1.3	N	PN	N	PN	PN	PN	PN	PN
	Assessor's Judgement	Low	Some concerns	Some concerns	Some cocerns	Some concerns	Some concerns	Some concerns	Low
2. Deviations from intended interventions	**2.1**	N	Y	Y	N	N	Y	Y	N
	2.2	N	Y	Y	Y	N	Y	Y	NI
	2.3	NA	PN	PN	N	NA	PN	N	PN
	2.4	NA	NA	NA	NA	NA	NA	NA	NA
	2.5	NA	NA	NA	NA	NA	NA	NA	NA
	2.6	Y	PY	Y	Y	NI	NI	Y	PY
	2.7	NA	NA	NA	NA	PN	PN	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Some concerns	Some concerns	Low	Low
3. Mising outcome data	**3.1**	Y	PN	Y	Y	NI	PN	PY	Y
	3.2	NA	N	NA	NA	N	N	NA	NA
	3.3	NA	PN	NA	NA	NI	PN	NA	NA
	3.4	NA	NA	NA	NA	PN	NA	NA	NA
	Assessor's judgement	Low	Low	Low	Low	Some concerns	Low	Low	Low
4. Measurement of the outcome	**4.1**	PN	PN	PN	N	N	PN	PN	N
	4.2	PN	PN	PN	N	N	PN	PN	N
	4.3	N	NI	N	NI	NI	N	N	N
	4.4	NA	PN	NA	PN	PN	NA	NA	NA
	4.5	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low
5. Selection of the reported result	**5.1**	PY	PY	PY	PY	PY	PY	PY	PY
	5.2	PN	PN	PN	PN	PN	PN	PN	PN
	5.3	PN	PN	PN	PN	PN	PN	PN	PN
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low
Overall	**Assessor's Judgement**	Low	Some concerns	Some concerns	Some concerns	High	High	Some concerns	Low
	General Note	-	Incomplete reporting for retraction rate (no SDs)	-	-	-	-	-	-

PN, probably no; N, no; NA, not applicable; NI, no information; PY, probably yes; Y, yes.

Supplementary Table 3.2 (continued). Detailed assessment of included randomized trials with the Risk-of-Bias 2.0 tool.

Domain	Reference	Taha 2020	Telatar 2020	Thomas 2021	Varella 2018	Wahab 2013	Wahab 2015	Yassaei 2016	Zeitounlouian 2021	Zheng 2021
1. Randomization process	**1.1**	PY	Y	Y	Y	PY	Y	NI	PY	Y
	1.2	NI	NI	Y	PY	NI	NI	NI	NI	NI
	1.3	PN	PN	N	PN	PN	PN	PN	PN	PN
	Assessor's Judgement	Some concerns	Some concerns	Low	Low	Some concerns	Some concerns	Some concerns	Some concerns	Some concerns
2. Deviations from intended interventions	**2.1**	Y	PY	Y	PN	NI	NI	NI	PY	NI
	2.2	Y	PY	Y	PN	NI	NI	N	PY	NI
	2.3	PN	PN	N	NA	PN	NI	PN	PN	PN
	2.4	NA	NA	NA	NA	NA	NI	NA	NA	NA
	2.5	NA	NA	NA	NA	NA	NI	NA	NA	NA
	2.6	Y	Y	Y	Y	Y	NI	PY	Y	NI
	2.7	NA	NA	NA	NA	NA	NI	NA	NA	PN
	Assessor's Judgement	Low	Low	Low	Low	Low	High	Low	Low	Some concerns
3. Mising outcome data	**3.1**	Y	Y	Y	NI	Y	NI	Y	Y	NI
	3.2	NA	NA	NA	N	NA	N	NA	NA	N
	3.3	NA	NA	NA	N	NA	PN	NA	NA	PN
	3.4	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low
4. Measurement of the outcome	**4.1**	PN	PN	N	N	PN	PN	PN	PN	N
	4.2	PN	PN	N	N	PN	PN	PN	PN	N
	4.3	N	N	N	NI	NI	NI	NI	N	NI
	4.4	NA	NA	NA	PN	PN	PN	PN	NA	PN
	4.5	NA	NA	NA	NA	NA	NA	NA	NA	NA
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low
5. Selection of the reported result	**5.1**	PY	PY	Y	PY	PY	PY	PY	PY	PY
	5.2	PN	PN	N	PN	PN	PN	PN	PN	PN
	5.3	PN	PN	N	PN	PN	PN	PN	PN	PN
	Assessor's Judgement	Low	Low	Low	Low	Low	Low	Low	Low	Low
Overall	**Assessor's Judgement**	Some concerns	Some concerns	Low	Low	Some concerns	High	Some concerns	Some concerns	High
	General Note	-	-	-	-	-	Incomplete reporting for retraction rate (no SDs)	-	-	-

PN, probably no; N, no; NA, not applicable; NI, no information; PY, probably yes; Y, yes.

Supplementary Table 3.3 Direct estimates (MD) from single trials on canine retraction rate not meta-analyzed in Table III.

aa	Study	Experimental	Comparison	Measurement	Time	MD (95% CI)	P
1	Abdelhameed 2018	MOP+LLLT	Control	Retraction increment (mm)	t0_1m	0.08 (-0.18, 0.34)	0.54
2	Abdelhameed 2018	MOP+LLLT	Control	Total retraction (mm)	t0_2m	0.01 (-0.33, 0.35)	0.95
3	Abdelhameed 2018	MOP+LLLT	Control	Total retraction (mm)	t0_3m	0 (-0.13, 0.12)	0.96
4	Abdelhameed 2018	MOP+LLLT	Control	Retraction increment (mm)	t1_2m	-0.07 (-0.30, 0.16)	0.54
5	Abdelhameed 2018	MOP+LLLT	Control	Retraction increment (mm)	t2_3m	-0.01 (-0.28, 0.26)	0.93
6	Abdelhameed 2018	MOP+LLLT	LLLT	Retraction increment (mm)	t0_1m	0.14 (-0.30, 0.58)	0.53
7	Abdelhameed 2018	MOP+LLLT	LLLT	Total retraction (mm)	t0_2m	0.09 (-0.47, 0.65)	0.75
8	Abdelhameed 2018	MOP+LLLT	LLLT	Total retraction (mm)	t0_3m	0.02 (-0.22, 0.26)	0.87
9	Abdelhameed 2018	MOP+LLLT	LLLT	Retraction increment (mm)	t1_2m	-0.05 (-0.42, 0.32)	0.79
10	Abdelhameed 2018	MOP+LLLT	LLLT	Retraction increment (mm)	t2_3m	-0.07 (-0.51, 0.37)	0.75
11	Deguchi 2007	ClearSnap bracket	Control	Retraction increment (mm)	t0_1m	0.97 (0.83, 1.10)	<0.001
12	Alfawal 2018	Piezocision	Control	Retraction; averaged (mm/month)	t0_4m	0.32 (0.27, 0.37)	<0.001
13	Alfawal 2018	Laser corticotomy	Control	Retraction; averaged (mm/month)	t0_4m	0.27 (0.23, 0.31)	<0.001
14	Alfawal 2018	Piezocision	LAFCG	Retraction increment (mm)	t0_1m	0.08 (-0.14, 0.30)	0.48
15	Alfawal 2018	Piezocision	LAFCG	Retraction increment (mm)	t1_2m	0.13 (-0.05, 0.31)	0.16
16	Alfawal 2018	Piezocision	LAFCG	Retraction increment (mm)	t2_3m	0.04 (-0.13, 0.21)	0.64
17	Alfawal 2018	Piezocision	LAFCG	Retraction increment (mm)	t3_4m	-0.02 (-0.10, 0.06)	0.63
18	Alfawal 2018	Piezocision	LAFCG	Retraction; averaged (mm/month)	t0_4m	0.05 (-0.03, 0.13)	0.21
19	Alfawal 2018	Piezocision	LAFCG	Total retraction (mm)	t0_4m	0.20 (-0.12, 0.52)	0.21
20	Alfawal 2018	Piezocision	LAFCG	Total retraction duration (months)	total	0.19 (-0.27, 0.65)	0.42
21	Alfawal 2018	Piezocision	LAFCG	Total retraction (mm)	t0_2m	0.21 (-0.17, 0.59)	0.27
22	Alfawal 2018	Piezocision	LAFCG	Total retraction (mm)	t0_3m	0.25 (-0.18, 0.68)	0.25
23	Deguchi 2007	ClearSnap bracket	Control	Retraction increment (mm)	t1_2m	1.00 (0.88, 1.12)	<0.001
24	Deguchi 2007	ClearSnap bracket	Control	Retraction increment (mm)	t2_3m	0.30 (0.14, 0.46)	<0.001
25	Deguchi 2007	ClearSnap bracket	Control	Total retraction (mm)	t0_2m	1.97 (1.73, 2.20)	<0.001
26	Deguchi 2007	ClearSnap bracket	Control	Total retraction (mm)	t0_3m	2.27 (1.91, 2.62)	<0.001
27	Deguchi 2007	ClearSnap bracket	Control	Total retraction duration (months)	total	-2.43 (-2.68, -2.19)	<0.001
28	Alqadasi 2020	Piezocision	MOP	Retraction increment (mm)	t0_1m	1.02 (0.39, 1.66)	0.002
29	Alqadasi 2020	Piezocision	MOP	Total retraction (mm)	t0_2m	0.51 (-0.13, 1.15)	0.12
30	Alqadasi 2020	Piezocision	MOP	Total retraction (mm)	t0_3m	0.51 (-0.15, 1.17)	0.13
31	Alqadasi 2020	Piezocision	MOP	Retraction increment (mm)	t1_2m	-0.51 (-1.15, 0.13)	0.12
32	Alqadasi 2020	Piezocision	MOP	Retraction increment (mm)	t2_3m	0 (-0.66, 0.66)	1.00
33	Araghbidikashani 2017	Coil spring	Laceback	Total retraction (mm)	t0_4m	1.65 (0.04, 3.26)	0.05
34	Babanouri 2020	MOP scheme 2	MOP scheme 1	Retraction increment (mm)	t0_1m	0.27 (0.09, 0.45)	0.004
35	Babanouri 2020	MOP scheme 2	MOP scheme 1	Retraction increment (mm)	t1_2m	0.28 (0.15, 0.41)	<0.001
36	Babanouri 2020	MOP scheme 2	MOP scheme 1	Retraction increment (mm)	t2_3m	0.24 (0.15, 0.33)	<0.001

37	Babanouri 2020	MOP scheme 2	MOP scheme 1	Total retraction (mm)	t0_2m	0.55 (0.26, 0.84)	<0.001
38	Babanouri 2020	MOP scheme 2	MOP scheme 1	Total retraction (mm)	t0_3m	0.79 (0.43, 1.15)	<0.001
39	Deguchi 2007	100g force	50g force	Retraction increment (mm)	t0_1m	0.60 (0.37, 0.83)	<0.001
40	Deguchi 2007	100g force	50g force	Retraction increment (mm)	t1_2m	0.60 (0.35, 0.85)	<0.001
41	Deguchi 2007	100g force	50g force	Retraction increment (mm)	t2_3m	0.20 (0, 0.40)	0.05
42	Deguchi 2007	100g force	50g force	Retraction; averaged (mm/month)	t0_3m	0.50 (0.27, 0.73)	<0.001
43	Deguchi 2007	100g force	50g force	Total retraction (mm)	t0_3m	1.50 (0.82, 2.18)	<0.001
44	Deguchi 2007	100g force	50g force	Total retraction duration (months)	total	-0.80 (-1.40, -0.20)	0.009
45	Deguchi 2007	100g force	50g force	Total retraction (mm)	t0_2m	1.20 (0.83, 1.57)	<0.001
46	Deguchi 2007	150g force	50g force	Retraction increment (mm)	t0_1m	0.60 (0.37, 0.83)	<0.001
47	Deguchi 2007	150g force	50g force	Retraction increment (mm)	t1_2m	0.60 (0.35, 0.85)	<0.001
48	Deguchi 2007	150g force	50g force	Retraction increment (mm)	t2_3m	0.10 (-0.13, 0.33)	0.39
49	Deguchi 2007	150g force	50g force	Retraction; averaged (mm/month)	t0_3m	0.80 (0.43, 1.17)	<0.001
50	Deguchi 2007	150g force	50g force	Total retraction (mm)	t0_3m	2.40 (1.29, 3.52)	<0.001
51	Deguchi 2007	150g force	50g force	Total retraction duration (months)	total	-1.30 (-1.99, -0.61)	<0.001
52	Deguchi 2007	150g force	50g force	Total retraction (mm)	t0_2m	1.20 (0.83, 1.57)	<0.001
53	Deguchi 2007	150g force	100g force	Retraction increment (mm)	t1_2m	0 (-0.30, 0.30)	1.00
54	Deguchi 2007	150g force	100g force	Retraction increment (mm)	t2_3m	-0.10 (-0.30, 0.10)	0.32
55	Deguchi 2007	150g force	100g force	Retraction; averaged (mm/month)	t0_3m	0.30 (-0.07, 0.67)	0.11
56	Deguchi 2007	150g force	100g force	Total retraction (mm)	t0_3m	0.90 (-0.22, 2.02)	0.11
57	Deguchi 2007	150g force	100g force	Total retraction duration (months)	total	-0.50 (-0.98, -0.02)	0.04
58	Deguchi 2007	150g force	100g force	Total retraction (mm)	t0_2m	0 (-0.38, 0.38)	1.00
59	Farid 2019	LLLT+ corticotomy	Corticotomy	Retraction increment (mm)	t0_1m	-0.35 (-0.73, 0.03)	0.07
60	Farid 2019	LLLT+ corticotomy	Corticotomy	Retraction increment (mm)	t1_2m	0.22 (-0.09, 0.53)	0.16
61	Farid 2019	LLLT+ corticotomy	Corticotomy	Retraction increment (mm)	t2_3m	-0.18 (-0.91, 0.55)	0.63
62	Farid 2019	LLLT+ corticotomy	Corticotomy	Retraction increment (mm)	t3_4m	0.60 (-0.07, 1.27)	0.08
63	Farid 2019	LLLT+ corticotomy	Corticotomy	Total retraction (mm)	t0_4m	0.25 (-0.65, 1.15)	0.59
64	Farid 2019	LLLT+ corticotomy	Corticotomy	Total retraction (mm)	t0_2m	-0.13 (-0.76, 0.50)	0.69
65	Farid 2019	LLLT+ corticotomy	Corticotomy	Total retraction (mm)	t0_3m	-0.31 (-1.44, 0.82)	0.59
66	Hassan 2016	SLB	CLB	Retraction; averaged (mm/month)	t0_3m	0.31 (0.24, 0.38)	<0.001
67	Jivrajani 2020	LLLT	Control	Retraction; averaged (mm/month)	t0_3m	0.42 (0.18, 0.67)	0.001
68	Siriphan 2019	Vibration	Control	Retraction; averaged (mm/month)	t0_3m	0.02 (-0.13, 0.16)	0.84
69	Siriphan 2019	Vibration (60Hz)	Vibration (30Hz)	Retraction; averaged (mm/month)	t0_3m	-0.05 (-0.26, 0.16)	0.65
70	Siriphan 2019	Vibration (60Hz)	Vibration (30Hz)	Total retraction (mm)	t0_3m	-0.15 (-0.79, 0.49)	0.65
71	Taha 2020	Vibration	Control	Retraction increment (mm)	t0_1m	0.27 (0.01, 0.53)	0.71
72	Taha 2020	Vibration	Control	Total retraction (mm)	t0_2m	-0.10 (-0.62, 0.42)	0.01
73	Taha 2020	Vibration	Control	Retraction increment (mm)	t1_2m	-0.54 (-0.97, -0.12)	0.87
74	Taha 2020	Vibration	Control	Retraction increment (mm)	t2_3m	0.04 (-0.44, 0.52)	0.33

