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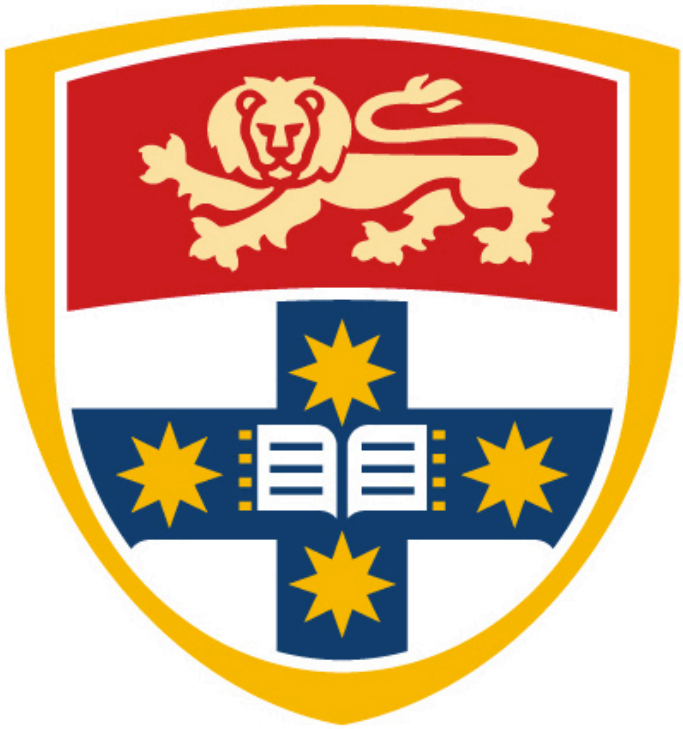

CYTOKINES IN GINGIVAL CREVICULAR FLUID AND ROOT RESORPTION: A MICROCOMPUTED TOMOGRAPHY STUDY

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A thesis submitted in partial fulfilment of the requirements for the degree of Doctor of Clinical Dentistry (Orthodontics)

September 2014

Declaration

CANDIDATE CERTIFICATION

This is to certify that the candidate carried out the work in this thesis,

in the Orthodontic Department, University of Sydney,

and it has not been submitted to

any other University or Institution for a higher degree.

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Jenkin J Chiu

Dedication

This work is dedicated to my wife, Elma, who supported me throughout the toughest times of my postgraduate studies. I am grateful for her deepest understanding for all the missed times we never could share in the first years of our marriage because of it. I love you.

I wish also to thank my parents who have given me all their support, every opportunity and every tool to allow me to become who am I today.

Acknowledgements

I wish to thank Professor M. Ali Darendeliler, Head and Chair of Orthodontics, for the opportunity of a lifetime to participate in postgraduate training in the Department of Orthodontics at the Sydney Dental Hospital. Not only have you been a great teacher, mentor and role model, but you have also become a great friend for many years to come.

I wish to thank Dr Öykü Dalci, Senior Lecturer and friend, for the confidence you have shown in me since my earliest days in the programme. Ever encouraging, ever understanding, and ever smiling, I will remember you as a 'smiling assassin' who pushed me hard to achieve the best that I could. I hope I have come close to reaching your expectations.

I am grateful to all the honorary specialists who donate their time and resources *pro bono* as tutors to teach my colleagues and myself in the Orthodontic Department, some travelling long distances to do so. You are a true inspiration to me and to our profession. I thank you all for showing me the way when I was lost, showing your patience when I tested it, and showing me humility when I was over-confident.

Thank you Mrs Rebecca Chan, my fellow postgraduate colleagues, Ms Maria Missikos and her team of technicians, Ms Yanti Suhartono and reception staff, Ms Elizabeth Mazzei, Ms Lorna Reyes and her team of dental assistants, friends and family for your help, support, friendship and all the good times we shared together.

List of Abbreviations

Ca, P, F	Calcium, phosphate, fluoride
CBCT	Cone-beam computed tomography
DPP	Dentin sialoprotein
DSP	Dentine phoshoprotein
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EPMA	Electron probe microanalysis
GCF	Gingival crevicular fluid
GM-CSF	Granulocyte and macrophage colony-stimulating factor
IFN-γ	Interferon-gamma
IL	Interleukin
LM	Light microscopy
MBAA	Multiplex bead array assay
micro-CT	Microcomputed tomography
MMP	Matrix metalloproteinase
MNGC	Multi-nucleated giant cell

OIIRR	Orthodontically induced inflammatory root resorption
OPG	Osteoprotegerin
OPT	Orthopantomogram
OTM	Orthodontic tooth movement
PA	Periapical radiograph
PDL	Periodontal ligament
PGE_2	Prostaglandin E_2
PP	Dentin phosphophoryn
RANK	Receptor activator of nuclear factor κB
RANKL	Receptor activator of nuclear factor κB ligand
RIA	Radioimmunoassay
RME	Rapid maxillary expansion
SEM	Scanning electron microscopy
SP	Substance P
TEM	Transmission electron microscopy
TIMP	Tissue inhibitor of metalloproteinase
TNF-α	Tumour necrosis factor-alpha
TRAP	Tartrate-resistant acid phosphatase

Table of Contents

1 Introduction

Root damage and root shortening is an unwanted side effect of orthodontic treatment. It is more properly termed *orthodontically induced inflammatory root resorption* (OIIRR). Despite decades of research, the truth about why and how it occurs still largely unknown. The occurrence and severity of OIIRR appears to be influenced by patient-related risk factors and treatment-related risk factors. There is no definitive way for an orthodontist to predict how susceptible or resistant a patient is to OIIRR, nor is there a way to effectively prevent its occurrence and severity with any certainty. Severe OIIRR, defined as root shortening of more than 4mm, or more than one third of starting root length, occurs in 2-5% of patients who received orthodontic treatment. Fortunately, the overall long-term effects, even if severe, do not harm the dental health of afflicted patients, from the perspective of both the patient and the dental professional. The worst reported outcome has been tooth mobility. Nevertheless, OIIRR is still a side effect or orthodontic treatment that needs to be avoided.

Most researchers have used two-dimensional X-rays in assessing OIIRR, which has its limitations. On the other hand, three-dimensional X-rays are better and more accurate in finding and measuring OIIRR. However, X-rays cannot identify whether the process is active or not. By identifying specific molecules (biomarkers) in the fluid collected from gum pockets around teeth (gingival crevicular fluid, GCF), orthodontists may have a new way to diagnose and monitor OIIRR. The ideal goal of this line of research is developing a rapid chair side test to spot specific biomarkers that predict the patient's susceptibility to OIIRR, and monitor it during treatment. More ambitiously, knowledge of GCF biomarkers may allow researchers to develop therapeutic measures to stop the active process of OIIRR.

2 Review of the Literature

2.1 History and Definition

Root resorption is defined as the destruction of formed dental tissue of the roots of permanent teeth[1]. Knowledge of root resorption dates back to 1856, when Bates[2] first described the phenomenon of "absorption" of permanent teeth. However, it was Ketcham (1927) who first demonstrated radiographic evidence of changes in the shapes of tooth roots before and after orthodontic treatment in a large number of treated cases. Ketcham showed that root resorption was a common and occasionally severe consequence of orthodontic treatment[3,4]. Recent research[5,6] into the process of root resorption related to orthodontic tooth movement in humans has revealed its inflammatory nature. Therefore, Brezniak and Wasserstein (2002) proposed that it be more accurately termed *orthodontically induced inflammatory root resorption* (OIIRR)[7].

2.2 Classification

Root resorption resulting from orthodontic treatment is one type of external root resorption. Andreasen's review[8] of root resorption defined the classification of external root resorption into surface resorption, inflammatory resorption or replacement resorption. Tronstad (1988) further divided inflammatory external root resorption into subgroups of transient or progressive[9]. Therefore, as described by Brezniak[7], OIIRR is classified as a *progressive external inflammatory root resorption*. There are three degrees of severity of OIIRR[7]:

Surface resorption. Only the outer cemental layers are resorbed, which are later completely regenerated or remodelled once the etiological factor is removed. It mimics the process of trabecular bone remodelling.

Deep resorption. Both cemental and outer dentinal layers are resorbed, and subsequently repaired by secondary cementum. Once repaired, the resulting form of the root surface may or may not be identical to the original, and a residual crater may be present.

Circumferential apical resorption. In this most severe variant, the resorption process completely involves both cemental and dentine layers of the root apex. The tridimensional nature of the process produce resorption craters that coalesce, which may also isolate islands of hard tissue from the root[10]. Consequently, the root shortens, and once this occurs, repair of the damaged root is impossible, although sharp edges may remodel.

2.3 Incidence and Prevalence

2.3.1 Incidence of OIIRR in treated populations

The literature cites varying figures on the incidence of OIIRR in treated populations. Reported values range from 6%[11], 29%[12] to 100%[13–15]. Lupi *et al* (1996) reported the incidence of root resorption in incisors in any degree to be 15% before treatment, increasing to 73% after treatment[16]. Such a huge range of incidence values is due to studies using different methods to identify root resorption ranging from scanning electron microscopy[14], through histology[17] to graded scales on panoramic[18] and periapical[11,12,16] radiographs. Due to the diverse methods and poorly defined criteria used to identify OIIRR, it is difficult to compare incidence data across different studies[19,20].

The reported incidence of OIIRR according to its severity also varied greatly. When scoring the degree of root shortening either subjectively or metrically, OIIRR of the most severe rating used in respective studies were reported to be 1.3%[21,22], 10.8%[23], 24.5%[16], or 41%[24] of patients. To the reader, the higher values reported[16,24] may be quite concerning. However, the authors[16,24] drew these values from severity stratification criteria that were less robust, either combining groups of moderate OIIRR with groups of severe OIIRR[16], or using lower threshold criteria[24]. Killiany (1999) reported the most indicative incidence values according to different amounts of root shortening, and showed the trend that as the measure of OIIRR severity increased, incidence values decreased. Root shortening of >3mm, >4mm, >5mm and >6mm occurred in 32%, 13.5%, 5% and 1.5% of patients, respectively[25]. Generally, severe OIIRR defined as exceeding 4mm or one third of the original root length generally occurs in 1-5% of the treated population[26].

Despite the lack of agreement on the overall incidence between studies, there is consensus that certain teeth experience OIIRR more than others. Of the entire dentition, maxillary incisors are the most commonly affected teeth in OIIRR in large samples of patients[27,28], and maxillary anterior teeth were affected twice as severely as mandibular anterior teeth[27]. When individual teeth were analyzed, the most severely resorbed teeth were the maxillary lateral incisors, followed by maxillary central incisors, maxillary canines, mandibular canines, mandibular central incisors, and mandibular lateral incisors[27,29]. Accordingly, most studies have focused on maxillary incisors when investigating OIIRR. Premolars are claimed to not attract as much clinical interest in OIIRR research because the amount of root shortening found in these teeth average less than 1mm[27]. Nevertheless, Apajalahti and Peltola (2007) observed apical root shortening of premolars in 8.5% of 316 non-extraction patients, and in half of these patients, the severity was graded as moderate to severe with loss of one-quarter or more of root length[28].

2.3.2 Prevalence of root resorption in untreated populations

Even in the absence of orthodontic treatment, root resorption can occur. The cited prevalence of idiopathic root resorption in untreated populations ranged from 0%[30], 86.4%[31] to more than 90%[32]. The extreme distribution is again due to the different methodologies adopted by different studies. Although Henry and Weinmann (1951) reported 90.5% of teeth showing microscopic resorption craters from autopsy material, all lesions showed evidence of repair[32]. Idiopathic external apical root resorption, which may have similar characteristics to OIIRR, can also affect untreated individuals[33]. As a very rare condition, only 32 case reports of idiopathic external apical resorption exist in the literature between 1965 and 2006[33].

There is evidence of a baseline process of root resorption occurring in the absence of force variables such as mastication, parafunction and pressure from oral and peri-oral soft tissues. In a human sample of unerupted third molars investigated *ex vivo*, Deane *et al* (2009) discovered evidence of root resorption craters. In comparison to teeth subject to light buccal and intrusive forces (25g), the roots of these third molars had craters with similar cubed volumes[34]. Although they used a small sample size, the authors suggested that physiological remodelling and turnover of radicular hard tissue occurs to produce minor to moderate root surface resorption without force application[34].

2.4 Etiology

Despite decades of research, the etiology of OIIRR is still poorly understood, and it is unknown how exactly orthodontic treatment influences its occurrence[26]. Essentially, OIIRR is a complex phenomenon that results from interplay between patient-related risk factors and orthodontic treatment-related risk factors[35]. There are currently no reliable measures to predict which patients will develop OIIRR or the severity of OIIRR[19,36].

2.4.1 Patient-related risk factors (systemic)

Individual susceptibility is a major contributor to the etiology and severity of OIIRR and idiopathic root resorption[26,27].

Genetics

Newman (1975) studied periapical radiographs of forty-seven patients and all first-degree relatives. Although the pedigree data that he was able to produce from the small sample size was limited, Newman tentatively suggested that root resorption might follow an autosomal dominant, autosomal recessive or polygenic mode of inheritance[37]. Harris, Kineret and Tolley (1997) examined external apical root resorption on panoramic radiographs of 103 sibling pairs treated by one orthodontist. He reported a highest heritability estimate of 70% for OIIRR to occur in the maxillary incisors, while the lowest heritability estimate was reported for mandibular incisors[38]. In a small sample of 16 monozygotic and 10 dizygotic twins, Ngan, Kharbanda and Byloff (2004) identified

OIIRR in maxillary incisors, mandibular incisors and mandibular molars. An overall heritability estimate of 34% suggested a genetic contribution to the etiology of OIIRR[39].