75	Telatar 2020	Vibration	Control	Retraction; averaged (mm/month)	t0_6m	0.18 (-0.18, 0.54)	0.32
76	Telatar 2020	Vibration	Control	Total retraction (mm)	t0_6m	1.08 (-1.07, 3.23)	0.04
77	Wahab 2013	SLB	CLB	Retraction increment (mm)	t0_1m	0.04 (-0.49, 0.58)	0.88
78	Wahab 2013	SLB	CLB	Total retraction (mm)	t0_2m	0.38 (-0.43, 1.19)	0.36
79	Wahab 2013	SLB	CLB	Retraction increment (mm)	t1_2m	0.34 (-0.17, 0.84)	0.19
80	Wahab 2013	SLB	CLB	Retraction increment (mm)	t2_3m	0.19 (-0.17, 0.55)	0.29
81	Yassaei 2016	LLLT	Control	Total retraction (mm)	t0_4m	0.03 (-0.04, 0.10)	0.41
82	Yassaei 2016	LLLT	Control	Retraction increment (mm)	t3_4m	-0.03 (-0.12, 0.06)	0.51
83	Zeitounlouian 2021	i-PRF	Control	Retraction increment (mm)	t4_5m	-0.55 (-0.79, -0.31)	<0.001
84	Karci 2021	PRF	Piezocision	Total retraction (mm)	t0_3m	-0.05 (-0.19, 0.09)	0.48

CLB, conventionally ligated bracket; i-PRF, injectable platelet rich fibrin; LAFCG, laser-assisted flapless corticotomy; LLLT, low laser light therapy; m, month; MOP, micro-osteoperforation; PRF, platelet rich fibrin; SLB, self ligated bracket.

Supplementary Table 3.4. Meta-regression / subgroup analyses for possible factor influencing the pooled average canine retraction duration and rate (indirect estimates from Table II) across identified randomized trials.

		Retraction month 0-1 (mm)			Retraction month 0-2 (mm)			Retraction month 0-3 (mm)			Retraction month 0-4 (mm)			Retraction month 1-2 (mm)			Retraction month 2-3 (mm)		
Factor	**Level**	**n**	**Effect (95% CI)**	**P**	**n**	**Effect (95% CI)**	**P**	**n**	**Effect (95% CI)**	**P**	**n**	**Effect (95% CI)**	**P**	**n**	**Effect (95% CI)**	**P**	**n**	**Effect (95% CI)**	**P**
Age	Per year	17	b -0.02 (-0.09, 0.05)	0.60	14	b -0.07 (-0.20, 0.07)	0.29	15	b -0.03 (-0.20, 0.14)	0.71	5	b -0.25 (-1.40, 0.90)	0.54	15	b -0.04 (-0.11, 0.03)	0.25	11	b -0.01 (-0.09, 0.08)	0.88
% male	Per 10%	18	b 0.05 (-0.11, 0.20)	0.55	15	b 0.08 (-0.17, 0.33)	0.49	17	b 0.05 (-0.32, 0.41)	0.79	6	b 0.46 (-0.56, 1.47)	0.28	16	b 0.01 (-0.12, 0.13)	0.92	13	b 0 (-0.16, 0.15)	0.95
Anchorage	TAD	10	1.11 (0.63, 1.58)	0.52	9	1.77 (1.44, 2.11)	0.94	10	2.39 (2.00, 2.78)	0.94	2	4.00 (-3.78, 11.79)	0.15	9	0.78 (0.59, 0.97)	0.72	8	0.71 (0.51, 0.91)	0.95
	TPA	8	1.11 (0.63, 1.58)		7	1.89 (1.04, 2.73)		7	2.55 (1.50, 3.61)		3	2.75 (-1.97, 7.47)		8	0.84 (0.51, 1.18)		7	0.76 (0.38, 1.14)	
	Braces	5	0.82 (0.39, 1.24)		4	1.87 (0.58, 3.15)		6	2.40 (1.58, 3.21)		1	5.13 (3.83, 6.42)		4	0.98 (0.18, 1.78)		2	0.75 (-2.49, 3.99)	
Active	Coil	22	-	-	19	-	-	22	-	-	5	-	-	20	-	-	17	-	-
	Power-chain	1	-		1	-		1	-		1	-		1	-		-	-	
Slot	22”	17	0.91 (0.68, 1.13)	0.22	14	1.72 (1.30, 2.13)	0.07	17	2.31 (1.91, 2.72)	0.003	5	-	-	15	0.82 (0.61, 1.04)	0.12	12	0.66 (0.44, 0.89)	0.06
	18”	4	1.16 (0.60, 1.72)		4	2.24 (1.54, 2.94)		3	3.41 (2.08, 4.75)		-			4	1.03 (0.74, 1.32)		3	0.96 (0.46, 1.46)	
Bracket	CLB	17	0.92 (0.73, 1.12)	0.53	15	1.90 (1.53, 2.27)	0.85	17	2.44 (2.03, 2.84)	0.82	5	-	-	16	0.89 (0.69, 1.08)	0.89	12	0.69 (0.46, 0.92)	0.68
	SLB	4	1.15 (0.03, 2.28)		3	1.77 (-1.14, 4.67)		6	2.54 (1.49, 3.56)		-			3	0.84 (-0.47, 2.15)		3	0.78 (0.01, 1.54)	
Force	50g	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	60g	-	-		-	-		1	-		-	-		-	-		-	-	
	100g	1	-		-	-		-	-		-	-		-	-		-	-	
	120g	-	-		-	-		-	-		-	-		1	-		1	-	
	150g	20	-		19	-		23	-		5	-		19	-		15	-	
	180g	1	-		1	-		1	-		-	-		1	-		1	-	

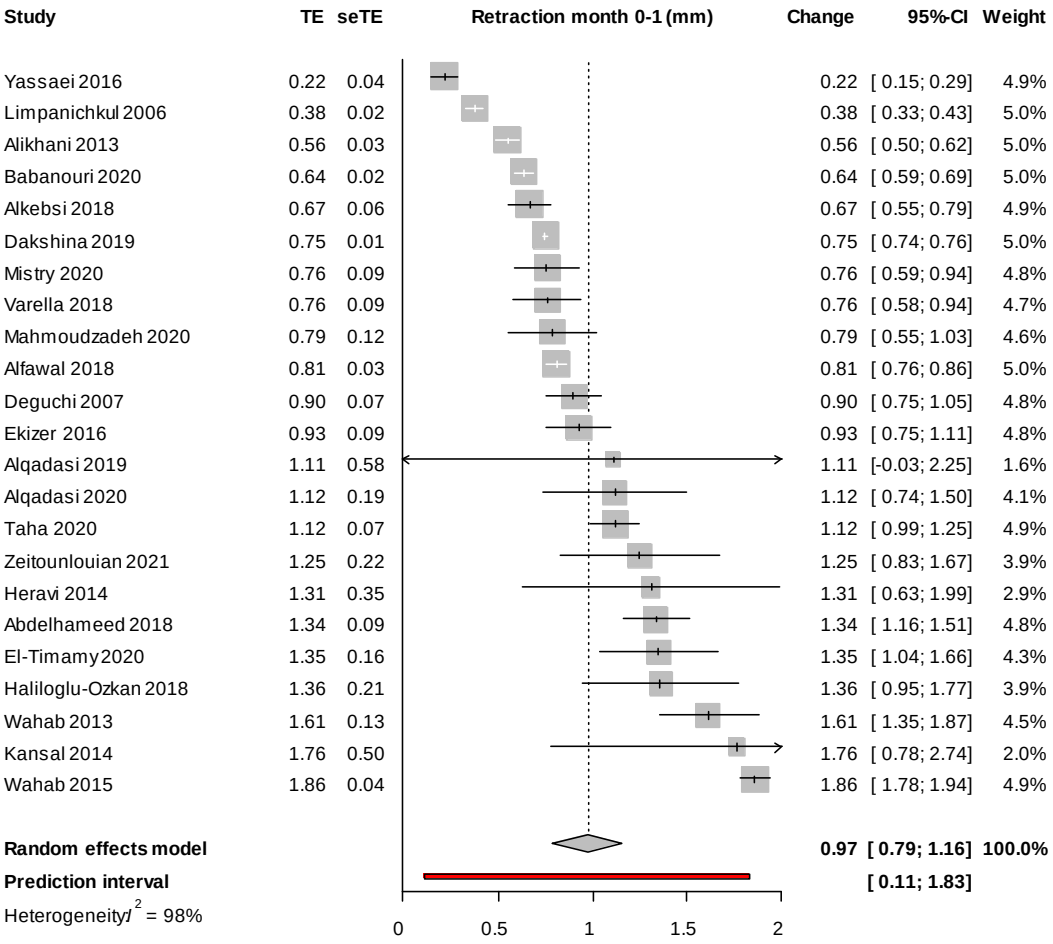
b, meta-regression coefficient; CI, confidence interval; CLB, conventionally ligated bracket; n, number of studies; SLB, self-ligating bracket; TAD, temporary anchorage device

Supplementary Table 3.5. Meta-regression / subgroup analyses for possible factor influencing canine retraction duration and rate (direct meta-analysis from Table III) across identified randomized trials.

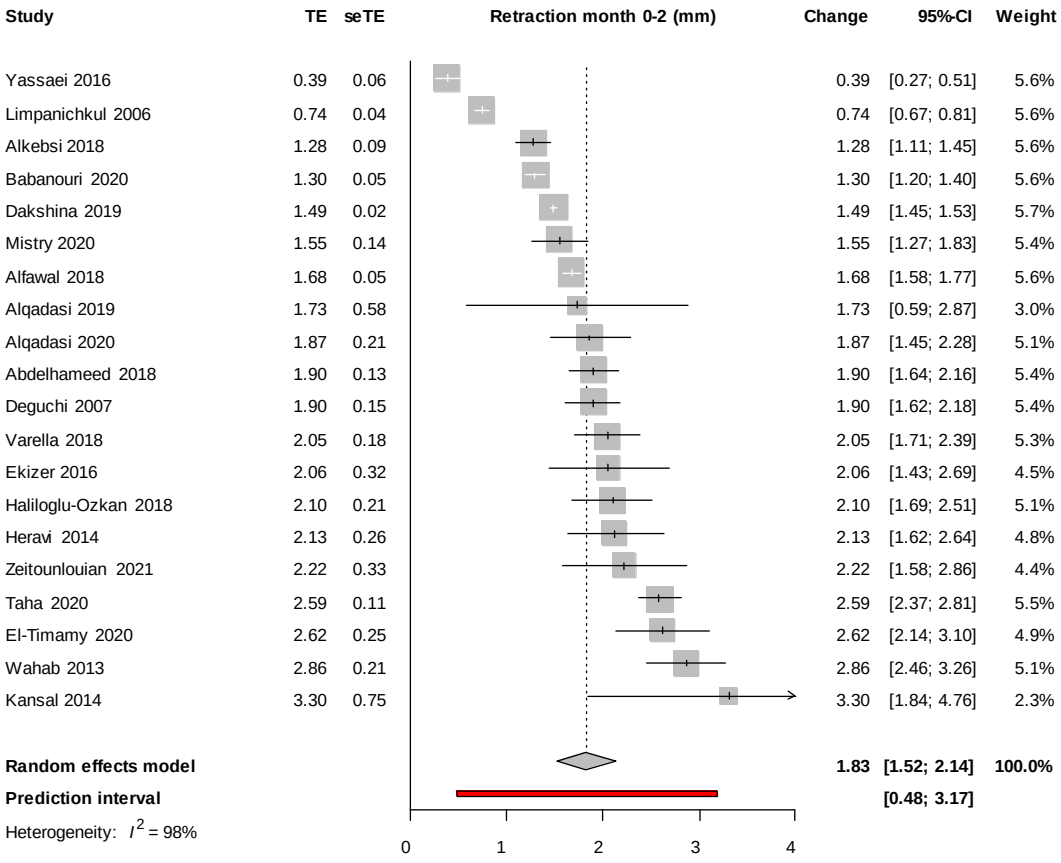
		LLLT vs control; retraction month 0-1 (mm)			Surgically-assisted orthodontics vs control; retraction month 0-1 (mm)			Surgically-assisted orthodontics vs control; retraction month 0-2 (mm)			Surgically-assisted orthodontics vs control; retraction month 0-3 (mm)			Surgically-assisted orthodontics vs control; retraction month 1-2 (mm)			Surgically-assisted orthodontics vs control; retraction month 2-3 (mm)		
Factor	**Level**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**	**n**	**Effect**	**P**
Age	Per year	6	b -0.16 (-0.40, 0.08)	0.14	9	b -0.01 (-0.10, 0.09)	0.90	7	b -0.02 (-0.21, 0.17)	0.82	6	b -0.02 (-0.28, 0.23)	0.82	8	b 0 (-0.09, 0.09)	1.00	7	b 0 (-0.09, 0.08)	0.98
Male	Per 10%	6	b 0.12 (-0.16, 0.40)	0.31	9	b -0.04 (-0.40, 0.33)	0.83	7	b -0.04 (-0.64, 0.55)	0.86	6	b 0.11 (-0.90, 1.12)	0.77	8	b 0.03 (-0.25, 0.32)	0.79	7	b 0.22 (0.03, 0.41)	0.03
Anchorage	TAD	3	MD 0.29 (-0.46, 1.04)	0.20	7	MD 0.33 (-0.02, 0.68)	<0.001	6	MD 0.28 (-0.10, 0.66)	<0.001	6	MD 0.43 (0, 2.94)	<0.001	6	MD 0.10 (-0.18, 0.38)	0.002	5	MD 0.09 (-0.08, 0.26)	0.27
	Braces	3	MD 0.29 (-1.00, 1.59)		1	MD 1.16 (0.92, 1.40)		-			-			-			-		
	TPA	3	MD 0.02 (0, 0.04)		2	MD 0.80 (0.29, 1.30)		2	MD 1.24 (-0.09, 2.57)		2	MD 1.36 (-0.23, 2.94		3	MD 0.51 (0.20, 0.81)		3	MD 0.30 (-0.48, 1.07)	
Bracket	CLB	7	MD 0.30 (-0.04, 0.63)	0.01	9			8			8			9			8		
	SLB	2	MD -0.05 (-0.33, 0.22)		-			-			-			-			-		
Slot	18”	2	MD 0.40 (-2.58, 3.37)	0.53	-			-			-			-			-		
	22”	6	MD 0.22 (-0.16, 0.61)		9			7			7			8			7		
Activ	Coil	8			10			8			8			9			8		
	Power-chain	1			-			-			-			-			-		
Force	100g	-			1			-			-			-			-		
	120g	-			-			-			-			1			1		
	150g	9			9			8			8			8			7		

b, meta-regression coefficient; CI, confidence interval; CLB, conventionally ligated bracket; MD, mean difference; n, number of studies; SLB, self-ligating bracket; TAD, temporary anchorage device; TPA, transpalatal arch.

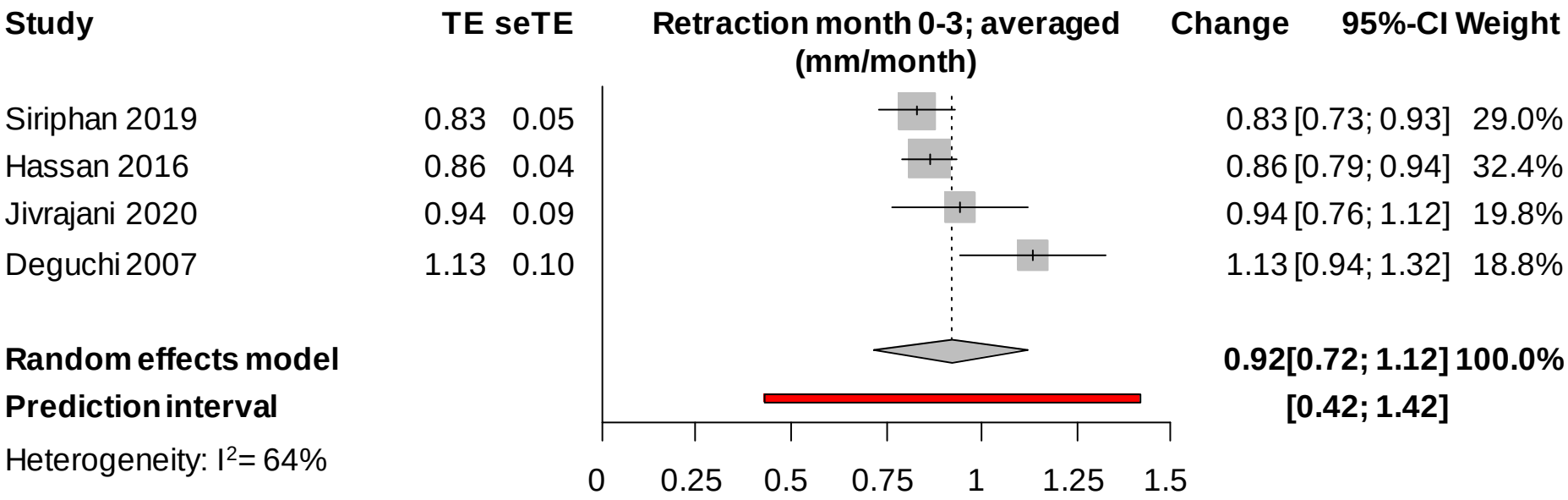
Appendix Figure 3.1 Forest plot for the indirect meta-analysis on retraction during month 0 to month 1.



Appendix Figure 3.2 Forest plot for the indirect meta-analysis on retraction during month 0 to month 2.

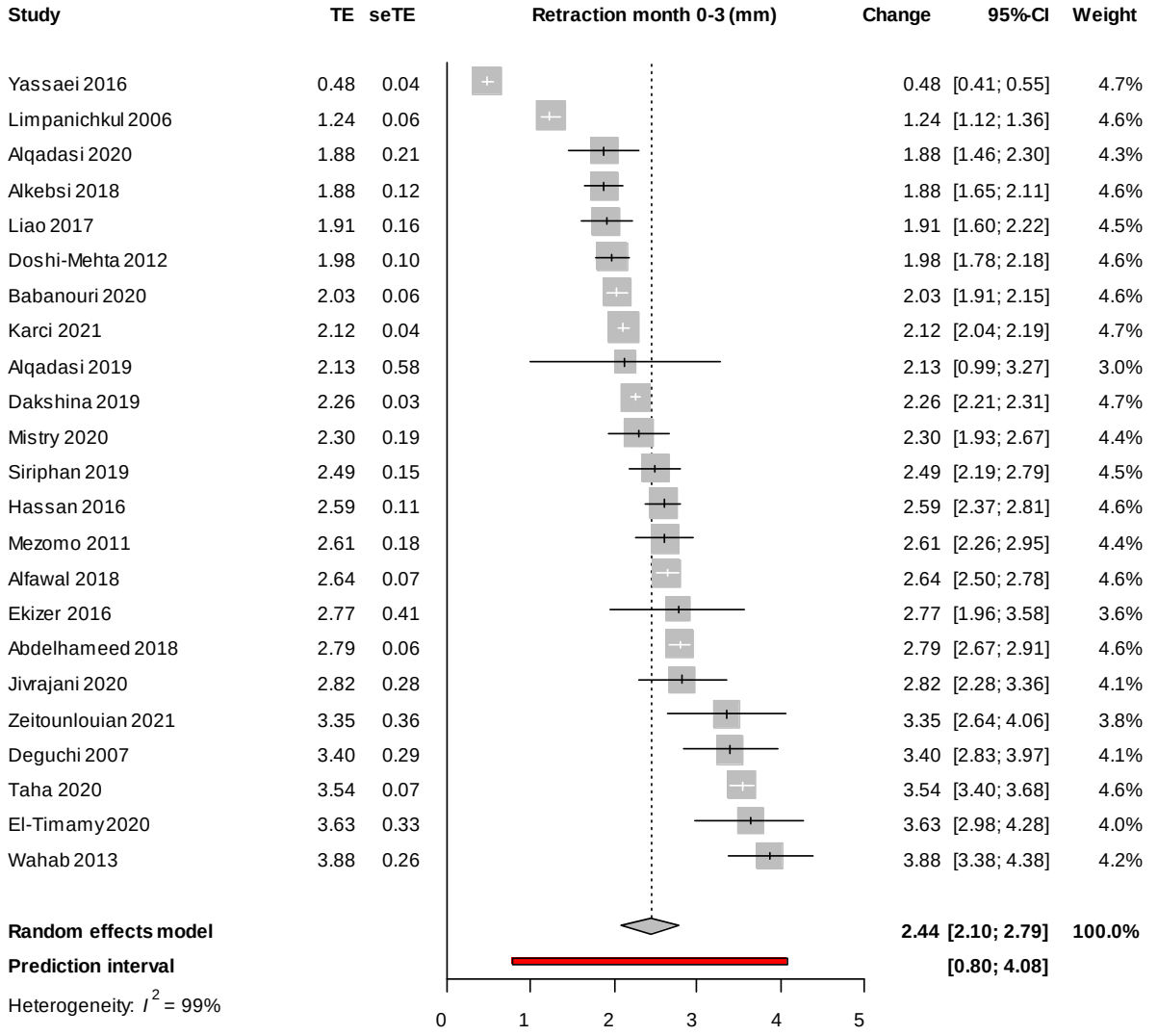

Study
TE
seTE
Retraction month 0-2 (mm)
Change
95%-CI
Weight
Yassaei 2016 0.39 0.06 0.39 [0.27; 0.51] 5.6%
Limpanichkul 2006 0.74 0.04 0.74 [0.67; 0.81] 5.6%
Alkebsi 2018 1.28 0.09 1.28 [1.11; 1.45] 5.6%
Babanouri 2020 1.30 0.05 1.30 [1.20; 1.40] 5.6%
Dakshina 2019 1.49 0.02 1.49 [1.45; 1.53] 5.7%
Mistry 2020 1.55 0.14 1.55 [1.27; 1.83] 5.4%
Alfawal 2018 1.68 0.05 1.68 [1.58; 1.77] 5.6%
Alqadasi 2019 1.73 0.58 1.73 [0.59; 2.87] 3.0%
Alqadasi 2020 1.87 0.21 1.87 [1.45; 2.28] 5.1%
Abdelhameed 2018 1.90 0.13 1.90 [1.64; 2.16] 5.4%
Deguchi 2007 1.90 0.15 1.90 [1.62; 2.18] 5.4%
Varella 2018 2.05 0.18 2.05 [1.71; 2.39] 5.3%
Ekizer 2016 2.06 0.32 2.06 [1.43; 2.69] 4.5%
Haliloglu-Ozkan 2018 2.10 0.21 2.10 [1.69; 2.51] 5.1%
Heravi 2014 2.13 0.26 2.13 [1.62; 2.64] 4.8%
Zeitounlouian 2021 2.22 0.33 2.22 [1.58; 2.86] 4.4%
Taha 2020 2.59 0.11 2.59 [2.37; 2.81] 5.5%
El-Timamy 2020 2.62 0.25 2.62 [2.14; 3.10] 4.9%
Wahab 2013 2.86 0.21 2.86 [2.46; 3.26] 5.1%
Kansal 2014 3.30 0.75 3.30 [1.84; 4.76] 2.3%
Random effects model 1.83 [1.52; 2.14] 100.0%
Prediction interval [0.48; 3.17]
Heterogeneity: I^2 = 98%
0
1
2
3
4

Appendix Figure 3.3 Forest plot for the indirect meta-analysis on retraction during month 0 to month 3; averaged per month.

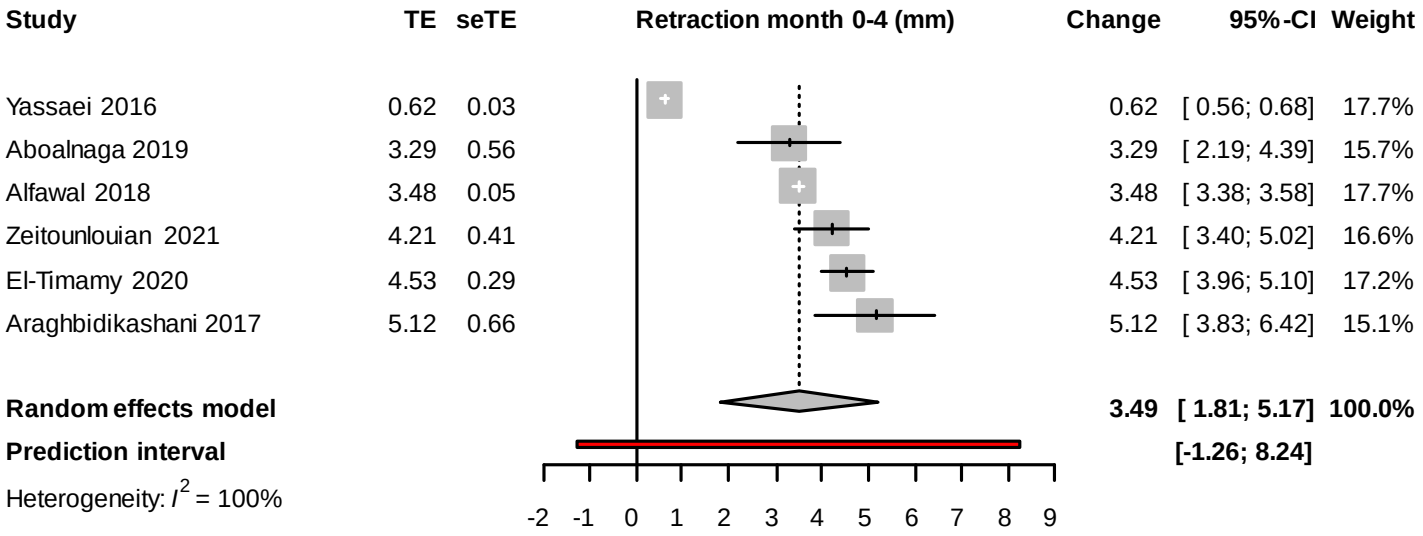


A

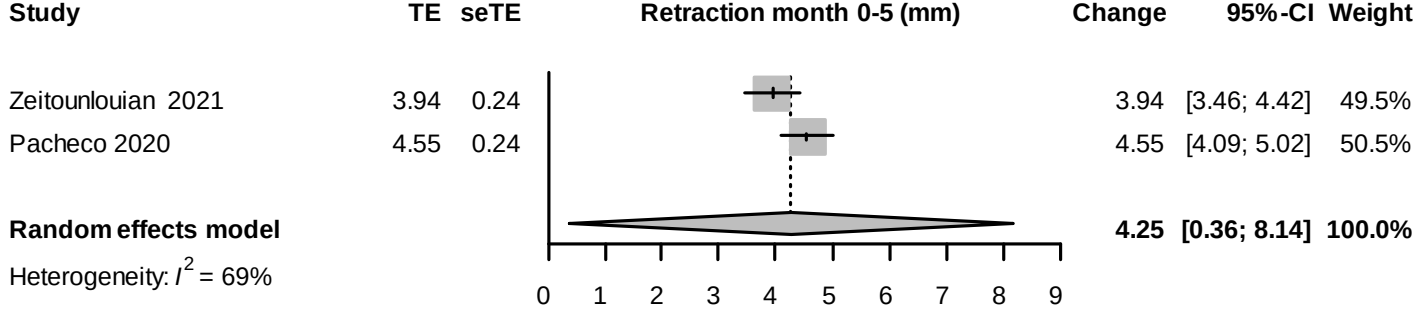
Appendix Figure 3.4 Forest plot for the indirect meta-analysis on retraction during month 0 to month 3.



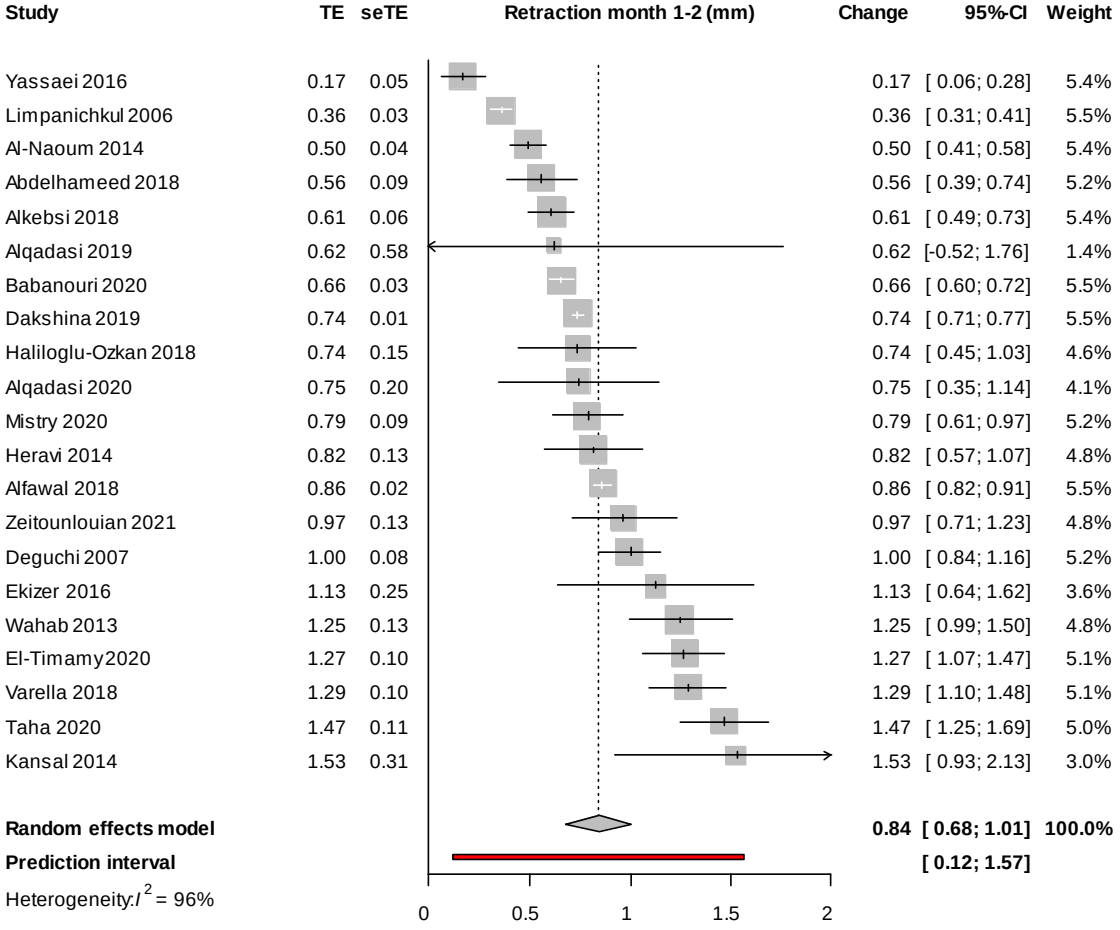
Appendix Figure 3.5 Forest plot for the indirect meta-analysis on retraction during month 0 to month 4.