Although investigations estimating heritability of OIIRR provide revealing evidence of a genetic etiology, estimates do not provide information on the number of genes involved. Recently, by studying parents and offspring within families, Al-Qawasmi *et al* (2003a) have demonstrated that OIIRR is linked to human genes that encode proteins involved in osteoclast recruitment and bone resorption during orthodontic tooth movement[40,41]. Individuals homozygous for the *IL-1B* allele 1, associated with decreased interleukin-1β (IL-1β) production, demonstrated a risk of OIIRR, with more than 2mm of root shortening, 5.6 times higher than patients who are not homozygous for the same allele[40]. Al-Qawasmi *et al* (2003b) further reported that *TNFRSF11A*, a candidate gene on chromosome-18 that encodes the receptor activator of nuclear factor-kappa B (RANK), is associated with OIIRR experienced by maxillary central incisors[41].

Ultimately, genetic factors that influence OIIRR are heterogenous, and account for at least 50% of the variation in OIIRR and other forms of external apical root resorption. Different genetic mechanisms operate in numerous combinations and extents in different affected patients, and may even produce varying OIIRR responses in different sites within the same individual[42].

Medical history

A speculated systemic immunologic mechanism to root resorption[43] may also explain why select patients experience more OIIRR than others. Davidovitch *et al* (2000) hypothesized that patients with medical histories that affect the immune system may be highly at risk of developing excessive OIIRR[44].

Systemic inflammatory mediators produced in asthma have been suspected to enter the periodontal ligament to initiate and enhance the process of external root resorption[45]. Owman-Moll

and Kurol (2000) histologically examined extracted premolars and reported results that were suggestive of a link between allergy and OIIRR, but the association was not statistically significant[46]. In stronger support of this theory, McNab *et al* (1999) reported a higher incidence of OIIRR in maxillary molars in asthmatic patients compared to healthy individuals. However, both groups exhibited similar degrees of severity of OIIRR[47]. With a similar methodology, the work of Nishioka *et al* (2006) also found asthma and allergies to be high-risk factors for OIIRR in a Japanese population, for which they reported odds ratios of approximately 4.4 and 2.8 respectively[48].

Endocrine imbalances such as hypothyroidism[49,50], hyperparathyroidism[51], hypopituitarism, hyperpituitarism[52,53] and hypophosphatasia[54] have been linked to root resorption. Other disorders such as Paget's disease of bone has also been reported to contribute to the etiology of root resorption[55]. However, Linge and Linge (1983) expressed doubt about the significance of the role that hormone imbalances played in the etiology of OIIRR, because they noted a marked variation in root resorption within the same individual[21]. Numerous case reports of severe idiopathic multiple root resorption, without orthodontic treatment, have also failed to associate it to systemic disorders[33]. Nutrition has also been suspected in the multifactorial etiology of OIIRR[56], and might modulate the effect of hormonal imbalances[57].

Drugs have been demonstrated to affect OIIRR. Nabumetone, a non-steroidal anti-inflammatory drug, was found to reduce the amount of OIIRR during intrusive tooth movement in humans[58]. Igarashi *et al* (1996) reported in rats that bisphosphonates reduce root resorption in a dose-dependent relationship[59]. However, Alatli *et al* (1996) showed that bisphosphonates inhibited the formation of cellular cementum, which rendered the root surfaces of rat molars susceptible to the resorptive process[60].

Davidovitch *et al* (1996) hypothesized that chronic alcoholism may cause root resorption. Alcohol prevents hydroxylation of vitamin D in the liver. Defective vitamin D then alters calcium

mobilization from the intestine, kidney and bone, which stimulates increased levels of parathyroid hormone. This results in enhanced resorption of mineralized tissues, including cementum[45].

Ethnicity

Sameshima and Sinclair (2001) documented a variation in the incidence of OIIRR according to ethnic groups. Asian patients experienced significantly less OIIRR compared to Hispanic and white patients[27]. However, from the statistical analyses that were carried out on the data, it was unclear whether this variation was due to genetic factors alone or due to treatment factors[61].

Age and gender

In their early review of the literature, Brezniak and Wasserstein (1993) described that root resorption was often reported to be more prevalent in adult patients. This was linked to adults' increased susceptibility to OIIRR due to their more avascular and aplastic periodontal membrane and bone[36]. However, more recent reviews[62,63] found no association between age and OIIRR, although Sameshima and Sinclair (2001)[27] reported that adults had more OIIRR than children in their sample.

There is consensus that OIIRR is not consistently associated with gender[27,38,62]. Nevertheless, there were controversial findings between some studies, which have reported OIIRR being more prevalent in either males[64] or females[65].

Systemic Fluoride

Fluoride may be a factor in the etiology of OIIRR. Foo, Jones and Darendeliler (2007) hypothesized that systemic fluoride incorporated into cementum renders it more resistant to OIIRR. The authors reported that resorption craters on rat molars were smaller in those animals fed with fluoridated water, but the differences were variable and not statistically significant[66]. In another rat model, Lim *et al* (2011) reported that exposure to 100ppm.F in fluoridated water resulted in

significantly smaller root resorption crater lesions, both in length and depth. The authors also discovered higher mineral content (fluoride, zinc and calcium) in rat molars of the fluoride-exposed group over those of the non-fluoridated group[67].

The effect of fluoride on root resorption has also been investigated in a human model. In a unique study design, Karadeniz *et al* (2011) recruited patients from two Turkish cities: one had a water supply with very high fluoride content (>2.0pmm), whilst the other had water supply with very low fluoride content (<0.05ppm). The authors concluded that fluoride was able to suppress the severity of root resorption that occurred under heavy forces (225g) that were applied for 4 weeks[68].

2.4.2 Patient-related risk factors (local)

Malocclusion

OIIRR has been found to be over-represented in certain types of malocclusion. There are published reports on the association between the amount of orthodontic tooth movement and the degree of OIIRR[35,42]. The amount of tooth movement carried out during orthodontic treatment is inherently related to the type and severity of malocclusion. For example, Beck and Harris (1994) demonstrated that the greater the overjet, the greater the amount of incisor retraction and the greater the amount of incisor resorption[69]. Taner, Ciger and Sençift (1999) reported that patients with Class II division 1 malocclusion experienced average root shortening of 2mm compared to 1mm in patients with Class I malocclusions after treatment[70]. Bollen (2002) further confirmed that large overjets and longer teeth were associated with more OIIRR. Patients requiring extractions for the treatment for their malocclusion experienced more root resorption compared to those not requiring

extractions[35]. Some preliminary evidence has also suggested that patients who have hyperdivergent skeletal growth patterns show tendencies towards experiencing more severe OIIRR[71].

Anterior open bite malocclusions have also been implicated as a strong risk factor for OIIRR. Kjær (1995) suggested that anterior open bites have a connection to root resorptions[65]. In a retrospective case-control study, Harris and Butler (1992) reported a higher prevalence of root resorption in anterior open bite cases treated at the Department of Orthodontics, University of Tennessee[72]. This is supported by the work of Kuperstein (2005), which showed a significant difference in the amount of maxillary incisor root resorption in patients with an anterior open bite (2.26mm) compared to patients with normal overbite (0.93mm, p=0.001)[73]. It is speculated that hypofunctional teeth in anterior open bite malocclusions have reduced resistance to external orthodontic stimuli, which explains the higher prevalence of OIIRR in these cases[74,75].

History of root resorption

The prevalence of root resorption in subjects who have not received orthodontic treatment ranges between 0%[30] to over 90%[32]. There is a high correlation between the amount and severity of root resorption present before treatment and to the root resorption found after completion of orthodontic treatment[23,76].

History of trauma

Trauma to the dental tissues may be a predisposing factor for root resorption during orthodontic treatment. Brin *et al* (1991) found that external root resorption was most prevalent (27.8%) in the patient cohort who experienced trauma to their upper incisors and underwent orthodontic treatment. This is in contrast to the prevalence of root resorption in patients who only had trauma to their upper incisors (7.8%) and those who received orthodontic treatment only without history of trauma (6.7%)[11]. However, Malmgren *et al* (1982) did not find evidence to support the hypothesis

that traumatized teeth have a higher tendency towards root resorption. They could only conclude that OIIRR was more likely in traumatized teeth showing signs of root resorption before orthodontic treatment[77].

Tooth type and tooth root qualities

Different tooth types experience different extents of OIIRR. Observational studies[27–29] have reported that maxillary anterior teeth are affected twice as severely as mandibular anterior teeth. Further, the most severely resorbed teeth are the maxillary lateral incisors, followed by maxillary central incisors, maxillary canines, mandibular canines, mandibular central incisors, and mandibular lateral incisors[27].

Abnormal root shapes have been reported to be risk factors of OIIRR. Pipette-shaped, pointed or dilacerated roots experienced the worst resorption, as reported by Sameshima and Sinclair (2001)[27]. This supports the findings of Levander and Malmgren (1988) that short, blunt, apically bent and pipette shaped roots significantly show more OIIRR during treatment[22]. However, other authors found no evidence that linked abnormalities in root shapes[78] or lengths[79] to a greater likelihood OIIRR. Kjær (1995) attempted to identify pre-treatment radiographic tooth characteristics that were associated with OIIRR in a sample of 107 patients presenting with "excessive" root shortening. Kjær established a strong correlation with OIIRR for invaginations, short roots, and taurodontism, and a weaker correlation was reported between ectopia, and agenesis with OIIRR[65].

Habits

Oral habits have been suggested to be an etiological factor in external root resorption[37]. Persistence of the habit during orthodontic treatment can therefore be expected to contribute to the occurrence of OIIRR. Harris and Butler (1992) found reason to suspect that tongue thrusting habits leading to anterior open bites increased the likelihood of OIIRR[72]. Severe nail biting was

significantly correlated with more severe external root resorption before and after orthodontic treatment in a large sample of adolescents[80]. Linge and Linge (1991) reported that a history of finger-sucking habits beyond the age of 7 contributed significantly to OIIRR[81]. On the other hand, a matched case-control study of treated cases with severe OIIRR by Sameshima and Sinclair (2004) reported that oral habits did not contribute to OIIRR as a variable[82].

Occlusal trauma

It has been speculated that heavy occlusal forces, occlusal trauma and chronic bruxism all increase the risk of root resorption[20,83,84]. Severe occlusal forces may lead to alveolar bone loss and, in some cases, root resorption when it has exhausted the capabilities of the periodontium to adapt to those forces[85]. Rawlinson (1991) described that occlusal trauma and bruxing are often caused by premature contacts in centric occlusion and lateral excursive movements, and these may exacerbate the root resorption process. Rawlinson also suggested that excessive fremitus may be associated with resorption of the root apex[86]. Improper occlusion from inadequate dental restorations and prosthetic appliances can also cause occlusal trauma and "jiggling" forces that promote root resorption[87]. Harris (2000)[20] cited references that reported a high frequency of external apical root resorption in roots of long-term abutment teeth. The corresponding authors speculated that this occurred because 'relatively normal teeth are carrying abnormally greater occlusal loads when used as bridge abutments'[20]. Such speculations were supported by evidence produced by Cakmak *et al* (2014). Using a split-mouth design, Cakmak and co-workers found premolar teeth that received occlusal trauma for 4 weeks from 2mm-thick light-cured glass ionomer cement experienced significantly more root resorption than the contralateral control premolars that did not receive any forces[88].

Density of alveolar bone

It is unclear how significant the role of bone density is in the etiology of OIIRR. Biological principles lead the observer to postulate that denser bone is associated with increased root resorption when a tooth is moved through it, due to higher forces and longer force application[89]. However, studies in both monkeys[90,91] and humans[92] have found no correlations between bone density and the extent or severity of OIIRR. Bone density may not be a primary risk factor for OIIRR.

It has also been suggested that a close proximity to cortical bone predisposes tooth roots to resorption. Goldson and Henrikson (1975) reported double the frequency and eight-fold increase in severity of OIIRR during Stage II of the Begg technique when roots are close to cortical bone[93]. Kaley and Phillips (1991) accounted a 20-fold increase in risk for root resorption to occur in maxillary incisors if they were in close proximity to the lingual cortical bone[94]. However, these methods and conclusions are questionable because only two-dimensional radiography was used to determine bone proximity. Other studies have failed to show an association between cortical plate proximity and OIIRR[91,92].

History of endodontic treatment

There was initially a belief that root-filled teeth were more susceptible to root resorption during orthodontic treatment. Wickwire *et al* (1974) observed a greater incidence of OIIRR in endodontically treated teeth compared to adjacent vital control teeth[95]. However, this observation was confounded by factors such as trauma and the timing of endodontic treatment, both of which either occurred before or during orthodontic tooth movement in different patients. Llamas-Carreras *et al* (2010) found no significant differences in OIIRR between root-filled and vital control teeth. However, when subdividing observations by tooth groups, the authors reported that root-filled incisors experienced more OIIRR than their contralateral vital controls[96]. It was suggested that the authors did not control for confounding factors because they did not examine the extent of root

resorption on the traumatized incisors prior to orthodontic treatment[97]. Other studies[98,99], literature reviews of the literature[100,101] and a systematic review[97] all report that root-filled teeth experience no more, or even less OIIRR, than vital teeth. Mirabella and Artun (1995) even speculated that endodontic treatment was a preventive factor against OIIRR[98]. It has been suggested that endodontically treated teeth are more resistant to OIIRR due to an increase in dentine hardness and density[102,103].

2.4.3 Treatment-related risk factors

Total treatment duration

It is suspected that longer treatment duration leads to increased incidence and increased severity of OIIRR. However, this correlation is an open controversy[62]. Rudolph (1940) found that after 1, 3, 4 and 7 years of active treatment, 40%, 70%, 80% and 100% of patients experienced some extent of root resorption, respectively[13]. In a meta-analysis of the literature, Segal, Schiffman and Tuncay (2004) showed that mean apical root resorption was strongly correlated to treatment duration (r = 0.852), having excluded studies with patients who had a history of root resorption and trauma[104]. DeShields (1969) claimed that the severity of root resorption is partially related to the duration of treatment in his study's sample of Class II division 1 malocclusions, but acknowledges that there may be confounding variables contributing to his observation[105]. In contrast, in another sample of subjects with Class II division 1 malocclusions, Taner, Ciger and Sençift (1999) found no correlations between OIIRR and the duration of active treatment[70]. Beck and Harris (1994) also found no significant association between treatment duration and OIIRR, despite some cases being in active treatment for up to 6 years[69].

Continuity of force application

Several authors have asserted that intermittent forces result in less OIIRR than continuous forces, because the pause in force application allows resorbed cementum to heal and prevent further resorption[36,106–108]. Prospective animal and human split-mouth studies support this belief. Konoo *et al* (2001) found significantly more root resorption in rat molars subjected to continuous force (24 hours per day) than those subjected to intermittent forces (1 hour per day)[109].

In humans, Acar, Canyürek, Kocaaga *et al* (1999) reported that discontinuous intrusive force application with elastics (12 hours per day) results in 54% less OIIRR than continuous intrusive force application with elastics (24 hours per day)[110]. Weiland (2003) designed a similar split mouth study, but produced continuous force on human premolars using superelastic wires, and intermittent force using stainless steel wires reactivated once every 4 weeks. Over the 12-week experimental period, Weiland observed that the perimeter, area and volume of resorption lacunae of teeth in the 'superelastic group' (continuous force) were 140% greater than on the teeth in the 'steel group' (intermittent force)[111]. Aras *et al* (2012) corroborated these findings. They reported that intermittent forces caused significantly less root resorption than continuous forces. This occurred regardless of whether the time interval was 2-weekly or 3-weekly activations. A 3-day pause from force application significantly reduced root resorption in teeth subjected to the intermittent force protocol[112]. Ballard *et al* (2009) had also found less root resorption volumes produced by intermittent forces compared to continuous forces, using a different pattern of intermittent force application (initial 2-week continuous force followed by weekly cycles of 4 days force application with 3 days of rest for 6 weeks)[113]. Furthermore, if only continuous force application is considered, Paetyangkul *et al* (2011) showed that the longer the duration of a continuous force (12 weeks vs. 8 weeks vs. 4 weeks), the greater the extent of root resorption. This was especially the case if heavy (225g) forces were used[114].

Magnitude of orthodontic force

According to Schwarz (1932), the risk of root resorption increased proportionally when the applied force led to pressures exceeding 20 to 26 grams per square centimetre on tooth roots[115]. Such heavy force represses the pressure in the blood capillaries in the periodontium[115]. Aseptic necrosis of the affected periodontal tissue follows, and undermining resorption occurs from the medullary space adjacent to the area of hyaline necrosis, as described by Carl Sandstedt[116,117]. The more this pressure is exceeded in the periodontal ligament, the more the soft tissues are crushed. The root surface is damaged by direct contact with the bone, and there is danger that the injured root surface may be resorbed[115]. Schwarz's observations were supported by the more recent research of Hohmann, Wolfram and Geiger *et al* (2007). These authors produced individual finite element analysis models of extracted human premolars to simulate the distribution of hydrostatic pressure in the PDL. After torque was applied to these teeth *in vivo*, teeth were extracted and scanned with micro-CT for resorption craters. The authors concluded that if the hydrostatic pressure in the PDL produced by orthodontic force exceeds typical human capillary blood pressure, the risk of root resorption increases[118].

There is general agreement between studies that stronger applied forces increase the incidence and severity of OIIRR. Harry and Sims (1982) described that apical resorptive lesions developed rapidly as the magnitude of forces increased from 50g to 200g, as investigated with scanning electron microscopy (SEM)[14]. A transmission electron microscopy study[119] has supported this by reporting that apical cementum was more severely resorbed proportional to the magnitude of applied force. More recent SEM studies compared the application of a heavy force (225g) versus a light force (25g), and recorded a 3.31-fold increase in resorption crater volumes in the heavy force group over the light force group[120–122]. This is also consistent with studies using micro-computed

tomographic radiology[114,123–129], where heavier forces produced greater mean volume of resorption craters. This was found true regardless of the type of tooth movement produced, whether it is buccal tipping[114,123,124], intrusion[125], extrusion[126], root tipping[127], root torque[128], or rotation[129]. On the other hand, other investigations into the effect of increased orthodontic force on the incidence and severity of OIIRR found no association[130,131]. However, the histological and light microscopic methods of Owman-Moll, Kurol and Lundgren (1996)[130,131] in quantifying resorption craters have been questioned due to inaccurate sectioning methods and parallax errors, leading to missed and miscalculated craters[120].

Type of tooth movement

Intrusion

When teeth are intruded or when their roots are torqued during treatment, there is a significant risk of OIIRR[26]. Some studies investigated OIIRR using light intrusive forces. When compared to an untreated control group, Dermaut and De Munck (1986) found obvious root shortening in upper anterior teeth after intrusion with a constant 100 grams of force for an average of 28 weeks[30]. McFadden *et al* (1989) also investigated the effect of light intrusive forces (25 grams) on root shortening. These authors reported less resorption, recording an average shortening by 1.84mm and 0.61mm for maxillary incisors and mandibular incisors, respectively[132]. However, no control group was used in this investigation, which subtracts from the significance of the findings. Costopoulos and Nanda (1996) intruded maxillary incisors with approximately 15 grams of force on each tooth. They found only slightly more root shortening in these experimental teeth compared to those in an orthodontically treated control group that did not receive intrusive forces (0.6mm compared to 0.2mm, respectively)[133].

When 100 grams of force was applied for 8 weeks in a study by Han *et al* (2005), intrusion of premolars led to four times the amount of root resorption than did extrusion of premolars in the

same individual. There was also a statistically significant difference between intruded teeth and control teeth[134]. Faltin *et al* (2001) utilized transmission electron microscopy in a split mouth study and qualitatively described resorption craters occurring after 4 weeks of continuous intrusive force. Teeth intruded with 100 grams of force showed more intense and more extensive craters in the apical mineralized cementum than teeth that were intruded with 50 grams of force[119]. More recently, a rigorously designed study by Harris, Jones and Darendeliler (2006) measured the volume of resorption craters on premolars subject to light (25 grams) and heavy (225 grams) intrusive forces. The authors observed a linear increase in resorption crater volumes from control to light to heavy intrusive forces (statistically significant), whilst mean volumes in the light and heavy groups were 2 and 4 times greater than in the control group, respectively[125].

When the reader collectively interprets these studies, it appears that if intrusive forces are kept light, the increase in OIIRR over controls is clinically insignificant, but statistically significant[30,133]. If the magnitude of intrusive force is increased to heavy, the increase in OIIRR becomes both statistically and clinically significant[119,125,134]. In the studies[30,132,133] which examined light intrusion forces on incisors, a weak[133] or no correlation[30,132] was found between the amount of root resorption and the duration of intrusion or the distance intruded.

Root Torque

The relationship between root torque and root resorption was first noted when Goldson and Henrikson (1975) reported that root resorption increased more for maxillary central incisors because they were subjected to root torque during treatment with the Begg technique[93]. Casa *et al* (2001) subjected premolars to 600 cN.mm and 300 cN.mm of torque and compared their resorptive lesions to those of non-moved premolars. Premolars moved with 600 cN.mm of torque after 4 weeks had severe resorption of cementum, and resorption areas were more numerous, wider and deeper than those on control teeth and teeth moved with 300 cN.mm of force[135]. Other authors have measured

root torque in degrees of change in the longitudinal root axis. Premolars administered with 2.5˚ and 15˚ of root torque did not show significant differences in total root resorption crater volumes between the groups after 4 weeks, but did have greater total crater volumes than the control group[128]. However, when craters were analyzed according to their location on the root, Bartley *et al* (2011) reported more root resorption at the apical region than at the middle and cervical regions in teeth that received torqueing forces[128].

There are two studies that attempt to compare the OIIRR risk of root torqueing and intrusion tooth movements to the OIIRR risk of other types of tooth movement. Kaley and Phillips (1991) investigated a series of 200 consecutively treated cases after treatment with the edgewise appliance. The authors designed a case-control study to estimate the risk of OIIRR associated with pre-treatment and treatment variables, comparing teeth with severe OIIRR against those without. They reported that root torqueing movements carried an odds ratio of 4.5, a higher risk for OIIRR than tipping, translation, round-tripping, intrusion and extrusion were[94]. A retrospective study designed by Parker and Harris (1998) used stepwise multivariate linear regression analyses to determine which types of tooth movements were most predictive of OIIRR. Parker and Harris found that measures of tooth movement accounted for 90% of the variance. They further reported that intrusion and increase in lingual root torque were the strongest predictors, while distal bodily retraction, extrusion or lingual crown tipping had no effect on the variance[136]. Concurring with these reports is the finding of Segal, Schiffman and Tuncay (2004), whose meta-analysis revealed a strong correlation (r = 0.852) between total apical displacement and the severity of OIIRR[104].

Jiggling

Unlike orthodontic forces, jiggling forces are not applied in any one direction for a sufficient duration to stimulate tooth movement[109]. Anecdotally, Proffit and Fields[137] believed that jiggling movements created by light-force rectangular archwires during the first stage of straight-wire

appliance treatment likely increase the risk of root resorption[62]. Other authors[83–87] have also speculated how root resorption is associated with jiggling forces from occlusal trauma, bruxing and fremitus. However, there has been no rigorous study designed and published to date that directly investigates this possible association. Konoo *et al* (2001) noticed, in their rat model, that forces applied intermittently for only 1 hour every 24 hours could stimulate osteoclastic activity but not tooth movement[109]. However, the authors did not find a concomitant occurrence of root resorption in their sample, speculating that the 23-hour rest periods imparted a protective effect against OIIRR[109]. Nevertheless, the reader would be tempted to think that their findings may suggest a possible link between jiggling forces and root resorption, because the cells involved in OIIRR (odontoclasts) are very closely related to osteoclasts[138–140].

Type of appliance and treatment technique

There has been interest in whether certain appliances or orthodontic techniques led to more external apical root resorption. According to Rygh[89], the heavy forces produced by rapid maxillary expansion (RME) can be expected to lead to root resorption, as forces applied to anchor teeth may reach to more than twenty pounds[141]. Numerous authors have histologically examined anchor teeth after the application of rapid maxillary expansion[142–145]. Extensive root resorption was reported as a common finding amongst these studies, and resorptive defects were repaired with cellular cementum during the retention period. Langford (1982) found no correlation between the period of RME, the length of retention and the total area of resorption affecting anchor teeth[146]. Different RME systems did not show significantly different effects on root resorption[145], although one study suggested that the Haas tissue-borne expansion appliance resulted in less resorption[147]. A cone-beam computed tomography study by Baysal *et al* (2012) compared pre-expansion and post-expansion root resorption volumes. The authors found a maximum root volume loss of 18.60 mm^3 on the mesio-buccal roots of the upper permanent first molars, but the percentage volume loss was

not statistically different between anchor teeth[148]. However, this study did not use a negative control group, nor did it compare the RME appliance with other appliances.

Removable orthodontic appliances are believed to pose a smaller risk for OIIRR than do fixed orthodontic appliances. A radiographic study on maxillary incisors conducted by Linge and Linge (1983) and established that the group treated with removable appliances had significantly less root resorption than the group treated with fixed appliances[21]. Patients treated with full edgewise appliances, Class II elastics and rectangular wires experienced more OIIRR than patients treated with removable activators, plates with clasps and vertical elastics[81]. A microcomputed-tomography study was conducted by Barbagallo *et al* (2008) to compare OIIRR induced by removable thermoplastic aligners versus fixed appliances producing light and heavy forces. The authors concluded that the removable aligners had the same effect on OIIRR as did light forces produced by fixed partial appliances, and resulted in less OIIRR compared to heavy fixed appliance forces[124].

Comparison has also been made between edgewise (Tweed) mechanics and light wire (Begg) mechanics on their effect on OIIRR. Beck and Harris (1994) found no significant difference in external apical root resorption in any tooth roots between patient groups treated with Begg or Tweed techniques[69]. This finding tempered earlier reports that Begg mechanics during Stages I and III were associated with increased external apical root resorption[93,149]. The method of ligation used in fixed appliances is a further variable that has been investigated for its influence on the incidence and severity of OIIRR. Blake, Woodside and Pharoah (1995) compared patients treated with a self-ligating fixed appliance (SPEED™ appliance) against patients treated with a conventional ligating straight-wire edgewise appliance. The authors hypothesized that the continuous forces generated by the Speed appliance influenced OIIRR differently to the interrupted forces produced by conventional ligation on the edgewise appliance. However, from periapical radiographs, Blake, Woodside and Pharoah found no significant difference in OIIRR outcome between the two groups[29].

2.4.4 Conclusion

Reports that refute or support each of the suspected risk factors of OIIRR can be found in equal numbers throughout the literature. Ultimately, no author has been able to accurately observe or calculate the relative contribution that each factor has on the variance of OIIRR, due to practical or ethical issues in experimental designs[150]. No study has yet been able to definitively prove the exact causation of OIIRR[35], and there exists a poor understanding on the way these factors interact to result in OIIRR. Nevertheless, the factors that seem to be most closely associated with OIIRR are a history of external apical root resorption, anterior open bite, nail biting, heavy continuous forces, intrusion, and root torque tooth movements. There is consensus in the literature that these factors influence OIIRR in conjunction with genetics and systemic factors that contribute to individual susceptibility to OIIRR.

2.5 Pathophysiology of OIIRR

In its essence, root resorption resulting from orthodontic treatment is a local inflammatory reaction[5,6], and is therefore termed orthodontically-induced inflammatory root resorption[7].