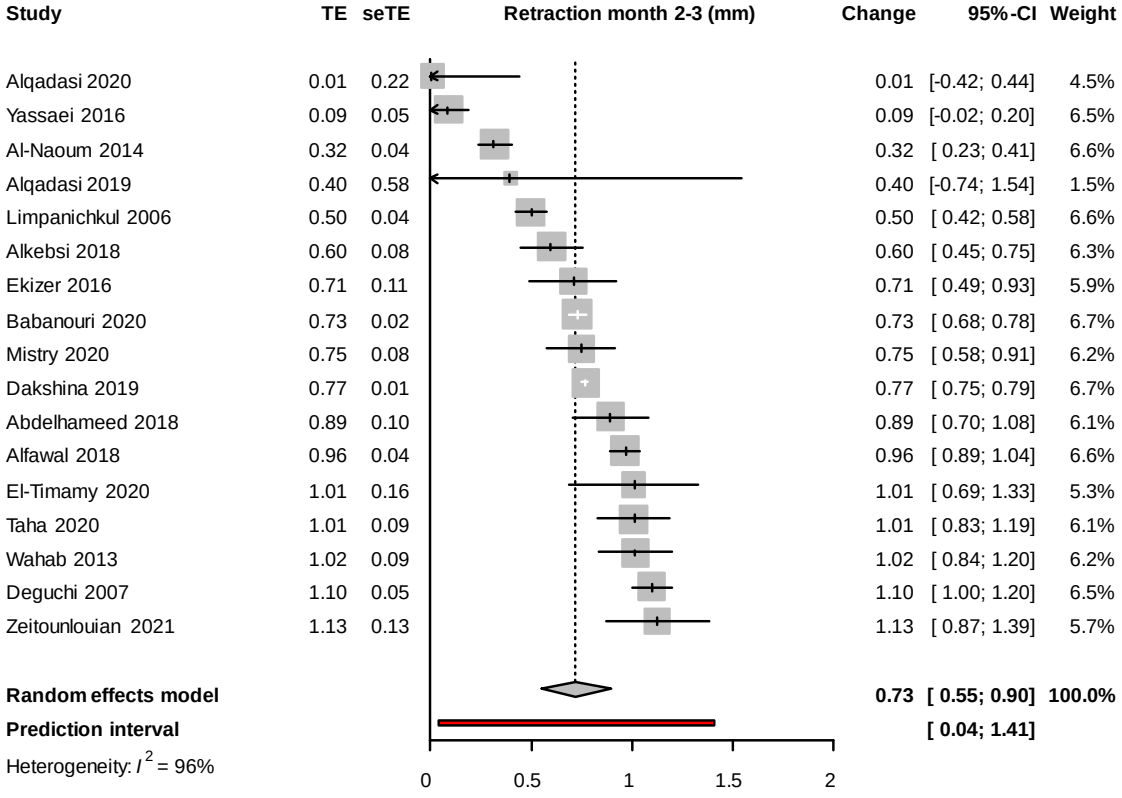
Appendix Figure 3.6 Forest plot for the indirect meta-analysis on retraction during month 0 to month 5.



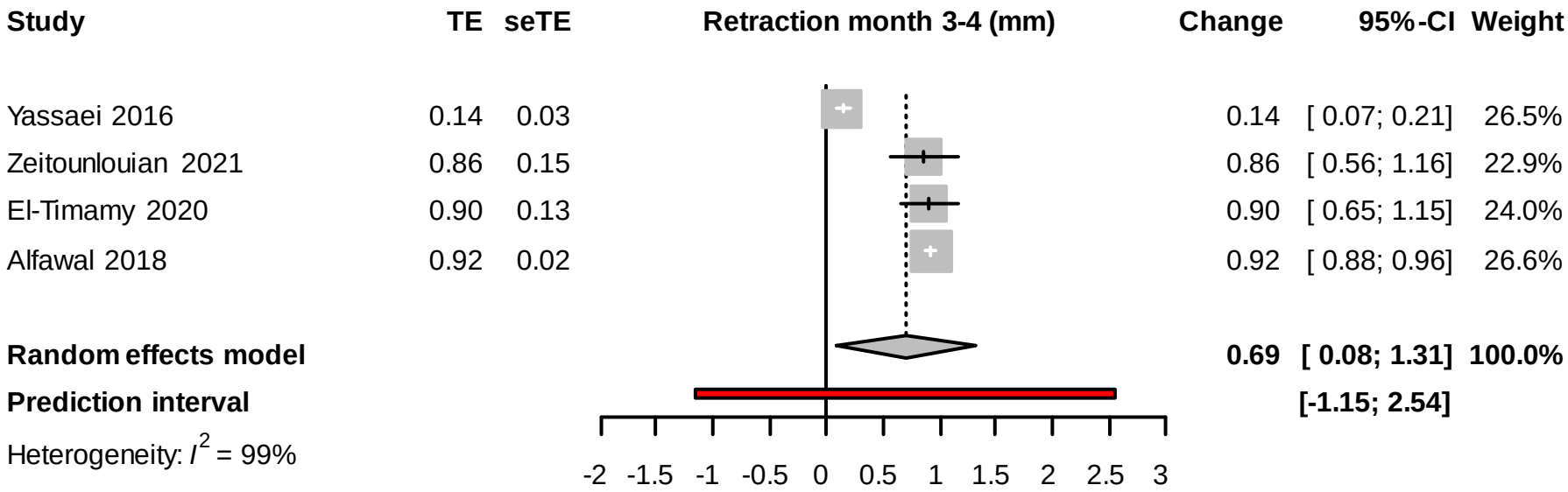
Appendix Figure 3.7 Forest plot for the indirect meta-analysis on retraction during month 1 to month 2.



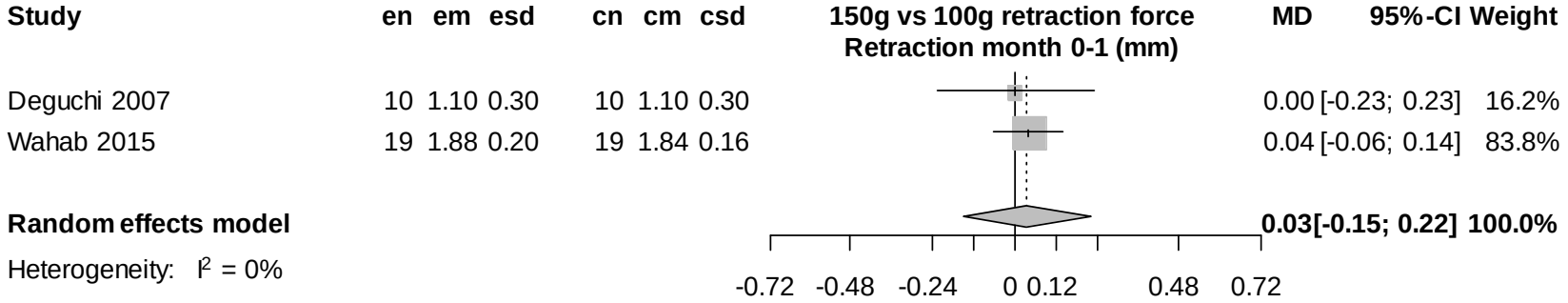
Appendix Figure 3.8 Forest plot for the indirect meta-analysis on retraction during month 2 to month 3.



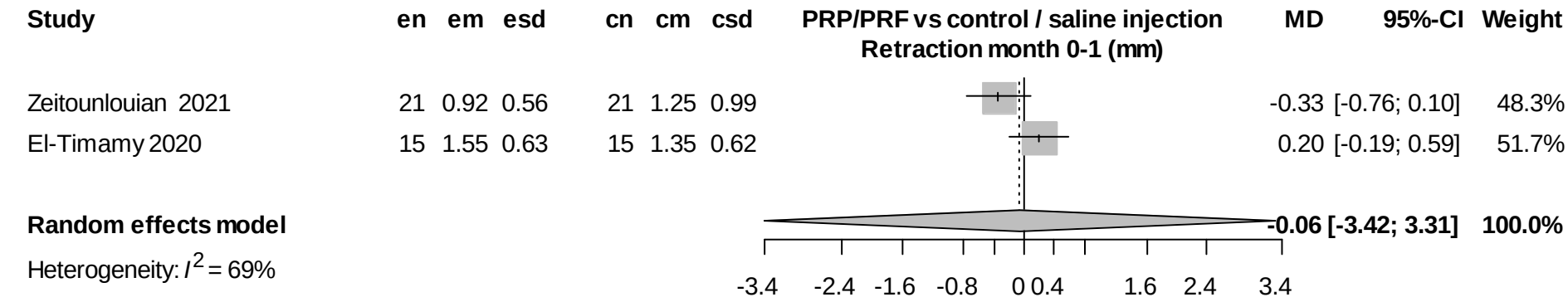
Appendix Figure 3.9 Forest plot for the indirect meta-analysis on retraction during month 3 to month 4.



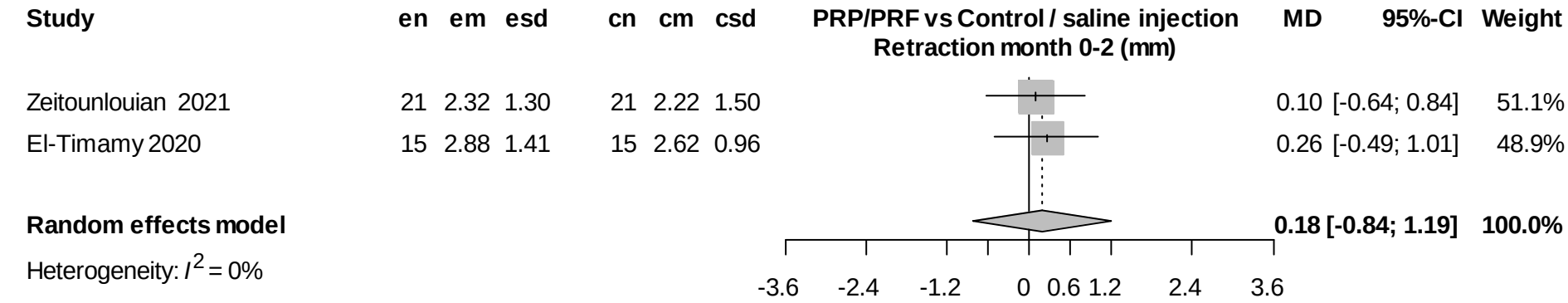
Appendix Figure 3.10 Forest plot for the direct meta-analysis on retraction during month 0 to month 1 between 150g-force group and 100g-force group.



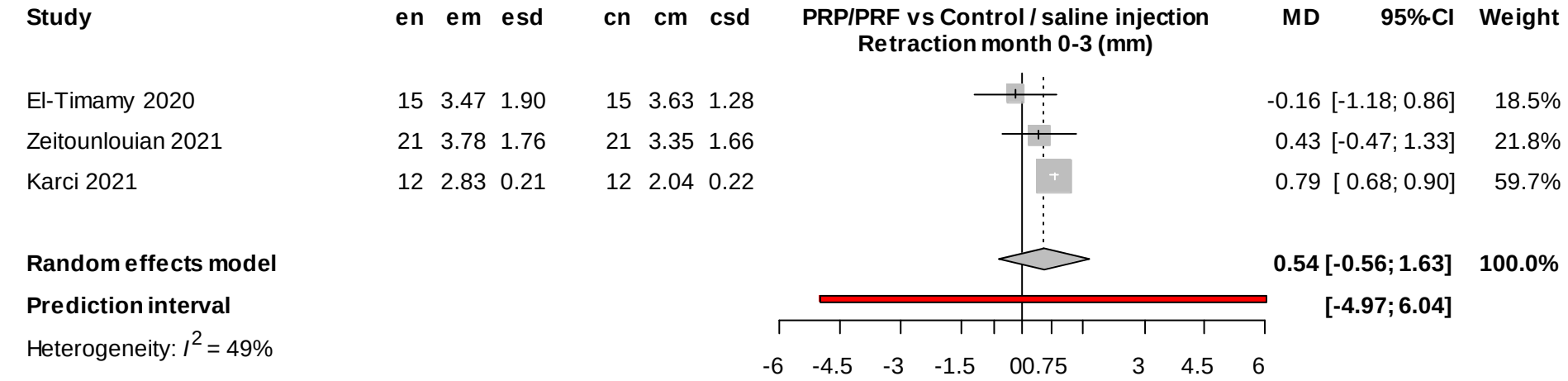
Appendix Figure 3.11 Forest plot for the direct meta-analysis on retraction during month 0 to month 1 between PRP/PRF group and control group.



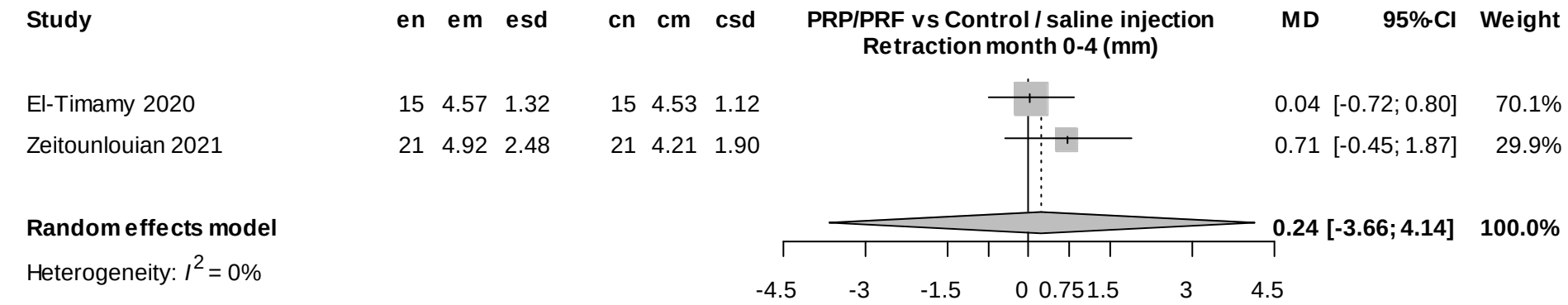
Appendix Figure 3.12 Forest plot for the direct meta-analysis on retraction during month 0 to month 2 between PRP/PRF group and control group.