2.5.1 Histopathology

Orthodontic force is the precipitating factor in the pathophysiology of OIIRR. It leads to formation of areas of tension and compression within the PDL. In the zones of compression, osteoclasts differentiate from progenitor cells and infiltrate to resorb bone, which allows tooth

movement to occur[151]. Cementum also receive these compressive forces and are susceptible to the action of clastic cells, but is more resistant to resorption than bone[138]. Orthodontic force may also lead to microtrauma (hyalinization and sterile necrosis) of the PDL, which activates a cascade of hormonal and cellular events that contribute to the pathophysiology of OIIRR and regulate odontoclast and osteoclast activity. Hyalinization is intimately related to root resorption[152], and always precedes the resorptive process[153]. With increasing orthodontic force, PDL soft tissue is crushed, the root surface contacts the hard alveolar bone and may be directly damaged. The greater the force, the greater the danger for the injured root surface to be resorbed[115]. The process of OIIRR begins when multinucleated cells colonize mineralized or denuded radicular surfaces[154]. This may occur when precementum becomes mineralized, when precementum is directly breached by mechanical damage and scraped off[9], or if the cementum and bone matrix surfaces are co-incident[155].

In the human model using transmission electron microscopy (TEM), Rygh (1977) suggested that unmineralized precementum (cementoid, 3-5μm thick) is a possible barrier preventing OIIRR, and that the microenvironment around hyalinized tissue is favourable for the induction of hard-tissue resorbing cells. Rygh demonstrated that elimination of hyalinized tissue led to the removal of the cementoid and mature collagen, which left the surface vulnerable to attack by odontoclasts. During the active process of OIIRR, resorption of cementum was observed, in his material, to occur from the rear as an undermining process, once resorption lacunae were established after an initial penetration of the outer cementum layer. This indicated that the outer cemental layer was more resistant to resorption than were the deeper cemental layer and dentine[138].

Brudvik and Rygh thoroughly described the histological events that occur during OIIRR using a combination of light microscopy[139,156] and TEM[140,157] techniques in a rodent model. Brudvik and Rygh, in their light microscopy studies, utilized the tartrate-resistant acid phosphatase (TRAP) stain

to identify osteoclasts, osteoclast-like cells and their precursors. They found that root resorption followed a pattern in its initial stages, and the authors made a distinction between two events that occurred[139,156]. Firstly, the process occurred circumferentially around necrotic hyalinized tissue in the PDL. Secondly, it began 3-4 days later in the central parts of the area of hyalinization. The cells that initially penetrated the root surface at the peripheries of hyalinized areas of PDL were not clast or clast precursor cells (TRAP-negative), and they originated from the adjacent healthy PDL[156]. TRAP-positive cells were first observed in the bone marrow spaces[156]. TEM investigation confirmed that mononucleated non-clast cells are involved in the initial local removal of precementum and mineralized acellular cementum at the peripheries of hyalinized PDL after 3 days[157].

The second distinctive histological stage of initial root resorption is when it occurs on the root surface directly beneath the main area of hyalinization, 3-4 days later. It was shown that there was an association between the presence and active removal of necrotic hyalinized tissue and root resorption in the rodent model using light microscopy. Root resorption was found to occur when invading cells responsible for removing necrotic PDL tissue were found close to the root surface. The majority of the cells were multi-nucleated and stained positive to TRAP, and were first observed in the channels between the bone marrow spaces and the PDL opening behind the necrotic tissue. There was no evidence that these cells were osteoclasts migrating from the marrow spaces. They were most likely mature macrophages that produced TRAP enzyme after being stimulated by mechanical force. From their observations, the authors therefore hypothesized that multi-nucleated TRAP-positive cells, after having removed hyalinized necrotic tissue, reached the subadjacent contaminated and damaged root surface and continued to the remove cementum off the tooth surface[139].

Odontoclasts are the cells responsible for the active removal of cementum and dentine during OIIRR[155]. They are multi-nucleated giant cells with ruffled borders[140] that stain TRAP-positive[139].

They were found to have damaged the root surface beneath the main mass of hyalinized tissue by thinning the cementum and severing the insertion of Sharpey's fibres, leaving the root naked[139]. The TEM study by Brudvik and Rygh (1994) revealed that multi-nucleated giant cells (MNGCs) without ruffled borders, as well as mono-nucleated macrophage-like cells, were responsible for removal of necrotic hyalinized PDL tissue and the superficial layers of cementum. The absence of ruffled cell borders from these cells was in contrast to the classical appearance of multi-nucleated odontoclasts. Odontoclasts have ruffled borders and were found only in the deeper parts of resorption lacunae, usually in contact with and actively resorbing the dentine surface. These observations are suggestive that the MNGCs without ruffled borders, osteoclasts and odontoclasts share the same progenitor cell, derived from the mono-nucleated phagocytic system[140].

2.5.2 Properties of root cementum

It has been hypothesized that the physical properties (hardness and modulus of elasticity) and mineral content of root cementum may be correlated to the occurrence of OIIRR. Reporting on results from a single tooth, Malek, Darendeliler and Swain (2001) described that apical cementum showed the lowest values for Young's modulus of elasticity and hardness, whereas cementum in the middle third of the root had higher values than the apical third[158]. Srivicharnkul, Kharbanda, Swain *et al* (2005)[159] and Chutimanutskul, Darendeliler, Swain *et al* (2005)[160] supported this finding and found that the mean hardness and modulus of elasticity of cementum reduced gradually from the cervical to apical regions in untreated teeth. This trend remained similar upon application of light and heavy forces[159,161]. Although Srivicharnkul, Kharbanda, Swain *et al* (2005)[159] and Darendeliler, Kharbanda, Chan *et al* (2004)[162] were unable to demonstrate any effect of force on cemental physical properties due to confounding variables, Chutimanutskul, Darendeliler, Shen *et al* (2006)

were able to show that heavy forces (225 cN) reduced the modulus of elasticity and hardness of cementum significantly more ($P < 0.01$) than light forces (25 cN) did[161].

The mineral content of cementum has also been speculated to be involved as a variable in the process of OIIRR, influencing a root's susceptibility or resistance to OIIRR. Cementum itself is a non-uniform mineralized connective tissue[163], yet is the most unmineralized dental tissue compared with dentine and enamel with a mineral content of approximately 65%[164]. Using electron probe microanalysis (EPMA) on untreated extracted premolars, Rex, Kharbanda, Petocz *et al* (2005) reported that there was a decreasing gradient of calcium (Ca), phosphorus (P) and fluoride (F) concentrations from the cervical to the apical third of the root. There was also a significant increasing gradient in Ca and P concentrations from the outer to inner third of cementum, whilst an opposite trend was found for F[165]. Upon force application, the same authors reported that light forces (25g) caused little change in the mineral composition of cementum, although they observed an inexplicable trend in increase of Ca, P and F at areas of PDL compression. Heavy forces (225g) produced more definite changes, decreasing the Ca and P concentrations significantly. It was difficult for the authors to correlate these findings directly to OIIRR, but they speculated that local tissue pH changes and mobilization of Ca and P during orthodontic tooth movement may affect the mineral content of cementum[166].

2.5.3 Repair of root resorption

If orthodontic force is discontinued, or falls under a certain level, resorption lacunae begin to be repaired[138]. Resorption lacunae increase the area of the involved root surface, which indirectly decreases the pressure exerted by the application of force. This decompression allows the root

resorption process to slow and stop, creating a favourable environment for cementum to be repaired[7]. Morphologically, different authors have described the repair process to begin at the periphery of the lacunae[138,167], from the base of the lacunae[168], or from both directions[169].

After 21 days of force application on the upper first molars in rats, Brudvik and Rygh (1995) hypothesized that the determinants of continued resorption versus repair was generally associated with the persistence of necrotic tissue. Using light microscopy, the authors found a process of repair started from the periphery in the resorbed lacunae where the PDL had re-established, while ongoing active resorption was observed beneath the existing hyalinized tissue[167]. The same authors also used TEM to demonstrate that the transition from active resorption to the process of repair was associated with invasion of fibroblast-like cells from the periphery into the root resorption site. New tooth supporting structures were seen in these areas, and the later stages of the repair process shared similar characteristics to early cementogenesis that occurs during tooth development. The structure of a new PDL was re-established, and Sharpey's fibres had inserted into the new cementum[170].

Clinically, OIIRR repair may occur very soon after force cessation. In a study on adolescents, Owman-Moll, Kurol and Lundgren (1995) observed repair of OIIRR after only 1 week of retention with a passive appliance. From 1-week retention to 8-weeks retention, the authors observed a three-fold increase in the average number of resorption lacunae that showed repair, increasing from 28% to 75%. Almost half of the resorption lacunae were completely repaired. In their sample, repair occurred almost exclusively with cellular cementum[168]. Langford and Sims (1982) had also described that resorption lacunae were repaired with cellular cementum, and further found that periodontal fibre bundles inserted directly into the repair cellular cementum matrix in their SEM study on histological sections[171]. In the rodent model, however, Hellsing and Hammarström (1996) found that

repair of resorption lacunae occurred with acellular cementum, and changes in the cementum surface and resorption cavities could be seen for as long as 6 weeks after force cessation[169].

Cheng, Türk, Elekdağ-Türk *et al* (2009) found that the resorption process of OIIRR continued for 4 weeks during the retention period after cessation of orthodontic force. After observing teeth for a further 4 weeks during retention, their study recorded differences between the groups that received light forces (25g) versus heavy forces (225g), with marked individual variations. Repair seemed to become steady between 4 to 8 weeks of passive retention if light forces were used, whereas more significant repair occurred during the same time period if heavy forces were used[172]. The finding of a continuation of the resorptive process after force cessation is in agreement with the report of Brudvik and Rygh (1995) that resorption continued in the area of where hyalinized tissue persisted even after active force had stopped on the rat molar[167].

2.6 Management of OIIRR

It is suggested that orthodontists should take all known measures to reduce the occurrence of OIIRR[62]. Prior to active treatment, patients need to be assessed for their risk factors that may contribute to their background susceptibility to OIIRR. Patients with a Hispanic background[27,35], family history of root resorption[38,39,42], previous root resorption experience[23,76], history of dental trauma[11], nail-biting[80], anterior open bite[72–75], reduced exposure to fluoride[68], and pointed or pipette-shaped root apices[22,27] may be more susceptible. These patients should be treated with greater caution to avoid excessive OIIRR.

During active treatment, habits like nail-biting should be controlled[80]. Treatment time should be limited, and treatment goals should be set accordingly[36,104]. Light[114,119–129] and intermittent[110–113]

forces may aid in reducing the risk of OIIRR, with longer intervals between force activations[62,173]. Heavy, continuous forces that torque the roots[94,136], intrude[119,125,134] or jiggle[83–87] the teeth should be avoided. Levander and Malmgren (1988) demonstrated the importance of taking a control radiograph after the first 6-9 months of treatment in patients suspected to be at risk of root resorption. The authors found a significant correlation between the amount of OIIRR seen on this radiograph and the severity of OIIRR evident at the end of treatment[22]. In agreement with Levander, Bajka and Malmgren (1998), Årtun and collaborators (2005 and 2009) have recommended the ideal standard time to monitor OIIRR to be after the first 6 months of active treatment, because patients with detectable OIIRR at this time were more likely to be identified to have severe OIIRR at the end of treatment[174–176]. A shorter three-month follow-up was recommended if teeth were identified to be at elevated risk of OIIRR[176].

If OIIRR is discovered during treatment, treatment with active forces should be paused for 2-3 months. In patients who were discovered to have OIIRR 6 months into treatment, Levander, Malmgren and Eliasson (1994) reported that root resorption was significantly less in the subgroup of patients that received a pause in treatment for 2-3 months, than those treated without interruption[177]. A pause in treatment allows repair to occur[138,168,170]. Where severe OIIRR has occurred, the clinician may need to re-assess treatment goals and accept compromises to shorten treatment duration[36,62,178] and finish as soon as possible. Brezniak and Wasserstein (2002) suggested pausing treatment in one arch for 3 months while continuing with the other to be a practical solution to avoid OIIRR without prolonging total treatment time[62]. Although the literature suggests many protective measures that the clinician may adopt, none of them can actually prevent OIIRR with any degree of certainty[62].

After treatment, the use of severely resorbed teeth as restorative abutments should be reconsidered and avoided[179], and retention with fixed appliances should be done with caution[62], as

occlusal trauma of the fixed teeth or tooth segments may exacerbate the OIIRR[86,87]. Severe cases of OIIRR should be followed up yearly until the resorptive process is quiescent[179]. In extreme cases of severe OIIRR at the completion of orthodontic treatment, endodontic therapy with calcium hydroxide may be indicated[63], especially if the resorptive process continues for a long time after completion of orthodontic therapy[180,181].

2.7 Long-term Clinical Significance of OIIRR

Root resorption can occur as early as one to three weeks after force application[153], but can also undergo a repair process[168]. The average orthodontic patient will experience shortening of approximately 2mm of the maxillary central incisors during comprehensive treatment[20,182]. Case reports are available to illustrate that despite causing severe root shortening in some cases, root resorption does not increase the risk of tooth loss, and affected teeth can remain in function up to more than 30 years later[183]. Vlaskalic, Boyd and Baumrind (1998) reported that only six accounts existed in the literature between 1914 and 1997 that discussed OIIRR as creating a problem for the patient and/or the clinician[19].

Using a computer graphics system to model a maxillary permanent central incisor, Kalkwarf, Krejci and Pao (1986) calculated the loss of periodontal attachment with each increment of 1mm root shortening. Their findings showed that 4mm of root shortening resulting from OIIRR (which is defined as severe by some authors[16,184]) equated to only 20% loss of periodontal attachment area. It can be gleaned that this has minimal effect on tooth loss, as the authors calculated that 3mm of apical root shortening was equivalent to 1mm of crestal bone loss[185]. In a prospective follow-up study of 100 patients at an average of 14 years post-treatment, Remington, Joondeph, Artun *et al*

(1989) observed hypermobility as the worst outcome, which was seen only in two cases[103]. Similarly and more optimistically, VonderAhe (1973) had reported that in 57 patients with varying degrees of OIIRR (mild, moderate and severe), there were no cases of hypermobility or other negative outcomes after an average post-retention period of 6.5 years[184]. However, at 5 to 15 years after active orthodontic treatment, there was a risk of tooth mobility in a maxillary incisor that experiences severe OIIRR if the remaining total root length was ≤9mm, as reported by Levander and Malmgren (2000). The risk of mobility was less if >9mm of root length remained[179]. In another cohort of patients, Jönsson, Malmgren and Levander (2007) reported similar findings 10-25 years after orthodontic treatment, but found 10mm of remaining root length as the threshold for long-term tooth mobility[186].