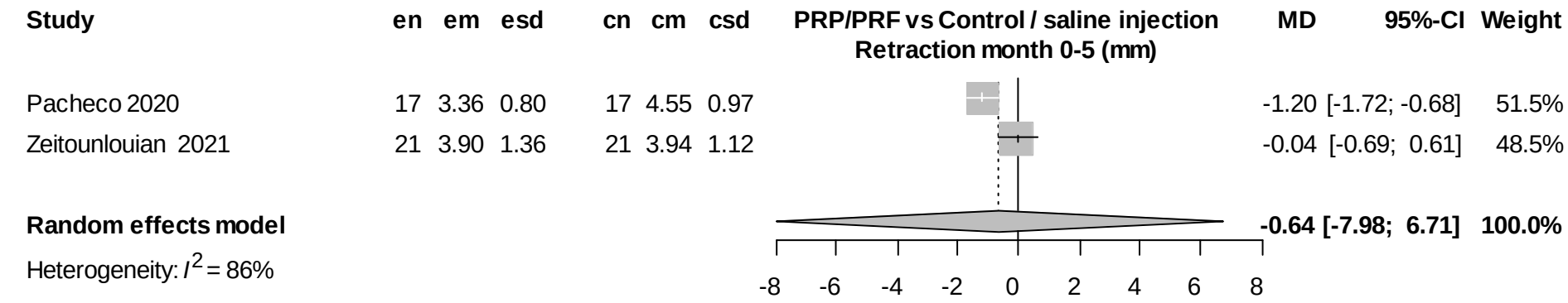
Appendix Figure 3.13 Forest plot for the direct meta-analysis on retraction during month 0 to month 3 between PRP/PRF group and control group.



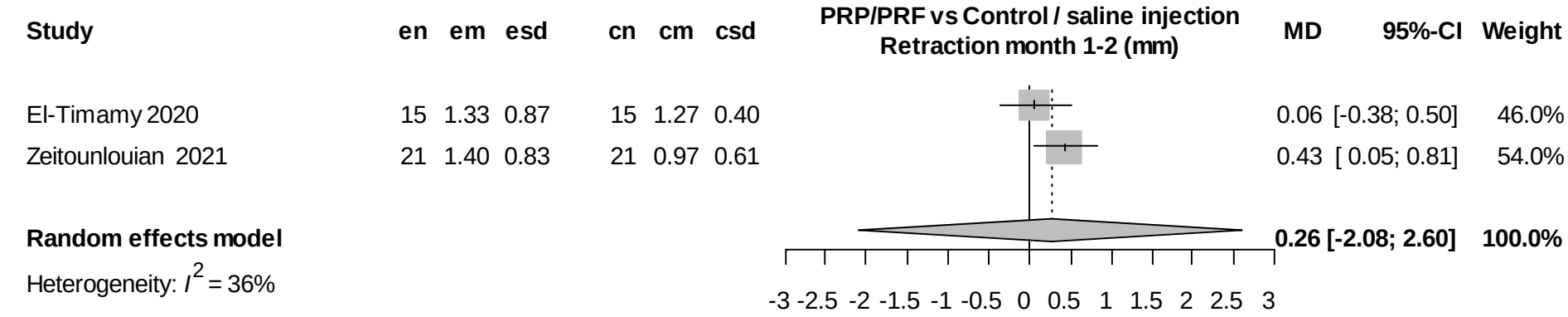
Appendix Figure 3.14 Forest plot for the direct meta-analysis on retraction during month 0 to month 4 between PRP/PRF group and control group.



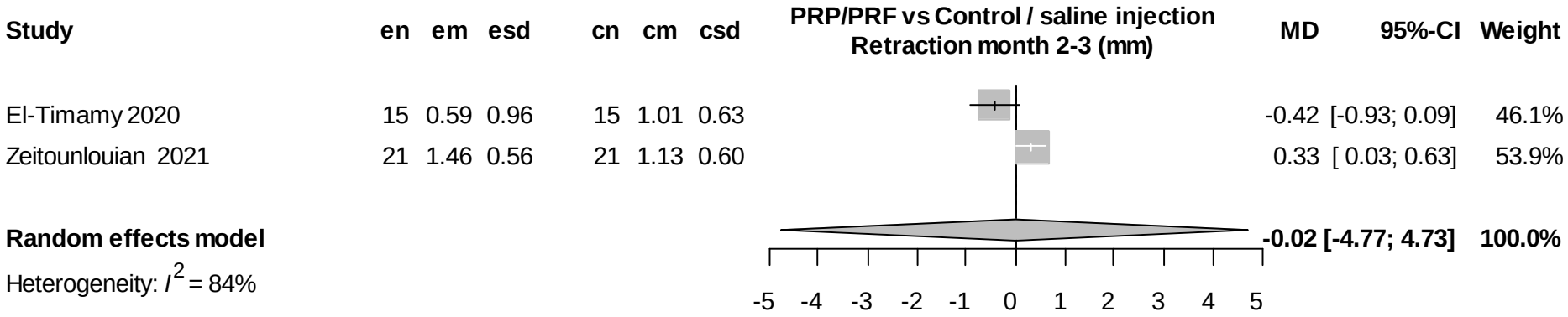
Appendix Figure 3.15 Forest plot for the direct meta-analysis on retraction during month 0 to month 5 between PRP/PRF group and control group.



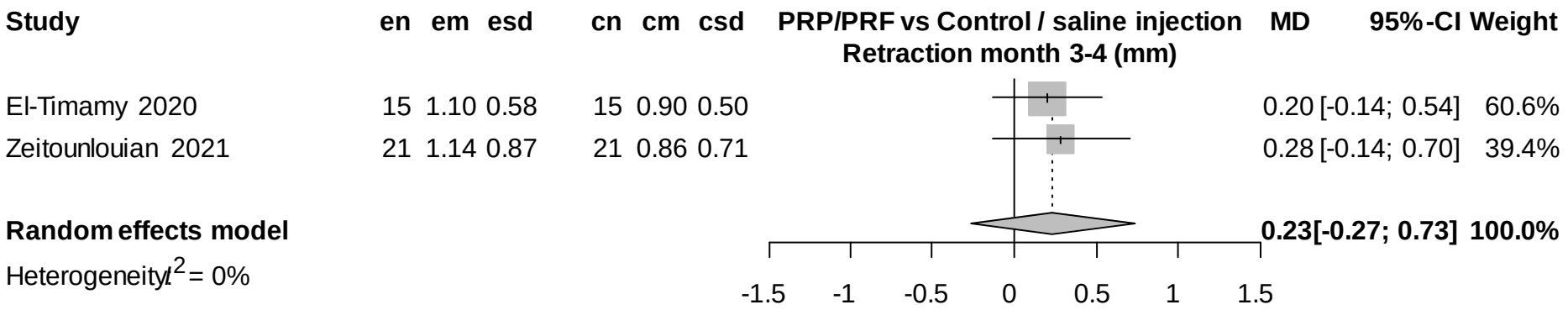
Appendix Figure 3.16 Forest plot for the direct meta-analysis on retraction during month 1 to month 2 between PRP/PRF group and control group.



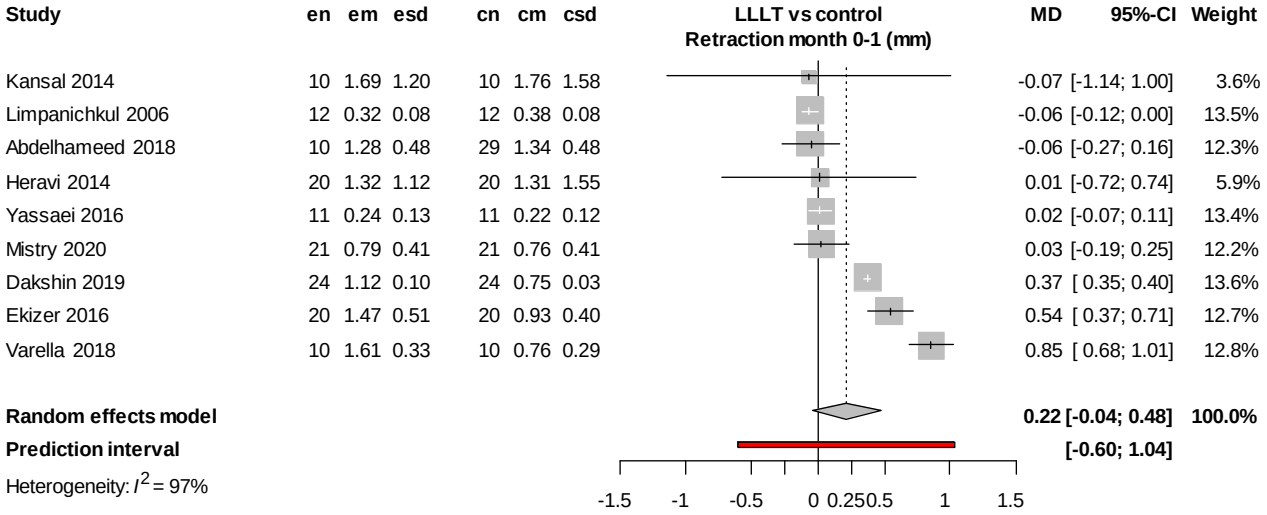
Appendix Figure 3.17 Forest plot for the direct meta-analysis on retraction during month 2 to month 3 between PRP/PRF group and control group.



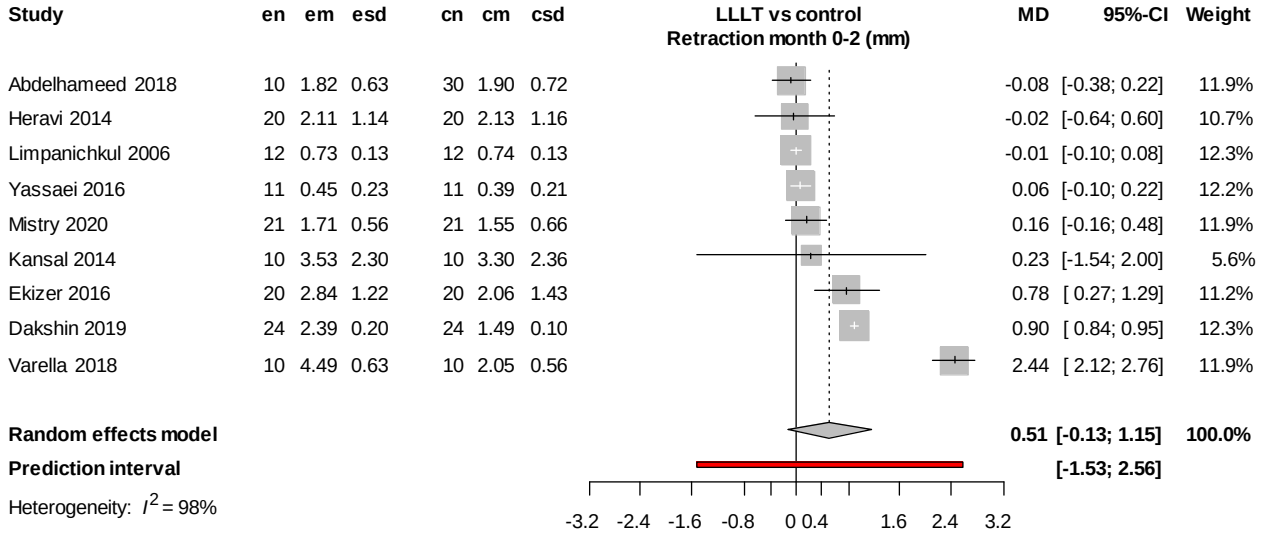
Appendix Figure 3.18 Forest plot for the direct meta-analysis on retraction during month 3 to month 4 between PRP/PRF group and control group.



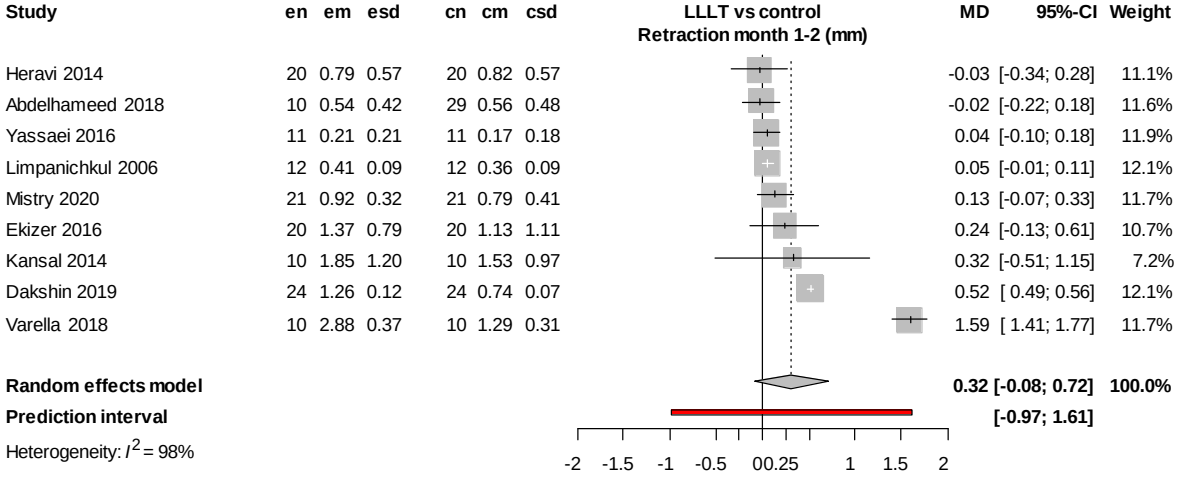
Appendix Figure 3.19 Forest plot for the direct meta-analysis on retraction during month 0 to month 1 between LLLT group and control group.



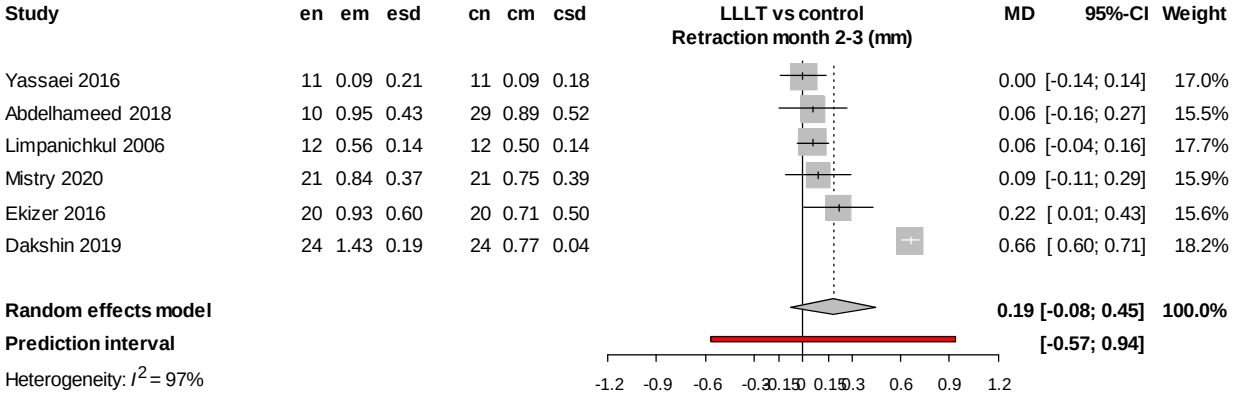
Appendix Figure 3.20 Forest plot for the direct meta-analysis on retraction during month 0 to month 2 between LLLT group and control group.



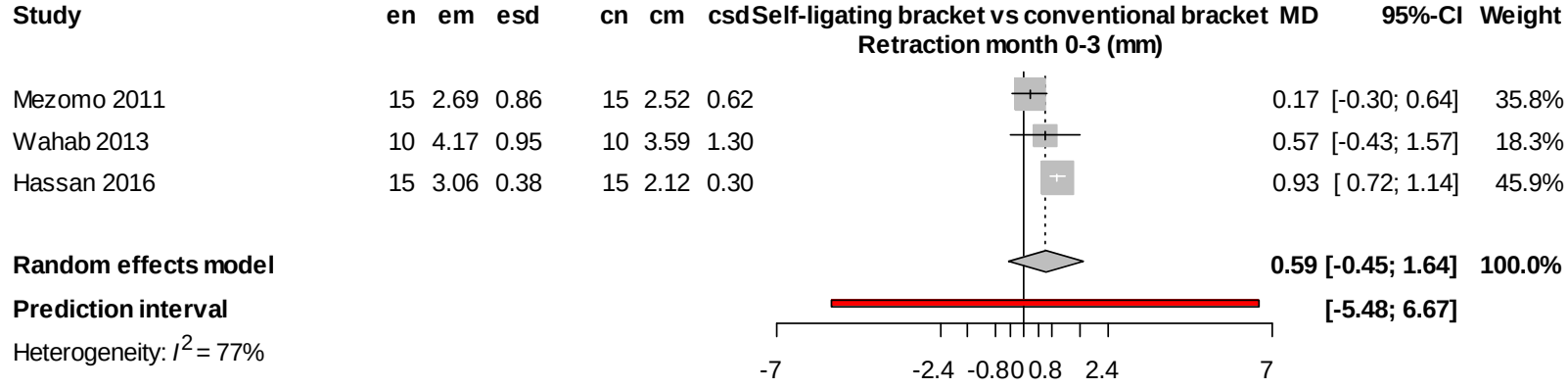
Appendix Figure 3.21 Forest plot for the direct meta-analysis on retraction during month 1 to month 2 between LLLT group and control group.



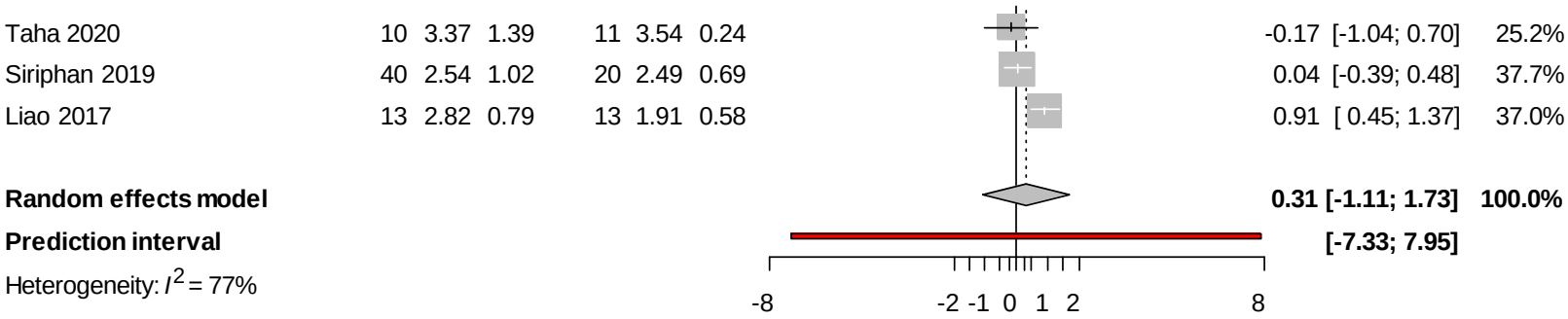
Appendix Figure 3.22 Forest plot for the direct meta-analysis on retraction during month 2 to month 3 between LLLT group and control group.



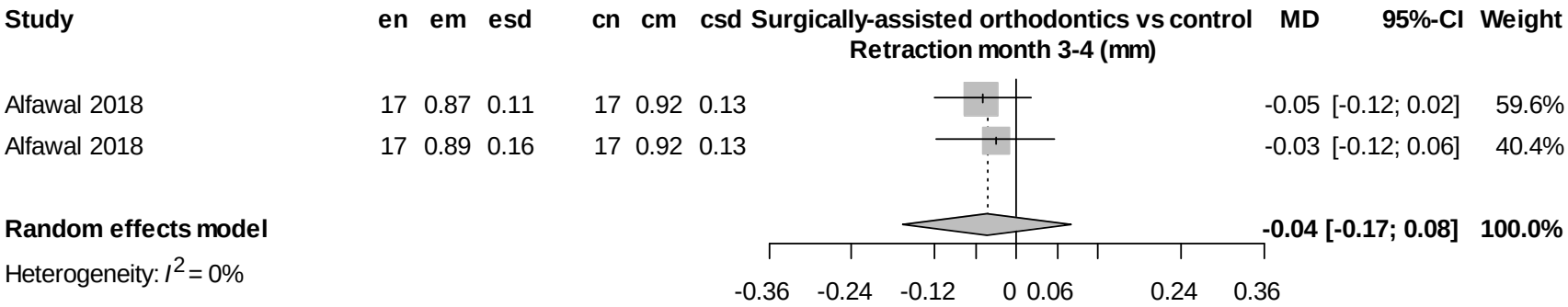
Appendix Figure 3.23 Forest plot for the direct meta-analysis on retraction during month 0 to month 3 between self-ligating bracket group and conventionally ligated bracket g



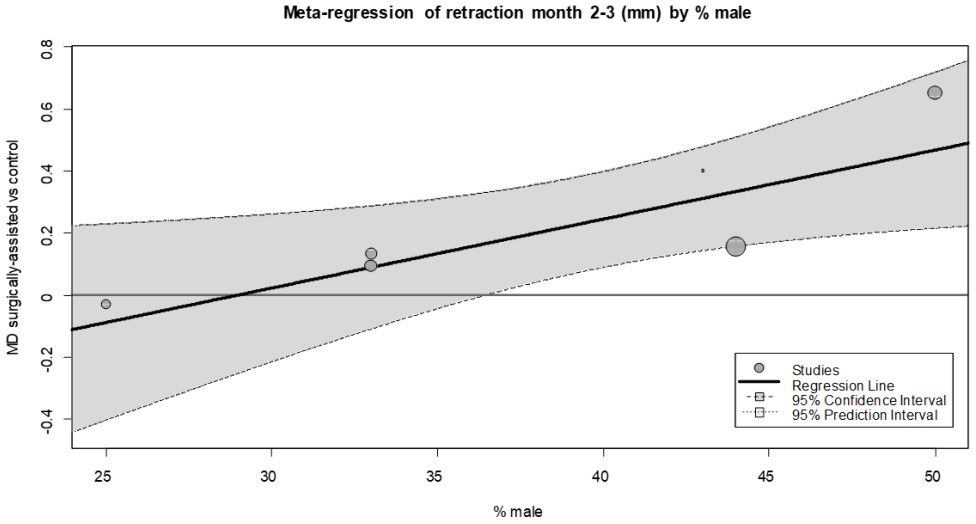
Appendix Figure 3.24 Forest plot for the direct meta-analysis on retraction during month 0 to month 3 between adjunct vibration group and control group.



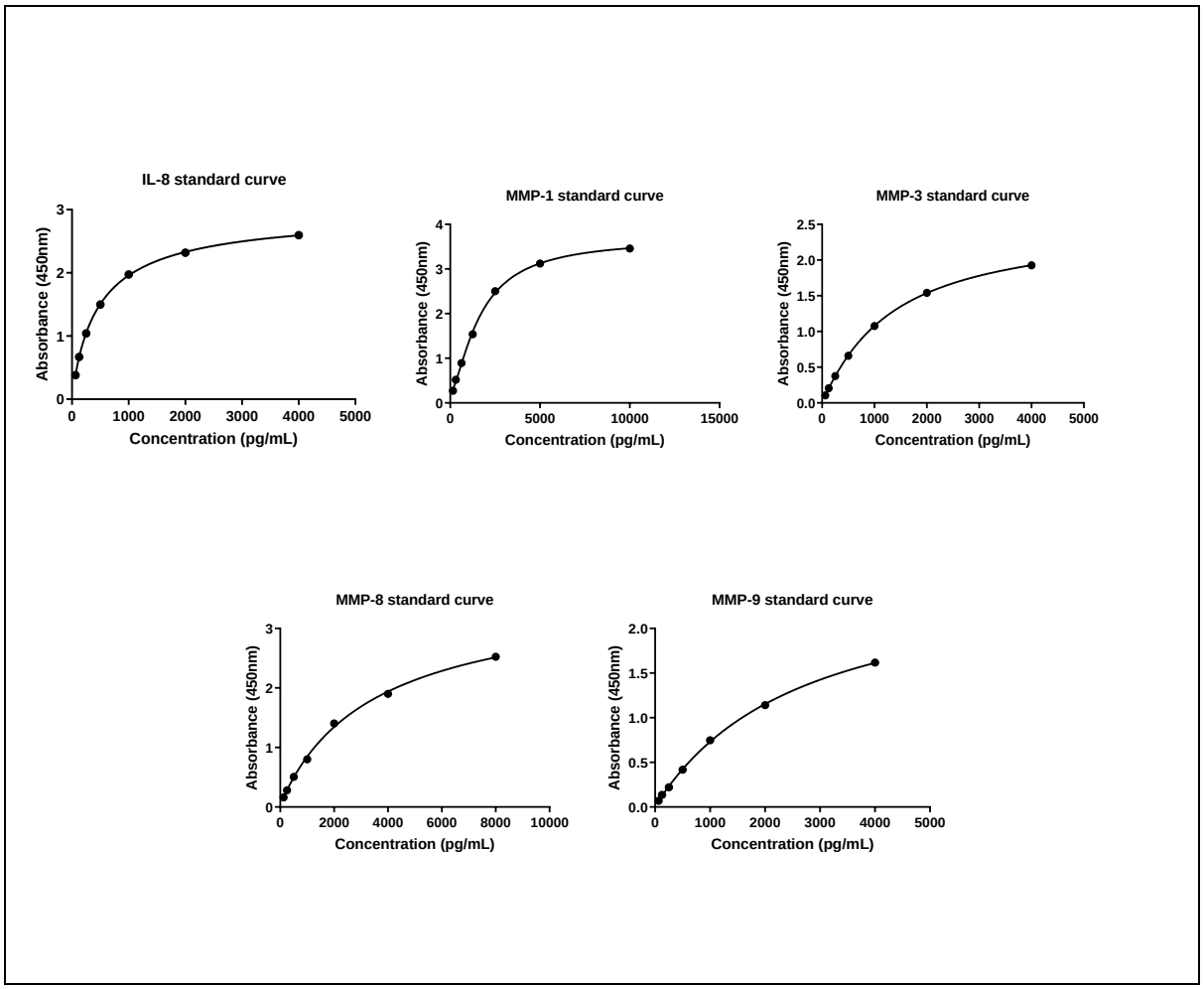
Appendix Figure 3.25 Forest plot for the direct meta-analysis on retraction during month 3 to month 4 between surgically-assisted group and control group.



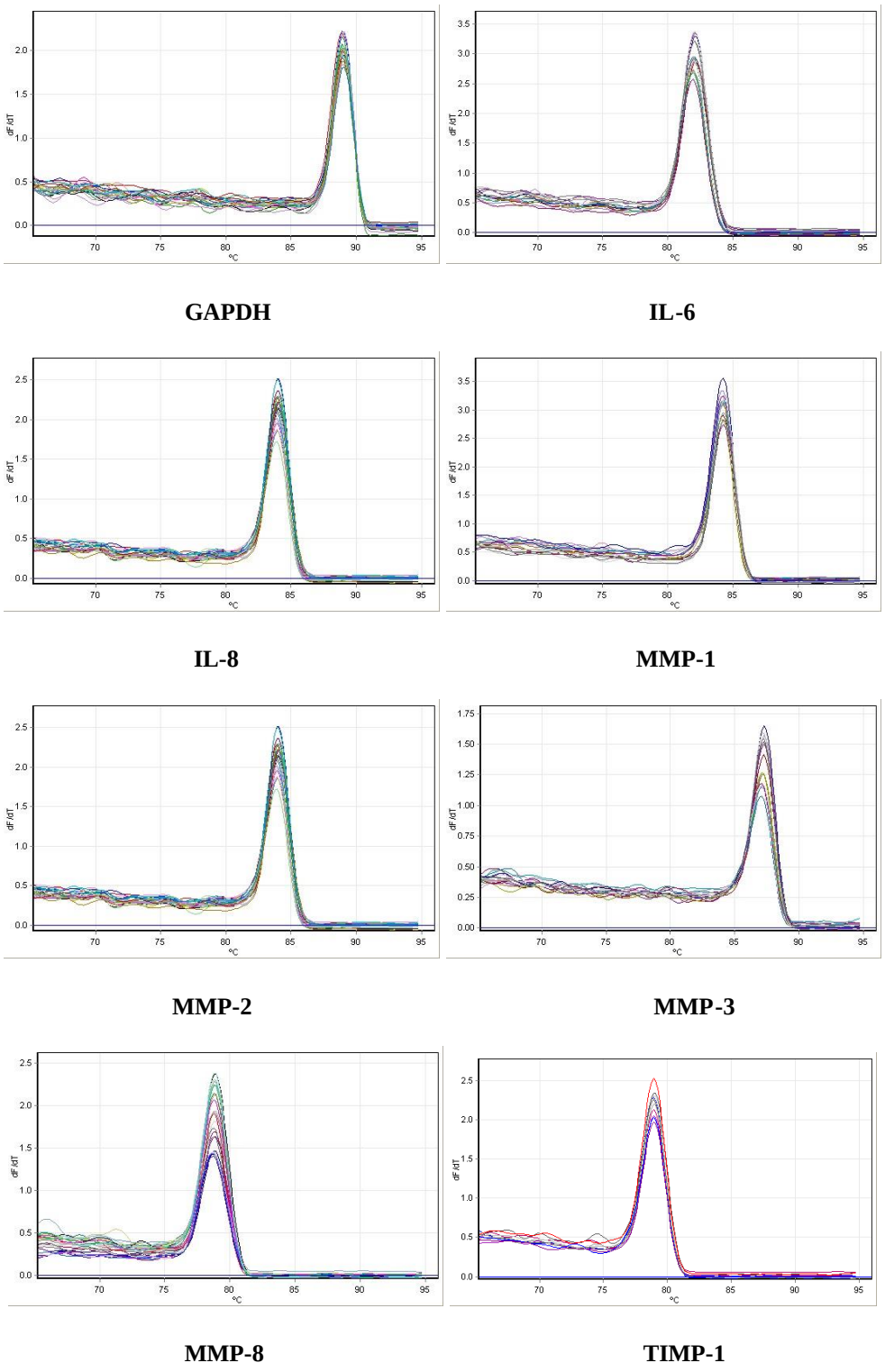
Appendix **Figure 3.26** Random-effects meta-regression for the effect of patient sex (% of male patients) on the MD of surgically-assisted group versus control group.