It can be concluded from the literature that the clinical consequence of OIIRR generally does not pose long-term harm to the dental health of the orthodontic patient[19]. Lee, Straja and Tuncay (2003) reported that dental professionals, overall, held favourable perceptions of OIIRR. General practitioners were more concerned than specialists, however, their knowledge was inconsistent and based on myths. Although most practitioners felt that 50% root loss due to OIIRR was detrimental to the stability of teeth, extraction and replacement was not a viable option[187].

2.8 Measures of OIIRR

2.8.1 Histological preparation with light microscopy

The detection and analysis of OIIRR was classically done with serial sectioning according to histologic techniques and light microscopy (LM), in both human[17,32] and animal models[139,156,167]. The literature reports the use of haemotoxylin and eosin stain[17] and tartrate-resistant acid phosphatase

(TRAP) stain[139,156,167] to appropriately investigate the cellular events of OIIRR. The group of Owman-Moll, Kurol and Lundgren[130,131,153,188] devised a severity scale based on histologic examination. These authors set an arbitrary unit to be 13.3µm, and graded the depth (shallow or deep) and width (small, medium or large) of resorption craters on histologic sections according to this arbitrary unit scale. However, parallax error and the method employed to section the teeth longitudinally most likely led to partially or totally missed craters[189]. Chan and Darendeliler (2004) questioned the true quantitative value of this method of assessing OIIRR[120].

2.8.2 Transmission electron microscopy (TEM)

This technique has been used in studying the regeneration of cementum and the PDL attachment following periodontal surgery[190]. The work of Brudvik and Rygh[139,156,167] attempted to identify the cells that were involved in the OIIRR process, but the use of TRAP staining was inadequate at differentiating cell identities. These authors therefore employed TEM[140,157,170] to provide ultrastructural evidence to differentiate the cell types that could not be done with standard histologic techniques with TRAP staining. In the context of OIIRR, TEM has only been used as a qualitative tool[138], and has not been used to quantify resorption lesions.

2.8.3 Scanning electron microscopy (SEM)

Kvam was one of the first authors to describe root resorption craters from OIIRR using the SEM technique[191,192]. Much of the other earlier investigations used SEM to qualitatively document their findings, describing the topographical changes on the root surface due to OIIRR[14,144]. Composite

electron micrographs were created from multiple acquired images in order to examine tooth roots as a whole. Acar, Canyürek, Kocaaga *et al* (1999) created composite electron micrographs to quantitatively study OIIRR, by calculating the resorbed area as a proportion of the total visible root area[110]. Chan and Darendeliler (2004) also questioned the adequacy of SEM used as a quantitative method in this way, due to possible parallax errors and craters that span past the edge of constituent micrographs[120].

Chan, Darendeliler, Petocz *et al* (2004) proposed a new method for volumetric measurement of OIIRR craters using the SEM technique. These authors described a protocol for capturing micrographs to create stereo images of resorption craters, application of shading correction to determine their depth, and using specialized computer software to calculate crater volumes[193]. The same group of authors was successful in validating two-dimensional measurements against three-dimensional measurements using SEM in the context of OIIRR investigated over 28 days[189]. This was because craters were shallow, and if the experimental period lasted longer, deeper craters may have been created, which may have made 2-D measurements inaccurate[120].

2.8.4 Radiography

Most studies have documented OIIRR using radiographs. Intra-oral periapical radiographs (PA) and panoramic radiographs (OPT) provide two-dimensional information on OIIRR. Computed tomography (CT), cone-beam computed tomography (CBCT), and micro-computed tomography (micro-CT) give three-dimensional information on OIIRR.

Two-dimensional radiography

PAs and OPT are classically the radiographic techniques most commonly used to investigate OIIRR. Authors who have used PAs have used graded scales to assess OIIRR. Newman (1975) was one of the first to describe a severity scale for root shortening[37]. Malmgren, Goldson, Hill *et al* (1982) proposed a root resorption index [77], whilst other authors used similar graded scales[16,194] for quantitative assessment of OIIRR. Mirabella and Årtun (1995) described formulae for calculating differences in tooth length measurements off "standardized" PAs to quantify OIIRR[24].

The accuracy of PAs relies on proper positioning of the film and X-ray source relative to the long-axis of the tooth/teeth, and the positioning needs to be reproducible for meaningful comparisons to be made[195]. However, accuracy is difficult to achieve with either the bisecting angle technique[103] or the paralleling technique[195]. PAs are inadequate to accurately diagnose OIIRR in its early stages[196–198], cannot detect resorption on buccal or lingual root surfaces[120], and root resorption assessment algorithms based on PAs are geometrically inaccurate[199]. Attempts have been made to increase the accuracy of PAs in assessing OIIRR, by using jigs to standardize magnification factors[133] or using mathematical reconstruction and digital subtraction radiography[200–202]. Digital radiography had a similar level of sensitivity in detecting OIIRR compared to conventional radiography[203].

Panoramic radiographs have also been used widely to evaluate OIIRR. Apajalahti and Peltola (2007) described an index against which the severity of OIIRR was judged, graded from 0 (no visible resorption), through 1 (mild resorption up to ¼ of the root) to 2 (moderate to severe resorption of ¼ or more of the root length)[28]. However, roots may be magnified or shortened if their angulations are different pre- to post-treatment, and sit in different places relative to the focal trough[195]. This limitation of OPT may explain why Sameshima and Asgarifar (2001) found that panoramic films overestimated the amount of root loss by 20% or more compared to PAs[204]. OPT was shown to

underestimate OIIRR compared to CBCT[205]. Armstrong, Kharbanda, Petocz *et al* (2006) reached the conclusion that OPT is not a reliable method to measure OIIRR and apical root shortening[206].

Three-dimensional radiography

CBCT was introduced into dentistry in period of the late 1990s[207], and was accepted due to its lower radiation doses compared to conventional computed tomography, high spatial resolution and affordability[208]. CBCT offers the ability of assessing OIIRR that has occurred on tooth surfaces that could not be imaged by conventional two-dimensional radiographs[209]. CBCT also has the advantage of producing images of whole teeth accurately despite changes in tooth/root position and angulation[209]. Sherrard, Rossouw, Benson *et al* (2010) used porcine heads to show that root lengths were underestimated by an average of 2.6mm on PAs, but by less than 0.3mm on CBCT images[210]. With intact dry human mandibles, Durack, Patel, Davies *et al* (2011) found that CBCT had higher sensitivity and almost twice the specificity than PAs in detecting simulated OIIRR cavities 0.5mm × 0.25mm in size[211]. Compared to conventional periapical radiographs, CBCT was shown to have a higher sensitivity at detecting OIIRR lesions in treated patients, doing the job more accurately at earlier stages of the resorption process, especially if a voxel size of 0.02mm or less is used[205,212].

Micro-CT is a highly accurate 3D imaging technique with very high spatial resolution and magnification used to visualize the external and internal microstructure of mineralized tissue. Due to the small size of the imaging gantry (68mm diameter) and long exposure times required (hours), it is only possible to analyze human dental material *ex vivo* using this technique[213]. Micro-CT allows direct quantitative analysis of OIIRR lesions on human teeth accurate down to 5µm[125], and has been used throughout the publications on root resorption originating from the University of Sydney, Sydney, Australia[34,66,68,88,112–114,123–129,172,214]. Wierzbicki, El-Bialy, Aldaghreer *et al* (2009) found that micro-CT was able to identify and measure microscopic OIIRR lesions on teeth without signs of macroscopic resorption[213]. Harris, Jones and Darendeliler (2006)[125] showed that micro-CT had

advantages even over the SEM technique described by Chan, Darendeliler, Petocz *et al* (2004)[193]. Micro-CT was able to differentiate true OIIRR craters from surface depressions that were openings of accessory root canals, and, unlike SEM images, micro-CT images were not graphic simulations[125]. Dudic, Giannopoulou, Martinez *et al* (2008) demonstrated micro-CT to had far superior sensitivity and specificity over PAs in detecting OIIRR after 8 weeks. These authors also suggested micro-CT to be a used as a criterion standard (gold standard) against which other OIIRR imaging techniques should be compared[215].

The literature continues to support radiography as a valuable diagnostic tool for qualitatively detecting OIIRR. However, the use of two-dimensional radiographs (PA and OPT) for quantitative measurements of OIIRR in 3D is relatively poor and should be avoided[120], despite efforts to increase its accuracy[133,200–202]. The application of high resolution three-dimensional radiographic methods (CBCT and micro-CT) is better suited to measure OIIRR, which is a three-dimensional phenomenon[205,212,215].

2.8.5 Possibility of a biochemical assay for biomarkers in OIIRR

Imaging techniques used to diagnose and study OIIRR, whether *in vivo* or *ex vivo*, carry inherent disadvantages. The more accurate techniques are invasive, and often require extraction of teeth (micro-CT, SEM, TEM, LM). The less invasive modalities (radiographic imaging) have problems with standardization, sensitivity, specificity, limited points of view (PA and OPT), and issues with radiation exposure (CBCT)[216]. All aforementioned methods are static and cannot indicate the activity of the OIIRR process, whether it is arrested or ongoing[216]. In recent years, researchers have entertained the idea that proteins found in gingival crevicular fluid (GCF) may serve as biological markers for

OIIRR[216,217], just like they may do for periodontal disease[218,219]. A *biomarker* is defined as a substance that can be measured objectively and evaluated as an indicator of a normal biological process, pathogenic process or a response to therapeutic intervention[220]. A future chair side diagnostic test based on GCF sampling that gives an immediate comprehensive profile of biomarkers and risk is the ultimate goal[220]. Mah and Prasad (2004) suggested that an assay of GCF biomarkers for detecting OIIRR to have many possible advantages: higher sensitivity, non-invasiveness, no radiation, the ability to identify at-risk individuals, and allowing the clinician to monitor OIIRR activity during treatment for better decision-making[216].

2.9 Gingival Crevicular Fluid

GCF is a transudate of gingival tissue interstitial fluid that is harvestable from the gingival sulcus or periodontal pocket[221]. More specifically, GCF is formed as a blood ultrafiltrate, but accumulates metabolic products from bacterial and host cells of the gingival crevice[222]. During a state of health, it is a transudate, but during disease, it becomes a true inflammatory exudate[223]. GCF is a complex mixture of antibodies[224], host enzymes[225], cytokines and inflammatory mediators[226], tissue degradation products[226], leukocytes[227], structural cells of the periodontium[227], and bacterial cells[227] and enzymes[228]. GCF has the two important characteristics of isolation and flushing: extrinsic substances do not easily penetrate the gingival sulcus, and are rapidly washed out if they do[222].

2.9.1 GCF proteins in periodontal disease

Studies on GCF extend over the last half-century, stemming from roots in the periodontal literature. Brill (1962) established an understanding of its physiology and composition[229], while Löe

and Holm-Pedersen (1964) explored the use of GCF as an indicator of periodontal disease activity[230]. Bang and Cimasoni (1971) were one of the first to study the presence of proteins and enzymes in GCF[231].

The periodontists lack precise clinical criteria and indices to accurately assess periodontal disease, and to precisely identify the underlying causes of the periodontal breakdown. Traditional clinical measures have shortcomings as measures (diagnostic tests) and predictors (prognostic tests) of periodontal disease[232–234]. This was (and still is) the stimulus for research towards finding biomarkers in GCF[235]. Researchers hoped that these biomarkers would serve as indicators of periodontal disease activity, measures of response to therapy, and prognostic indicators for future disease and high-risk patients[235,236].

Interleukins IL-1α, IL-1β, IL-6, IL-8 and interferon-gamma (IFN-γ) were present at elevated levels in active periodontitis [237–240], and IL-1β levels in GCF increased significantly after plaque accumulation for 3 weeks[241]. Graves (2008) suggested that some of these cytokines may even play an active role in promoting periodontal tissue destruction[242]. Bone-specific matrix molecules resulting from bone turnover or breakdown detected in GCF have been suggested as biomarkers for periodontal disease[243]. Periodontally diseased patients also expressed higher ratio of receptor activator of nuclear factor κB ligand (RANKL) to osteoprotegerin (OPG) concentrations in GCF than healthy patients[244]. Work has also been directed at cytokine gene expression during peri-implantitis: mRNA levels were elevated for IL-10, IL-12 and tumor necrosis factor-alpha (TNF-α), and reduced for IL-4 during active disease[245]. Cytokines and other biomarkers in GCF have also been used to monitor the effects of initial non-surgical[246,247], antibiotic[248], laser[249] or surgical therapy[250]. Researchers[219] have proposed that other biomarkers in GCF be used as predictors of future periodontal disease, such as prostaglandin E_2 (PGE_2)[251], lysosomal β-glucuronidase[252], IL-1β, C-reactive protein[253], glycosaminoglycans[254], and matrix metalloproteinases (MMPs)[255]. Embery and Waddington (1994)

proposed that "no single marker would fulfil all criteria necessary for the assessment of the clinical state of the periodontium", and efforts should be made to identify packages of biomarkers that will fulfil the role[235].

2.9.2 GCF proteins in orthodontic tooth movement

Over the last two decades, there has been interest in biomarkers in GCF that reflect events occurring in the PDL during orthodontic tooth movement (OTM). Where candidate biomarkers for periodontal disease may identify disease activity and risk, candidate biomarkers for OTM may monitor and may even enhance the effectiveness and efficiency of orthodontic treatment[256]. Proteins that orchestrate the cellular inflammatory response during bone resorption and deposition, and enzymes have been candidate biomarkers for OTM[256,257].

A review of the literature by Ren and Vissink (2008) revealed that interleukin-1β (IL-1β) has been the most investigated cytokine in research of OTM[258]. It has been consistently reported that IL-1β levels increase significantly in response to applied forces, and that a significant peak in levels of IL-1β occurred at 24 hours after orthodontic force application, with a decline to baseline readings after 7 days or more[259–264]. Other reports described the peak in IL-1β to occur at 3 days after for application, but still found a trend to return toward baseline levels beyond 7 days[265,266]. The common trend in a decrease of cytokine levels after an initial short-term increase has been suggested to be the result of the periodontal system stabilizing at a new physiological homeostasis[262]. Heavy (250g), interrupted forces resulted in significant decreases in IL-1β levels to baseline, as shown by the research groups of Uematsu[263] and Yamaguchi[264]. This was in contrast to light continuous forces used by the groups of Lee[261] and Iwasaki[265] which maintained relatively high

IL-1β levels for a longer period. Ren and Vissink interpreted these trends as evidence at the cellular level for optimal orthodontic forces to be light and continuous, because these induce longer lasting levels of cytokines that are needed for continuous remodelling of bone during OTM[258]. Further, there is early evidence that elevated IL-1β levels occurring with decreased interleukin-1 receptor antagonist (IL-1RA) were correlated to faster tooth movement[267,268].

Other cytokines and inflammatory mediators present in GCF have also been investigated during OTM. Interleukins IL-2, IL-6, IL-8, and TNF-α (cytokines) were found to follow a similar trend in concentration levels as IL-1β, increasing above baseline upon force application[269–271] and peaking after 24 hours[262,263,272]. In patients who were in comprehensive orthodontic treatment for at least 12 months, mean IL-4 levels in GCF tended to be lower than untreated adolescents, but did not reach statistical significance[271]. Inflammatory mediators PGE_2 and substance P (SP) were found in significantly higher amounts in the GCF during OTM, and peaked at 24 hours and remained elevated[259,260], although PGE_2 showed inconsistent changes[261]. There were also interactions between cytokines and inflammatory mediators. Studies that investigated PGE_2 or SP simultaneously with IL-1β have remarked on complex interactions and feedback mechanisms between cytokines and extracellular inflammatory mediators during OTM: SP induced IL-1β production[264], whilst PGE_2 inhibited it[261]. Patient age[273] and other biological interactions between cytokines[274] may further complicate the interpretation of cytokine level trends in GCF during OTM.

The RANKL-OPG system is involved in the regulation of bone resorption. RANKL is responsible for the induction of osteoclastogenesis, whilst OPG is a decoy receptor that competitively binds RANKL to inhibit its action[275]. During OTM, GCF levels of RANKL were significantly higher by a factor of 16.7, whilst OPG levels were significantly lower by a factor of 2.9 in experimental canines being distalized than in control teeth[276]. Furthermore, OPG was found to decrease in a time-dependent manner over 3 months of canine distalization[277]. Age was also found to have a diminishing effect on

the amount of OPG detected in GCF during OTM[278]. Therefore, monitoring local OPG and RANKL concentrations in GCF has been presented in the literature as a possible biomarker to indicate the effectiveness and efficiency of treatment in achieving optimal tooth movement[277].

Other possible biomarkers of metabolic activity during OTM cannot be ignored. Non-collagenous components of the PDL extracellular matrix (ECM), proteoglycans, have been detected in GCF in association with OTM[279]. Waddington and Embery's review of the literature described early evidence that chondroitin 4-sulphate and heparin sulphate appeared in GCF during orthodontic treatment[280]. Proteases involved in the remodelling of ECM, matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) have also been identified in GCF during OTM[281]. However, findings on MMPs and TIMPs have been inconsistent: comparisons in levels between control and experimental sides ranged from no difference[282] to a 12-fold increase on the force application side[283]. Some authors further reported time-dependent changes in the levels of MMPs in GCF[284,285] during OTM. Multiple extracellular enzymes have also been investigated in GCF during OTM as potential biomarkers to monitor progress of orthodontic treatment. Levels of lysosomal β-glucuronidase[286], alkaline phosphatase[287], aspartate aminotransferase[288,289], cathepsin B[290], lactate dehydrogenase[291,292] and myeloperoxidase[293] were all increased upon the application of orthodontic force compared to controls. Manipulation of chemokines and growth factors (epidermal growth factor[263], transforming growth factor[294] and insulin-like growth factor[295]) have also been suggested to be other pathways to modulate OTM in the future, although their exact functional role in OTM is yet undiscovered[256]. Developing knowledge about the proteins and molecules found in GCF may possibly allow orthodontist to monitor the outcome of OTM, accelerate OTM, selectively increasing anchorage at particular sites, and increase stability of orthodontic results[256].

All studies investigating biomarkers in GCF during OTM shared common drawbacks in their designs[258]. They suffered from large fluctuations in levels of biomarkers, and recruited small sample

sizes of patients with varying ages and gender. Age[273] and gender[296] may be variables affecting cytokine levels. Each collected samples at different time points, used different methods of molecular analyses, and reported data using dissimilar measurement scales and styles. Repeated GCF sampling and different sampling protocols may have affected the concentration of biomarkers found between studies[297]. Authors often collected GCF samples only from the pressure side of experimental teeth, or they did not discriminate at all whether samples were collected from the pressure or tension side. Apart from one study[271], all reports only describe short-term changes in biomarker levels in GCF, which may be a surrogate measure and not reflect the true nature of orthodontic treatment which takes much longer time. Ren and Vissink (2008) advocated that future studies in this field should focus on overcoming these limitations, and use common GCF sampling and reporting protocols to allow more meaningful comparisons to be made between studies[258].

2.9.3 GCF proteins in root resorption

In an attempt to develop a more sensitive and specific test to diagnose and monitor OIIRR, Mah and Prasad (2004) were one of the first to investigate biomarkers associated with OIIRR. These authors targeted dentine phosphoproteins (DPP) as local organic matrix proteins released into the GCF during resorption of dentine. They recruited three groups of teeth: (1) untreated healthy permanent central incisors (control), (2) mildly resorbed permanent central incisors under active orthodontic treatment, and (3) deciduous second premolars with half their roots resorbed. By showing that DPP levels in GCF were higher in the deciduous group ($P = 0.001$) and mildly resorbed treated group ($P = 0.046$) than in the control group, Mah and Prasad identified a possible biomarker for OIIRR in GCF[216]. Other dentine breakdown products have also been identified as possible suitable biomarkers of OIIRR. Balducci, Ramachandran, Hao *et al* (2007), in their cross-sectional

study, reported that dentin phosphophoryn (PP) and dentin sialoprotein (DSP) were found in statistically higher amounts in severe OIIRR (>2mm root shortening) than in mild OIIRR (<2mm root shortening). PP and DSP were also found in small amounts in the GCF of untreated control patients, but in lower levels than both OIIRR groups[217]. Kereshanan, Stephenson and Waddington (2008) also reported elevated levels of DSP at 12 weeks after commencement of orthodontic therapy[298]. The findings of dentine breakdown products in the controls support findings that tooth roots undergo physiologic remodelling and turnover with baseline root resorption[34].

However, the search of single biomarkers for OIIRR shed into GCF may have low a probability for success because it requires the individual separation, identification and testing of each candidate biomarker. It is also difficult to develop antibodies against dentine breakdown products for immunoassay, because dentine proteins are heavily phosphorylated, making them less antigenic and less reactive with antibodies[299]. Rody Jr, Holliday, McHugh *et al* (2014) utilized a new proteomic technology that combines mass spectrometry with liquid chromatography to profile a panel of over two thousand proteins in GCF collected from resorbing deciduous molars and non-resorbing permanent molars. There was significant characteristic up-regulation and down-regulation of proteins in deciduous teeth with resorption. Some were novel proteins identified in GCF, and the authors suggested that these might be used as a single biomarker or as part of a panel of biomarkers for OIIRR[299].

The OPG/RANK/RANKL system has an established role in the process of physiological root resorption of deciduous teeth. Receptor activator of nuclear factor κB (RANK) and its ligand (RANKL) are both expressed by odontoclasts that resorb deciduous roots, and osteoprotegerin (OPG) suppresses the RANKL-induced activation of odontoclasts[300]. Therefore, it was logically suggested that the RANKL:OPG ratio may contribute to the process of OIIRR[301]. George and Evans (2009) conducted a cross-sectional study to show that both the concentration of RANKL and the

RANKL:OPG ratio in GCF of mild and severe OIIRR patients were significantly higher than in negative untreated controls ($P < 0.05$), implicating RANKL as a possible biomarker for OIIRR[302]. However, because the regulatory role of the OPG/RANK/RANKL system is common to both the osteoclast and odontoclast[303], more evidence is required to demonstrate how RANKL concentrations and the RANKL:OPG ratio in GCF can be differentially interpreted to indicate bone remodelling rate during OTM or OIIRR.

Compared to the research on biomarkers of OTM, the research into biomarkers for OIIRR is newer and fewer. However, there is already a common basic limitation amongst these studies. They have used conventional two-dimensional radiographic techniques (PA) to identify and stratify patients into their OIIRR groups according to severity (mild or severe)[217,302]. As discussed earlier, two-dimensional radiography (PA) has limitations in accurately identifying OIIRR[103,195,199], especially in the early stages[196–198] and if resorptive lesions are located on the buccal or lingual root surfaces[120]. Otherwise, studies have investigated root resorption in the context of exfoliation of the primary dentition, instead of the context of orthodontic force application[216,298,299]. Future investigations into biomarkers in GCF for OIIRR need to focus on addressing these issues in study design, so that the relevant hypotheses can be properly addressed.

2.9.4 Candidate cytokines and their biological roles

Cytokines are extracellular signalling proteins for cell-to-cell communication that act at low concentrations in a paracrine or autocrine mode[151]. In the context of orthodontics, cytokines are involved in bone remodelling and inflammatory processes during OTM, by directly and/or indirectly facilitating the activation and differentiation of PDL cells and bone cells[304]. Due to similarities

between osteoclasts and odontoclasts[138,303], these cytokines may also possibly modulate the process of root resorption and OIIRR; however, strong evidence is still lacking.

Pro-inflammatory cytokines

IL-1β, IL-2, IL-6, IL-7, IL-8, GM-CSF and TNF-α

IL-1β directly stimulates osteoclast function, and has potent actions in promoting bone resorption and inhibiting bone formation[265,305]. IL-2 stimulates macrophages, natural killer cells and osteoclastic activity[270,306]. IL-6 stimulates the formation of osteoclasts and the bone-resorbing potential of preformed osteoclasts in an autocrine and paracrine fashion[307]. However, IL-6 can also have both positive and negative effects on osteoblast and osteoclast differentiation[308]. IL-7 induces osteoclastogenesis through the activation of T-cells that produce more RANKL, and is a significant mediator of bone loss in inflammatory conditions, such as rheumatoid arthritis and periodontitis[309]. IL-8 is a potent pro-inflammatory cytokine, a chemoattractant that recruits and activates neutrophils[310,311]. Colony-stimulating factor related to both granulocyte and macrophages (GM-CSF) is potent at stimulating bone marrow cells to produce osteoclasts[312], but by themselves may not be able to induce terminal osteoclast differentiation[307]. TNF-α is produced primarily by activated monocytes, macrophages and osteoblasts. TNF-α directly induces bone resorption by stimulating osteoclast differentiation from precursors and up-regulating RANKL[256,313]. IL-1α, IL-1β, IL-6, IL-8 and TNF-α were present at elevated levels during active periodontitis[237–239,245] and after non-surgical periodontal treatment to resolve inflammation, levels of IL-2 and IL-7 reduced[247]. Pro-inflammatory cytokines can also act together synergistically. IL-1β, IL-6, IL-8 and TNF-α each enhance the actions of the others to promote osteoclastogenesis and activation of osteoclasts[256,313]. Cytokines may also

be co-dependent: IL-6 potentiates IL-7 expression314, and the production of IL-6 and IL-8 has been shown to depend upon the presence of IL-1β, relying on autocrine or paracrine mechanisms[274,315].

Anti-inflammatory cytokines

IL-4, IL-5, IL-10, IL-12p70, IL-13 and IFN-γ

IL-4 and IL-13 are closely related cytokines that inhibit bone resorption by inhibiting the differentiation and activity of osteoblasts[307]. In both an animal[316] and a human model[317], IL-4 and IL-13 induced the expression of OPG, reduced the expression of RANKL, and reduced the expression of RANK in osteoclast progenitor cells. IL-5 promotes bone formation by mobilizing osteoblast progenitor cells and inhibiting activated osteoclasts[318]. IL-10 directly inhibits osteoclastogenesis at an early stage, preventing differentiation of osteoclast progenitors to preosteoclasts[319]. IL-10 suppresses OPG[320] and inhibits the production of pro-inflammatory cytokines IL-1, IL-6, IL-8, TNF-α and GM-CSF[256,321]. IL-10 has more powerful anti-inflammatory actions than IL-4 due to different mechanisms of suppression of pro-inflammatory mediators[321]. IL-10 also contributes to the maintenance of tissue integrity and ECM deposition by modulating the balance of MMPs and TIMPs[322]. IL-10 may also have anti-inflammatory actions in periodontal tissue, since treating periodontitis results in the increase of levels of IL-10, but other authors presented conflicting results[248]. IL-12p70 is the biologically active heterodimer consisting of two subunits, p35 and p40. IL-12p70 regulates T-cell differentiation and proliferation, promotes the Th1 cell-mediated immune response, and regulates the adaptive immune response[323]. Overall, IL-12p70 has the effect of reducing bone loss, mainly through the induction of IFN-γ[313], and is found in higher concentrations during reduced periodontal inflammation[238]. IFN-γ is an extremely potent cytokine that inhibits both osteoclast formation and bone resorption[307]. IFN-γ has further anti-inflammatory actions in bone

metabolism by inhibiting the activity of IL-1 and TNF-α[305,324]. However, during periodontal disease, IFN-γ has been shown to display potent pro-inflammatory actions[240], and non-surgical periodontal therapy to resolve inflammation resulted in decreased levels of IFN-γ found in GCF[246].