Chapter 4

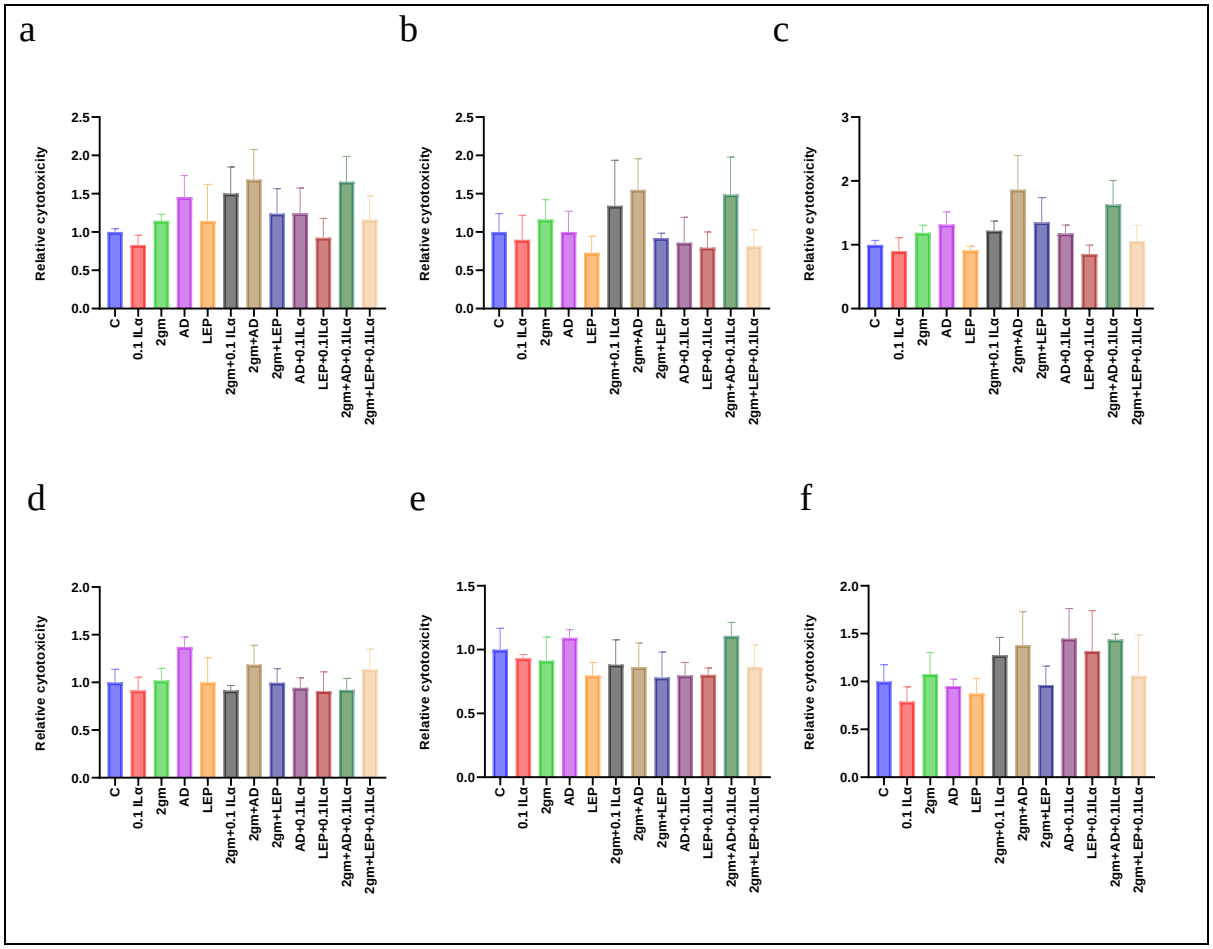


Appendix Figure 4.1 ELISA standard curves.



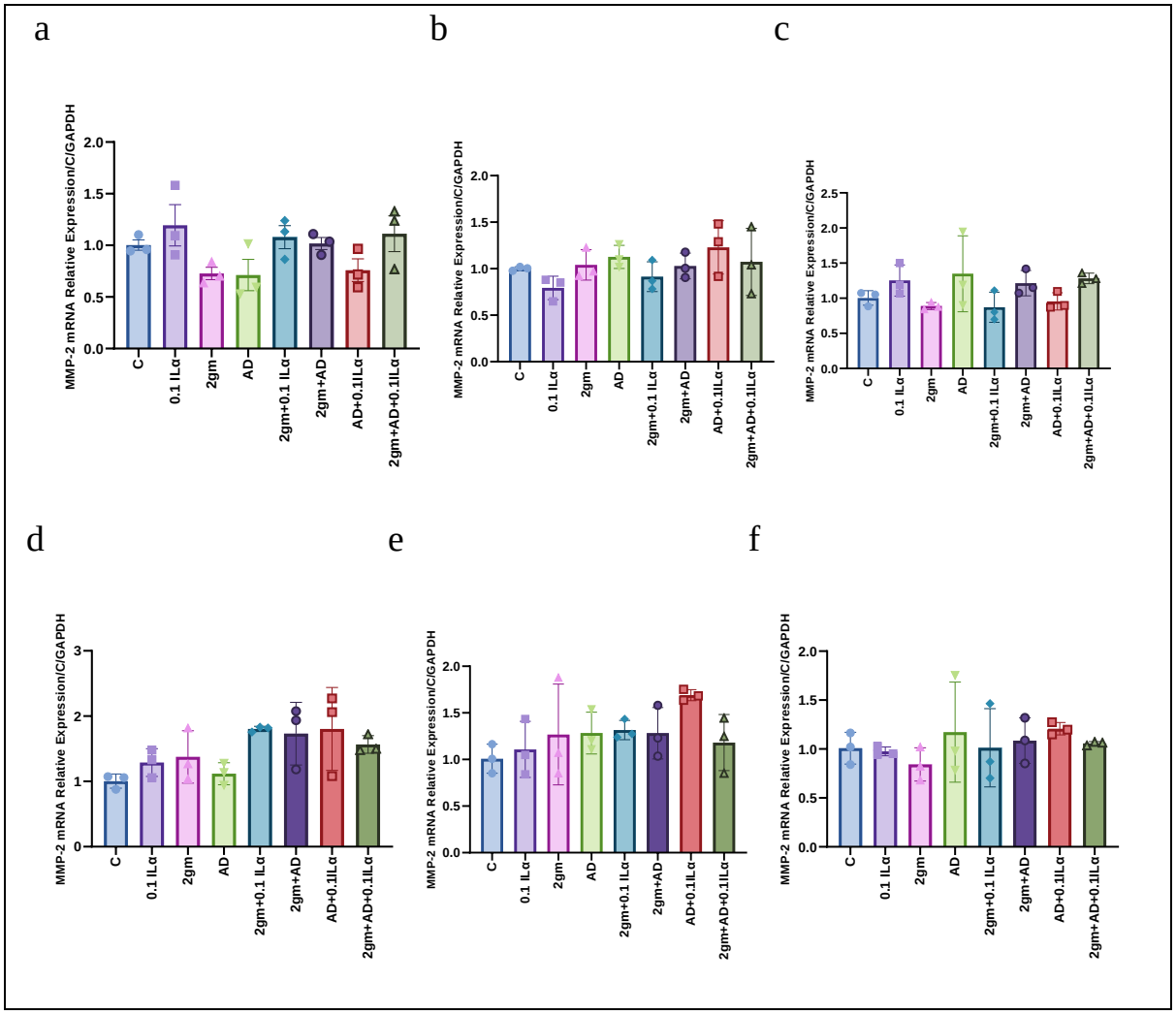
Appendix Figure 4.2 Melting curves of primers used in the study.

Primer specificity was checked by melt curve analysis, and the Melting curves of all primers showed a single peak indicating an absence of primer dimers.



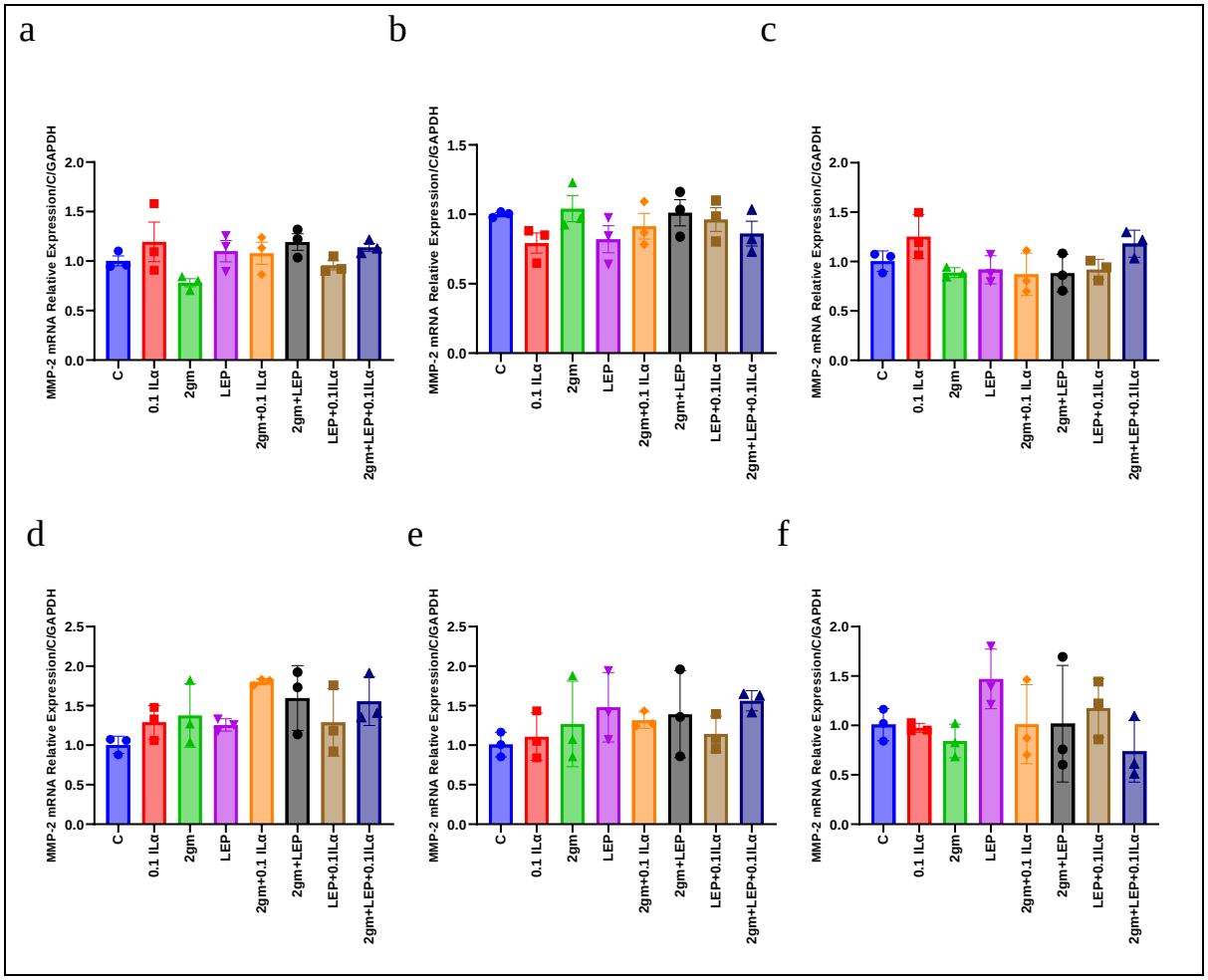
Appendix Figure 4.3 The effect of IL-1α, leptin, AdipoRon, and force on the cytotoxicity of hPDLFs and hGFs.

hPDLFs (a, b, & c) and hGFs (d, e, & f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm2), leptin (10 µg/ml), and AdipoRon (40 µM) alone or in combination. Cytotoxicity of hPDLFs was assessed using LDH cytotoxicity assay after 24 (a), 48 (b), and 72 hours (c) of stimulation. Cytotoxicity was expressed as a fold change relative to the unstimulated cells. C, unstimulated cells; O.1 ILα, 0.1 IL-1α; AD, AdipoRon; LEP, leptin; 2gm, 2 gm/cm2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Dunnett-corrected).



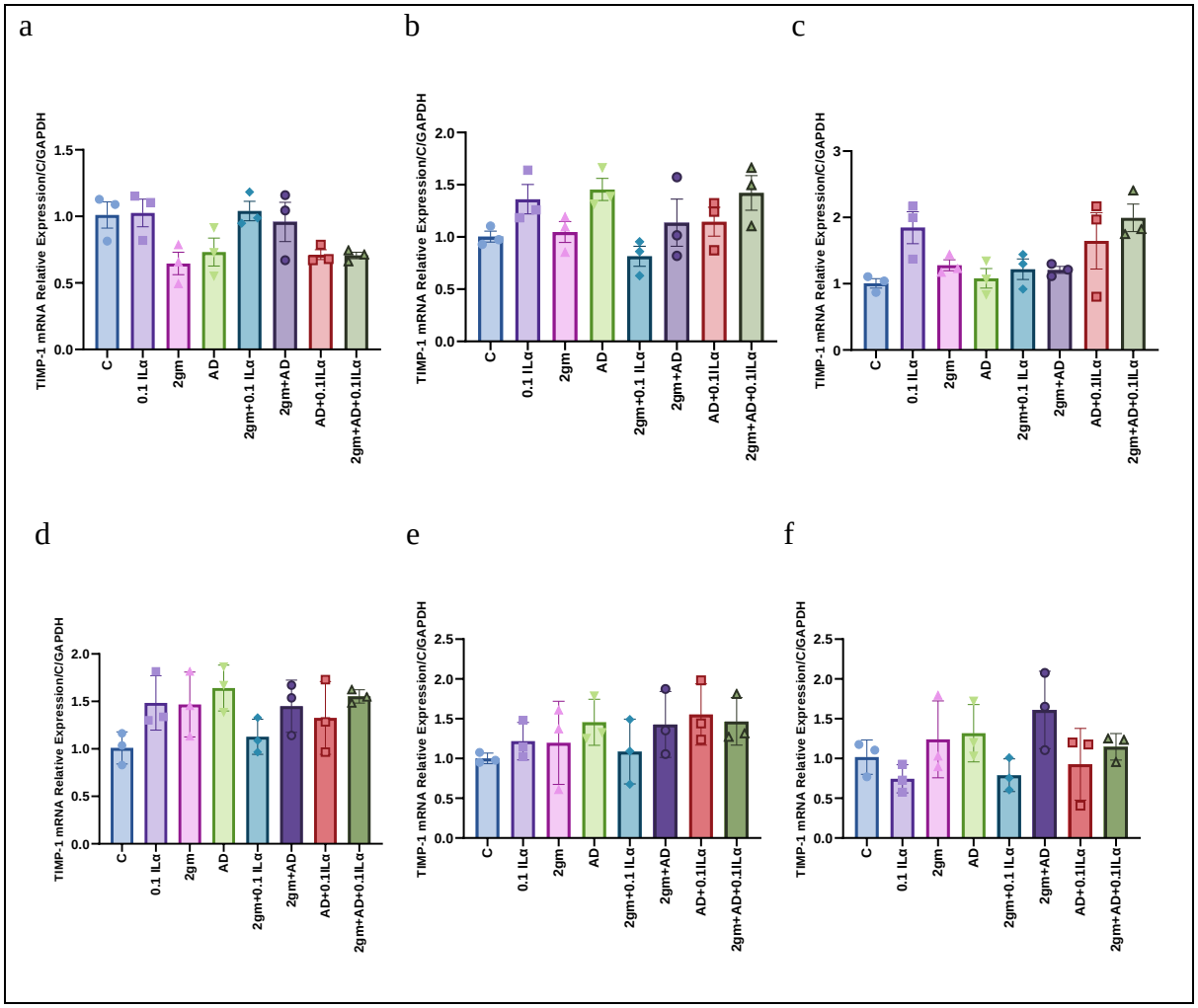
Appendix Figure 4.4 Relative MMP-2 mRNA expression in hPDLFs and hGFs.

hPDLFs (a-c) and hGFs (d-f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and AdipoRon (40 μM) alone or in combination for 24 (a & d), 48 (b & e), and 72 (c & f) hours. MMP-2 mRNA expression was measured using the 2-ddCt method using GAPDH as the housekeeping gene and relative to the unstimulated cells. C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; AD, AdipoRon; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected).