2.10 Methods for Molecular Analysis of Cytokines in GCF

2.10.1 Enzyme-linked immunosorbent assay

ELISA technique uses an immobilized antibody that captures a soluble ligand (the target protein of investigation). Detection and quantification of target proteins is possible through further binding of a reporter antibody to the complex, followed by enzyme amplification of a colorimetric substrate[325]. ELISA is widely accepted and used in clinical and laboratory settings[216,255,277], and has long been the criterion standard for quantitative analysis of cytokines and biomarkers[326,327]. This technique can only investigate one analyte at a time, with the disadvantage of low data throughput and requirement for large sample amounts, but the advantage of not suffering problems associated with multiplexing and cross-reactivity of reagents[328].

2.10.2 Multiplex bead array assay

This assay technique was conceptualized in 1977, and has been used in the investigation of proteins in GCF in periodontal disease research[247–249] and OTM research[262,282]. It allows users to simultaneously identify and quantify many more proteins in a sample than ELISA can, and can number as many as forty-eight[296]. MBAA has the advantages of being able to analyze small volumes, easy to perform, is time- and cost-effective, reproducible, has the capacity for multiplexing[329], and

decreases experimental variability[296]. MBAA also carries disadvantages. MBAA may be of limited sensitivity[326], while multiplexing may lead to cross-reactivity of anti-cytokine antibodies with other cytokines, cross-species antibodies or interfering substances (the 'matrix effect')[328]. However, this issue is adequately addressed by most contemporary commercial MBAA kits, which have been optimized to minimize or eliminate any or all artefacts that may result from the matrix effect[327]. Assays done with MBAA have good to excellent correlations to ELISA for most, but not all, cytokines[330–333]. Any differences observed during poorer correlations were attributed to dissimilarities in capture antibodies, reported antibodies, sample diluents[331,332], or kits sourced from different manufacturers[334]. MBAA kits from different vendors yielded different absolute quantities of cytokines, although cytokine levels followed similar qualitative trends between kits. However, absolute values for some cytokines were similar between ELISA and MBAA using kits from the same manufacturer[334]. Overall, MBAA performs just as well as ELISA[327].

2.10.3 Other methods

The western blot technique has also been used to investigate proteins in GCF[217,298]. This technique separates and identifies proteins from a mixture of many, based on molecular weight through gel electrophoresis. Proteins are transferred to a solid support membrane, washed and marked with antibodies, then detected by developing the membrane film[335]. The radioimmunoassay (RIA) technique was used during the earlier years of periodontal research of proteins in GCF[336], and more recently in orthodontic GCF research[273]. RIA is a very sensitive tool for measuring the concentration of a protein using detector antibodies pre-bound to a radioactively labelled antigen. When a sample with an unknown amount of target protein is added, it competes with and displaces the labelled antigen. The now-free labelled antigen is then measureable using a gamma counter[337]. What RIA was able to achieve can now be accomplished by contemporary ELISA techniques[338].

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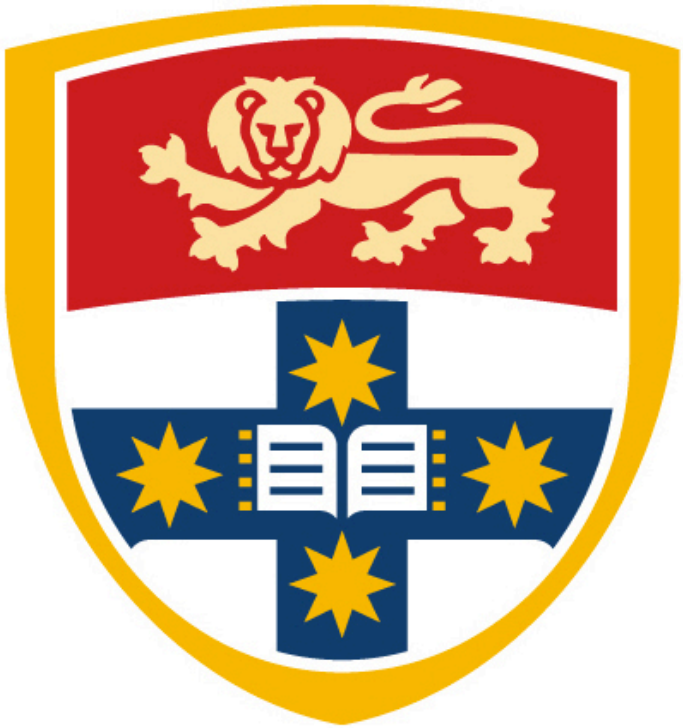

Physical properties of root cementum: Part 25. Cytokines in gingival crevicular fluid and root resorption: A microcomputed tomography study

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A condensed version of this manuscript will be submitted for publication in the AMERICAN JOURNAL OF ORTHODONTICS & DENTOFACIAL ORTHOPEDICS (AJODO)

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Number of pages: 30

Number of figures: 8

Number of tables: 1

4.1 Abstract

Introduction: Orthodontically induced inflammatory root resorption (OIIRR) is a treatment complication in 2-5% of orthodontic patients. Our aim in this randomized prospective clinical trial was to investigate candidate anti-inflammatory and pro-inflammatory cytokines in gingival crevicular fluid (GCF) that may be biomarkers for an underlying immunologic factor in the OIIRR process.

Methods: This split-mouth study included 34 maxillary permanent first premolars, indicated for extraction from 17 adolescent, prospective orthodontic patients. Patients were allocated into two groups. Group I (light force) patients received 25g of buccally tipping force on one premolar. Group II (heavy force) patients received 225g of force. Contralateral premolars served as controls. Two samples of GCF were collected from the mid-buccal sulcus of each maxillary first premolar at baseline, then at 3 hours, 24 hours, 3 days, 7 days and 28 days after force application. GCF samples were measured for volume and analyzed for thirteen cytokines using multiplex bead array assay. After 28 days, the premolars were carefully extracted, imaged with a microcomputed tomography system and analyzed with software designed for volumetric measurement of resorption craters.

Results: Patients identified to have more severe root resorption had significantly lower levels of IL-4 and higher levels of IL-10 ($P \leq 0.001$). Levels of IL-13 in these patients were affected by force magnitude. Heavy forces reduced IL-2 levels. No differences in cytokine levels were found between time points. Force magnitude had a statistically significant effect on the total volume of resorption craters.

Conclusions: Levels of IL-4, IL-10 and IL-13 in GCF appeared to correlate with OIIRR. Further investigation is required to elucidate what seems to be a complex relationship between OIIRR severity, force magnitude and cytokine levels in GCF.

4.2 Introduction

Orthodontically induced inflammatory root resorption (OIIRR) is a type of progressive, external inflammatory root resorption[1]. It can occur in all orthodontically treated patients[2], but does so to a severe degree in up to 5% of patients, who experience more than one-third[3] or >5mm of root shortening[4]. OIIRR is influenced by a multifactorial etiology[5], a complex combination of systemic and local patient-related risk factors[6] and treatment-related risk factors[7]. The exact cause is elusive, and there is no definitive understanding of how risk factors combine and interact in individual patients that allows clinicians to predict or prevent OIIRR with any certainty.

Despite the best efforts to avoid severe OIIRR, why are up to 5% of patients afflicted? Furthermore, why do some individuals experience external apical root resorption even without treatment[8]? There is evidence of genetic predisposition to OIIRR, with heritability estimates of 34%[9] to 70%[10]. Al-Qawasmi and collaborators identified that risk of OIIRR was linked to the *IL-1B* allele 1[11] and the *TNFRSF11A* gene[12]. Ultimately, genetic factors that influence OIIRR are heterogeneous, and account for at least 50% variation in OIIRR and other forms of external apical root resorption[13].

Identifying gingival crevicular fluid (GCF) biomarkers that are indicative of OIIRR has been suggested as an improved method for its early diagnosis[14], which is essential for identifying the risk of severe OIIRR in patients so that it may be properly managed[3]. Biomarkers may have advantages over the diagnostic limitations of radiographic techniques in early detection[15,16], give information on the activity of the resorptive process, and possibly identify at-risk individuals[17].

During orthodontic tooth movement (OTM), forces produce zones of compression within the periodontal ligament (PDL), leading to hyalinization in some of these areas. The cellular process removing hyalinized tissue also removes the protective cementoid layer[18,19], and is intimately linked to the cellular process of OIIRR[20,21]. Cytokines are extracellular proteins for intercellular signalling

that affect bone metabolism and tissue remodelling during these processes[22]. They have been found in GCF during OTM and suggested to be a diagnostic tool to monitor orthodontic treatment[23]. However, there are no studies to date that have directed attention at cytokines in GCF as biomarkers of OIIRR.

Therefore, the aim of the current randomized prospective clinical trial was to investigate candidate pro-inflammatory and anti-inflammatory cytokines in GCF associated with the occurrence of OIIRR. This may reveal biomarkers that may be used in diagnosing OIIRR, identifying at-risk patients, and possibly further our understanding of contributing patient-related systemic risk factors. Microcomputed tomography (micro-CT) and multiplex bead array assay (MBAA) techniques were used. The following null hypotheses were proposed:

1. There is no difference in the levels of candidate cytokines in GCF collected from teeth receiving a heavy force and from teeth receiving a light force.
2. There is no distinct time-related trend in the levels of candidate cytokines in GCF collected from teeth receiving either light or heavy forces.
3. There is no difference in total crater volumes on roots between teeth receiving light or heavy orthodontic forces and control teeth.
4. If hypothesis (3) is rejected, there is no difference in the levels of candidate cytokines in GCF collected from teeth that have external root resorption craters of greater total volume resulting from orthodontic forces, compared to those with craters of lesser total volume.

4.3 Material and Methods

Subjects

Ethical approval was received from the Human Research Ethics Committee of the Sydney Local Health Network and University of Sydney (Royal Prince Alfred Hospital Zone) (Protocol No. X11-0028 and HREC/11/RPAH/37). Seventeen adolescent, prospective orthodontic patients who required planned bilateral extractions of maxillary first permanent premolars were included in this study. Maxillary premolars were chosen due to their higher susceptibility to OIIRR compared to mandibular premolars[24]. Patients were recruited according to previously established selection criteria[25], and without medical history of allergies or asthma. All patients were issued full information pamphlets regarding the clinical study, and completed written consent forms (or completed by parents/guardians where applicable). Patients were randomly allocated into one of two groups. Group I (light force) consisted of 9 patients (5 female and 4 male, mean age 14.8 ± 1.05 years), and Group II (heavy force) consisted of 8 patients (2 female and 6 male, mean age 16.5 ± 1.58 years).

Experimental design

Strict oral hygiene instructions were given to every patient, and oral hygiene was monitored for at least 2 weeks prior to and during the experimental period. Using a split-mouth design in each patient[26], one maxillary first premolar was randomly selected to receive a buccally tipping force (force-side), whilst the contralateral first premolar was used as the control (control-side). Active self-ligating brackets with 0.022-in slot (SPEED™; Strite Industries, Cambridge, Ontario, Canada) were bonded buccally to force-side and control-side premolars, and SPEED™ tubes were bonded to

the force-side ipsilateral first permanent molars (Fig 1, *A*). According to previous protocol[27], patients in Group I each received 25 grams to the force-side premolar, applied with a 0.016-in TMA® cantilever spring (ORMCO, Glendora, CA). Patients in the Group II each received 225 grams to the force-side premolar, applied with a 0.017×0.025-in Beta III Titanium cantilever spring (3M Unitek, Monrovia, CA). Compomer material (Transbond™ Plus; 3M Unitek, Monrovia, CA) was bonded to the mandibular first permanent molars as occlusal stops such that maxillary first premolars did not receive occlusal forces (Fig 1, *B*). Forces were activated once at the beginning of the 28-day experimental period. The premolars were carefully extracted after 28 days and immediately stored in de-ionized water (Milli-Q™; Merck Millipore, Billerica, MA) until analysis[28].

GCF Collection

During the experimental period, GCF was collected using sterile absorbent paper strips (Periopaper™; Oraflow, Smithtown, NY) from the buccal sulcus of both force-side and control-side premolars in every patient (Fig 1, *C*). After careful removal of traces of supragingival plaque (if necessary) with a sterile probe without contacting the gingiva, each site was isolated with cotton rolls, washed with water and gently dried with compressed air for 3 seconds. One paper strip was inserted 1mm into the gingival sulcus on the mid-buccal aspect for 30 seconds, and after a pause of 60 seconds, a second paper strip was inserted at the same site for the same length of time[29]. Strips contaminated by blood were discarded and resampled. Each GCF sample was immediately measured for its volume using a Periotron 8000® (Oraflow) (calibrated with bovine serum albumin[30]), and then immediately sealed in a 1.5mL polypropylene Safe-Lock Tube® (Eppendorf AG, Hamburg, Germany). Samples were stored at −80°C until the day of analysis[29]. Plaque index, gingival index and probing depths around the premolars were recorded after sample collection at

each visit. GCF samples were collected at baseline just before bonding the appliance, then at 3 hours, 24 hours, 3 days, 7 days and 28 days after force application, prior to extractions[23].