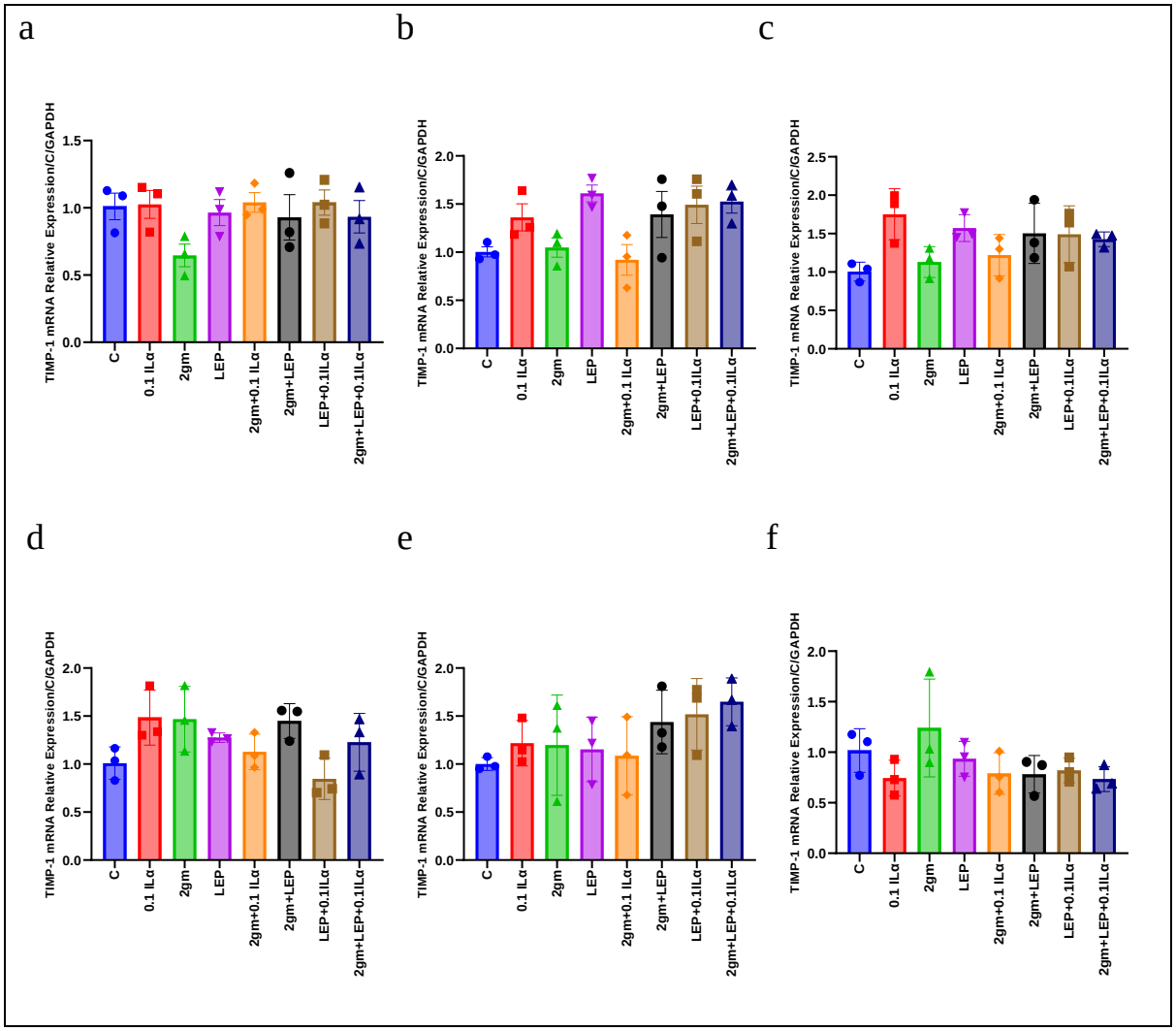
Appendix Figure 4.5 Relative MMP-2 mRNA expression in hPDLFs and hGFs.

hPDLFs (a-c) and hGFs (d-f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm2), and leptin (10 µg/ml) alone or in combination for 24 (a & d), 48 (b & e), and 72 (c & f) hours. MMP-2 mRNA expression was measured using the 2-ddCt method using GAPDH as the housekeeping gene and relative to the unstimulated cells. C, unstimulated cells; O.1 ILα, 0.1 IL-1α; LEP, leptin; 2gm, 2 gm/cm2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected).



Appendix Figure 4.6 Relative TIMP-1 mRNA expression in hPDLFs and hGFs.

hPDLFs (a-c) and hGFs (d-f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and AdipoRon (40 μM) alone or in combination for 24 (a & d), 48 (b & e), and 72 (c & f) hours. TIMP-1 mRNA expression was measured using the 2^{-ddCt} method using GAPDH as the housekeeping gene and relative to the unstimulated cells. C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; AD, AdipoRon; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected).



Appendix figure 4.7 Relative TIMP-1 mRNA expression in hPDLFs and hGFs.

hPDLFs (a-c) and hGFs (d-f) were stimulated with IL-1α (0.1 ng/ml), compressive force (2 gm/cm^2), and leptin (10 µg/ml) alone or in combination 24 (a & d), 48 (b & e), and 72 (c & f) hours. TIMP-1 mRNA expression was measured using the 2^{-ddCt} method using GAPDH as the housekeeping gene and relative to the unstimulated cells. C, unstimulated cells; 0.1 ILα, 0.1 IL-1α; LEP, leptin; 2gm, 2 gm/cm^2 compressive force; n=3. Data are shown as mean ± SD. Data were analysed by One-way ANOVA (Tukey-corrected).

Chapter 5

Appendix Table 5.1 The top 15 common proteins and percentage of peptides for each identified at each time-point.

Protein ID	Protein Name	T1					
		P1	P2	P3	P4	P5	Mean
PIGR_HUMAN	Polymeric immunoglobulin receptor	0.42	0.86	0.34	1.60	0.68	0.78
PRP1_HUMAN	Basic salivary proline-rich protein 1	23.55	20.80	15.54	30.19	24.30	22.88
PRB2_HUMAN	Basic salivary proline-rich protein 2	19.99	19.99	18.51	27.45	22.38	21.66
PRB3_HUMAN	Basic salivary proline-rich protein 3	5.94	7.22	9.49	4.34	8.68	7.13
PRB4_HUMAN	Basic salivary proline-rich protein 4	5.48	8.09	12.63	8.49	11.72	9.28
PRPC_HUMAN	Salivary acidic proline-rich phosphoprotein 1/2	8.74	11.95	11.83	14.8	9.73	11.41
SMR3B_HUMAN	Submaxillary gland androgen-regulated protein 3B	8.16	4.68	5.94	4.22	6.51	5.90
STAT_HUMAN	Statherin	6.27	4.48	2.29	1.32	3.16	3.50
HIS1_HUMAN	Histatin-1	3.47	1.53	3.14	1.70	2.98	2.56
HIS3_HUMAN	Histatin-3	1.25	0.81	1.77	2.55	1.12	1.50
PRR27_HUMAN	Proline-rich protein 27	0.42	0.20	0.17	0.19	0.12	0.22
PROL4_HUMAN	Proline-rich protein 4	0.71	0.76	0.23	0.0	0.06	0.35
CO1A1_HUMAN	Collagen alpha-1(I)	0.04	0.25	0	0.38	0.19	0.17
CO2A1_HUMAN	Collagen alpha-1(II)	0.18	0.15	0.11	0.19	0.12	0.15
TR_HUMAN	Uncharacterized protein OS	8.36	11.55	12.06	0.09	0	6.41

Protein ID	Protein Name	T2					
		P1	P2	P3	P4	P5	Mean
PIGR_HUMAN	Polymeric immunoglobulin receptor	1.11	1.19	0.7	1.47	1.03	1.10
PRP1_HUMAN	Basic salivary proline-rich protein 1	18.82	19.32	14.68	22.03	25.59	20.09
PRB2_HUMAN	Basic salivary proline-rich protein 2	16.34	18.16	16.42	22.11	21.96	19.00
PRB3_HUMAN	Basic salivary proline-rich protein 3	5.69	5.01	8.58	3.94	5.8	5.80
PRB4_HUMAN	Basic salivary proline-rich protein 4	3.21	4.98	10.26	6.87	7.69	6.60
PRPC_HUMAN	Salivary acidic proline-rich phosphoprotein 1/2	9.83	9.8	9.07	8.79	8.03	9.10
SMR3B_HUMAN	Submaxillary gland androgen-regulated protein 3B	7.51	6.64	5.82	4.69	4.22	5.78
STAT_HUMAN	Statherin	7.57	7.64	6.77	7.04	6.21	7.05
HIS1_HUMAN	Histatin-1	3.21	2.35	4.11	2.43	1.78	2.78
HIS3_HUMAN	Histatin-3	0.22	0.28	0.98	1.17	0.41	0.61
PRR27_HUMAN	Proline-rich protein 27	1.7	1.06	0.98	0.42	0.48	0.93
PROL4_HUMAN	Proline-rich protein 4	1.48	1.6	1.44	0.54	0.38	1.09
CO1A1_HUMAN	Collagen alpha-1(I)	1.11	1.1	0.77	0.63	0.41	0.75
CO2A1_HUMAN	Collagen alpha-1(II)	0.56	0.53	0.58	0.88	0.45	0.6
TR_HUMAN	Uncharacterized protein OS	9.3	9.46	9.59	8.46	7.75	8.91

Protein ID	Protein Name	T3					
		P1	P2	P3	P4	P5	Mean
PIGR_HUMAN	Polymeric immunoglobulin receptor	1.78	1.75	0.77	1.64	1.18	1.424
PRP1_HUMAN	Basic salivary proline-rich protein 1	14.57	18.1	17.78	22.2	24.79	19.49
PRB2_HUMAN	Basic salivary proline-rich protein 2	12.75	18.22	19.28	21.15	22.06	18.69

PRB3_HUMAN	Basic salivary proline-rich protein 3	8.12	6.39	7.31	5.62	4.66	6.42
PRB4_HUMAN	Basic salivary proline-rich protein 4	6.10	6.36	8.7	8.66	7.21	7.41
PRPC_HUMAN	Salivary acidic proline-rich phosphoprotein 1/2	14.36	11.93	9.85	11.67	8.17	11.20
SMR3B_HUMAN	Submaxillary gland androgen-regulated protein 3B	5.18	5.73	4.14	3.59	4.85	4.70
STAT_HUMAN	Statherin	5.38	7.04	6.19	4.53	5.97	5.82
HIS1_HUMAN	Histatin-1	1.34	1.75	3.38	1.76	0.9	1.83
HIS3_HUMAN	Histatin-3	0.1	0.34	0.0	1.13	0.28	0.37
PRR27_HUMAN	Proline-rich protein 27	1.02	0.41	0.94	0.55	0.4	0.66
PROL4_HUMAN	Proline-rich protein 4	0.82	1.0	1.15	0.43	0.53	0.79
CO1A1_HUMAN	Collagen alpha-1(I)	0.21	0.44	0.87	0.94	0.37	0.57
CO2A1_HUMAN	Collagen alpha-1(II)	0.34	0.31	0.28	0.2	0.19	0.26
TR_HUMAN	Uncharacterized protein OS	13.54	11.58	10.16	11.39	7.92	10.92

Protein ID	**Protein Name**	**T4**					
		P1	**P2**	**P3**	**P4**	**P5**	**Mean**
PIGR_HUMAN	Polymeric immunoglobulin receptor	0.83	1.11	0.5	1.91	0.67	1.00
PRP1_HUMAN	Basic salivary proline-rich protein 1	23.28	20.08	14.28	24.48	24.36	21.30
PRB2_HUMAN	Basic salivary proline-rich protein 2	19.93	19.36	15.99	22.93	21.29	19.90
PRB3_HUMAN	Basic salivary proline-rich protein 3	5.74	12.43	9.91	6.01	8.83	8.58
PRB4_HUMAN	Basic salivary proline-rich protein 4	5.96	12.69	12.56	7.55	11.47	10.05
PRPC_HUMAN	Salivary acidic proline-rich phosphoprotein 1/2	10.18	14.72	12.4	19.84	11.23	13.67
SMR3B_HUMAN	Submaxillary gland androgen-regulated protein 3B	6.92	5.10	4.54	3.73	6.44	5.35
STAT_HUMAN	Statherin	4.44	3.92	3.43	1.36	3.25	3.28
HIS1_HUMAN	Histatin-1	2.31	0.78	3.21	1.73	2.82	2.17
HIS3_HUMAN	Histatin-3	0.83	0.0	1.72	2.91	0.43	1.18
PRR27_HUMAN	Proline-rich protein 27	0.65	0.46	0.22	0.0	0.18	0.30
PROL4_HUMAN	Proline-rich protein 4	0.83	1.24	0.39	0.27	0.12	0.57
CO1A1_HUMAN	Collagen alpha-1(I)	0.13	0.13	0.22	0.27	0.31	0.21
CO2A1_HUMAN	Collagen alpha-1(II)	0	0.2	0.11	0.27	0.18	0.15
TR_HUMAN	Uncharacterized protein OS	9.88	0.0	12.73	0.0	0.0	4.52