Measurement of cytokines in GCF

GCF was thawed and eluted from the paper strips with 70µl of 0.05% Tween 20 in phosphate buffered saline (PBS) and protease inhibitor cocktails for human (Sigma-Aldrich, St. Louis, MO) by water bath sonication for 15 minutes, and centrifugation at 20,000g for 10 minutes[31]. Supernatants were collected and analyzed for thirteen cytokines (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, TNF-α, IFN-γ and GM-CSF) by multiplex bead array immunoassay using high sensitivity human cytokine magnetic bead kits (premixed) (Cat. # HSCYMAG60SPMX13) (Milliplex® MAP; Merck Millipore). The standards and samples were assayed on a robotic liquid handling workstation (epMotion 5075; Eppendorf AG). Plates were washed with Bio-Plex Pro II wash station (Bio-Rad, Hercules, CA) and read with the Bio-Plex Systems 200 (Bio-Rad) as previously reported[32]. Samples were analyzed, and standard curves [Log(x) – Linear(y)] were generated using Bio-Plex Manager software (version 6.0; Bio-Rad). Data for cytokine levels were reported not only as concentration, but also as total protein amount. The presentation of concentration data alone may be misleading due to the intrinsic problems of accurate determination of GCF volume[31,33].

Measurement of resorption craters

Extracted teeth were scanned individually with a desktop microcomputed tomography system (SkyScan 1172; SkyScan, Aartselaar, Belgium) from approximately 2mm coronal to the cementoenamel junction to approximately 2mm beyond the root apex. X-radiation was generated at 60kV voltage, 167µA current and 10W power[34]. All acquisition was performed with no filter at a resolution of 17.66µm, 2 × 2 camera binning, a rotation step of 0.15° over 180°, and an exposure

time of 0.59 seconds. Radiographic images were saved in 16-bit tagged image file format (TIFF), and reconstructed into a series of cross-section images using NRecon software (version 1.6.8.0; SkyScan). The cross-section images were visualized in 3-dimensions with VG Studio Max software (version 2.0; Volume Graphics, Heidelberg, Germany), and resorption craters were identified (Fig 2, A and B). A specifically developed convex hull macro for Fiji software35 (a distribution of ImageJ, version 1.48s; NIH, USA) was used to measure the cubic volume of each identified crater (Fig 2, C), which were summed to give a total crater volume for each tooth. One author (J.C.) identified and measured the craters on all teeth, blinded to the force-side or control-side identity of each premolar. For each patient, we divided the total volume of root resorption craters (μm^3) of the force-side premolar by that of the control-side premolar, to account for baseline root surface remodeling[36]. This produced a value that we termed the resorption index (R Index).

Statistical analysis

Data was modelled with a mixed effects model, which examined the interaction between variables nominated as fixed effects and those nominated as random effects. This model allowed random effects to counter the effect of non-independence in the data set due to repeated samples taken per patient per time point. Post-hoc comparisons between patient groups were made simply using a two-tailed t-test ($P < 0.05$), and paired two-tailed t-test ($P < 0.05$) where appropriate. P-values were adjusted for false discovery rate, according to the method described by Holm[37]. Statistical analyses were performed using R statistical computing software (version 3.0.3; R Development Core Team, Vienna, Austria)[38].

4.4 Results

Plaque index, gingival index and probing depths remained low for all patients and were not significantly different between time points per patient. There were 3 patients in Group I (light force), and 2 patients in Group II (heavy force) who had significantly higher R Index values (P = 0.0001). These 5 patients were deemed to have *higher severity* OIIRR (HS), while the other 12 patients were deemed to have *lower severity* OIIRR (LS) (Table I). There were significant differences in the R Index between Group I and Group II, and between HS and LS patients, as shown in Figure 3. The concentrations and total protein amounts of cytokines in GCF were compared between groupings of patients. Comparisons were made between Group I patients and Group II patients, and between HS and LS patients. Comparisons were also made between HS patients and LS patients within Group I or II, and between Group I patients and Group II patients controlling for R Index severity. No statistically significant differences were found between time-points in any comparisons, so GCF data was pooled across all time-points per tooth per patient. Cytokine levels in GCF were interpreted as two data sets: (1) control-side cytokine levels provided background information, and (2) force-side cytokine levels divided by control-side cytokine levels provided information about the effects of therapeutic intervention, similar to the "activity index" described by Iwasaki *et al*[40].

HS patients had less background levels of interleukin-4 (IL-4) (4 to 20-times in concentration, $P <$ 0.001), and more background levels of interleukin-10 (IL-10) (2 to 6-times in concentration, $P \leq$ 0.001) than LS patients. Differences were strongest when Group I HS patients were compared to all LS patients ($P < 0.001$) (Fig 4 and Fig 5). No differences were found when the two Group II HS patients were compared to other patient groupings. Levels of interleukin-13 (IL-13) were higher in the GCF of HS patients under the influence of light (25g) forces (1.8-times in concentration, $P \leq$ 0.004). However, the trend was opposite under the influence of heavy (225g) forces: IL-13 levels

were lower in HS patients (1.8-times in concentration, $P \leq 0.052$). This trend in IL-13 under heavy forces was more statistically significant in terms of total protein amount ($P < 0.001$) than concentration ($P \leq 0.052$) (Fig 6). When cytokine levels were reported as total protein amounts, all the aforementioned trends were corroborated and statistically significant (Figs 4-6).

Heavy forces resulted in less interleukin-2 (IL-2) among LS patients (3-times in concentration, $P < 0.001$, and 1.4-times in total protein amount, $P = 0.027$), and among HS patients (1.9-times in concentration, $P = 0.113$, and total protein, $P < 0.001$) (Fig 7). When data was examined in terms of total protein amount, there were statistically significant trends that were not evident in the concentration data. Under heavy forces in HS patients, interleukin-5 (IL-5) levels were higher and interleukin-8 (IL-8) levels were lower (Fig 8).

4.5 Discussion

Only null hypothesis 2 was not rejected by the present investigation. Force magnitude seemed to affect the levels of certain cytokines collected from GCF. Heavy forces produced higher total crater volumes than light forces, which was in agreement with previous studies[24,39]. There also seemed to be differences in the level of certain candidate cytokines in GCF between HS and LS patients.

A biomarker is defined as a substance that can be objectively measured and evaluated as an indicator of a normal physiologic process, a pathologic process, or a response to therapeutic intervention[41]. To indicate OIIRR, local dentine breakdown products in GCF resulting from the resorptive process have been suggested as potential biomarkers, such as dentine phophoproteins[17], dentin sialoprotein[14,42] and dentin phosphophoryn[14]. These proteins were found at elevated levels in

GCF taken from teeth undergoing orthodontic treatment[42], resorbing deciduous teeth[17] or teeth with more than 2mm of root shortening[14]. Receptor activator of nuclear factor κB ligand (RANKL) and osteoprotegerin (OPG) have been other candidate GCF biomarkers for OIIRR[43]. George and Evans conducted a cross-sectional study to show that both the concentration of RANKL and the RANKL:OPG ratio in GCF of patients with mild and severe OIIRR were significantly higher than in negative untreated controls ($P < 0.05$), implicating RANKL as a possible biomarker for OIIRR[44]. However, because the regulatory role of the OPG/RANKL system is common to both the osteoclast and odontoclast[45], more evidence is required to demonstrate how RANKL concentrations and the RANKL:OPG ratio in GCF can be differentially interpreted to indicate bone remodelling rate during OTM or OIIRR[43].

Cytokines also have potential to be biomarkers of OIIRR, because they are innate to the linked cellular processes of hyalinization, bone resorption and root resorption during OTM[18–22]. There is very limited knowledge about the association between cytokines in GCF and the occurrence of OIIRR. The present randomized prospective clinical trial revealed possible biomarkers of OIIRR. Results showed that there were strong trends of significantly less IL-4 and significantly more IL-10 in the GCF collected from the control teeth of HS patients who had higher R Index values. IL-13 appeared to have opposing trends in HS patients dependent on force magnitude; lower levels under heavy forces, yet higher levels under light forces. IL-5 and IL-8 also showed trends, although weaker, and may also be possible biomarkers of OIIRR. IL-2 levels were lower under heavy force application.

In bone metabolism, IL-4, IL-10 and IL-13 are considered to be anti-inflammatory cytokines. IL-4 and IL-13 are closely related cytokines that inhibit bone resorption by inhibiting the differentiation and activity of osteoblasts[46]. In both an animal[47] and a human model[48], IL-4 and IL-13 induced the expression of OPG, reduced the expression of RANK and RANKL in osteoclast progenitor cells. IL-10 directly inhibits osteoclastogenesis at an early stage, preventing differentiation of osteoclast

progenitors to preosteoclasts[49]. IL-10 has more powerful anti-inflammatory actions than IL-4 due to different mechanisms of suppression of pro-inflammatory mediators[50].

The morphological and functional similarities between osteoclasts and odontoclasts[19,21,45] provide a starting point for understanding how these cytokines may modulate the process of OIIRR in possibly a similar fashion to bone resorption. The present results suggest that IL-4 has a background inhibitory effect on odontoclasts, as it does on osteoclasts. However, IL-10 may have counter-intuitive mechanisms in OIIRR. Due to increased background levels in HS patients, IL-10 either indirectly predisposes HS patients to OIIRR by inhibiting bone resorption to tip the balance towards root resorption, or directly acts to promote the action of odontoclasts. IL-13 levels appeared to be affected in different ways by force magnitude in HS patients. Under heavy forces, IL-13 may directly inhibit odontoclasts (HS patients showed lower levels), but under light forces IL-13 may inhibit osteoclasts (higher levels in HS patients to indirectly worsen OIIRR).

However, it may be too simplistic to interpret the current results using the existing knowledge about the role of cytokines in bone resorption. It is evident that there was a complex relationship between the severity of OIIRR and force magnitude and cytokine levels in GCF. The present study revealed other, albeit weaker, trends in cytokine levels in GCF. IL-2 levels (concentration and total protein amount) tended to be lower under heavy force application, while IL-8 and IL-5 levels (total protein amount only) were lower and higher, respectively, in Group II HS patients. IL-2 stimulates osteoclastic activity[51], and IL-8 is a potent pro-inflammatory cytokine and chemoattractant that recruits and activates neutrophils[52]. Both these cytokines were found to be elevated in GCF during OTM[51]. IL-5 promotes bone formation by mobilizing osteoblast progenitor cells and inhibiting activated osteoblasts[53]. With this knowledge, the currently reported trends, however, were inexplicably opposite to what may be expected. It may be that IL-2, IL-5 and IL-8 in GCF modulate the process of OIIRR differently to bone metabolism and OTM.

Interferon-gamma (IFN-γ) is an exemplar cytokine that appears to be indicative of opposing actions in different biological processes: bone metabolism and periodontal disease. IFN-γ is an extremely potent anti-inflammatory cytokine that inhibits both osteoclast formation and bone resorption[46]. IFN-γ has further anti-inflammatory actions in bone metabolism by inhibiting the activity of pro-inflammatory cytokines IL-1 and TNF-α[54]. However, IFN-γ displayed potent pro-inflammatory actions during periodontal disease[55], and non-surgical periodontal therapy performed to resolve inflammation resulted in decreased levels of IFN-γ found in GCF[56].

Therefore, it may be reasonable to suspect that IL-2, IL-5, IL-8, IL-10 and IL-13 each similarly might have different actions in OIIRR and in bone metabolism. Despite the similarities between osteoclasts and odontoclasts[19,21,45], the current findings suggested that pro-inflammatory and anti-inflammatory cytokines could have different influences on odontoclasts in contrast to osteoclasts. This needs further investigation. IL-4 seemed to be the exception from the current findings: its anti-inflammatory and inhibitory influence may apply to odontoclasts in OIIRR as it does to osteoclasts in bone resorption.

Studies on biomarkers of OIIRR in GCF have used two-dimensional radiographic techniques to measure and stratify patients according to resorption severity[14,44]. These techniques are inaccurate at identifying OIIRR, especially in the early stages[15,16] and if resorptive lesions are located on the buccal or lingual root surfaces[57]. Otherwise, these studies[17,42,58] have investigated root resorption in the context of exfoliation of the primary dentition, instead of the context of orthodontic force application. Future investigations into biomarkers in GCF for OIIRR need to consider these issues in study design, so that the relevant hypotheses can be properly and accurately addressed.

A split-mouth design was chosen for the study because it had the advantage of allowing a small sample size to be used. It was suitable because patient-dependent outcomes were measured, because no carry-across effects were expected, and because any between-site differences were

clinically insignificant or managed through randomization[59]. Micro-CT was chosen as the technique for identifying and measuring OIIRR in the present study. It had the advantage of being able detect microscopic root resorption craters on root surfaces that have no macroscopic signs otherwise[60]. It had superior sensitivity and specificity over periapical radiographs[61] and scanning electron microscopy[39], and is considered to be the criterion standard for assessing OIIRR[61].

Enzyme-linked immunosorbent assay (ELISA) has long been the criterion standard for quantitative analysis of cytokines and biomarkers in medical research[62]. It has been used by preceding studies on biomarkers of OIIRR[14,17,44]. However, it requires large sample volumes, can only measure one analyte at a time, and has low data throughput at high cost[63]. The MBAA technique was chosen for its advantages over ELISA in analyzing cytokine levels in GCF samples. It can analyze small sample volumes, obtain quantitative results, simultaneously detect numerous proteins from low sample volumes, is reproducible, has lower experimental variability and is cost-effective[32,64,65]. MBAA performs just as well as ELISA, and there are generally good to excellent correlations between the two techniques[62], especially if kits using the same capture and reporting antibodies are sourced from the same manufacturer[66].

The current study suffered from limitations that are common to investigations analyzing cytokines in GCF in the context of orthodontics. A small sample size of patients with varying ages and gender was used: age[29] and gender[32] may be variables that affect cytokine levels. There were large fluctuations in the levels of cytokines over time, and the pooling of data performed across time points may be too simplistic, which may weaken the power of analysis[23]. We identified only 2 Group II HS patients, which led to some imbalanced statistical comparisons. Repeated sampling of GCF, which is very technique sensitive[31], was necessary to collect enough volume of sample for analysis. However, from the initial GCF sample to subsequent samples, the protein concentration rises from being comparable to interstitial fluid to being comparable to serum[33]. To counter this effect, we used an established, standardized GCF collection protocol[29] at commonly used time points[23], and

reported data in terms of concentration and total protein amount[31]. A short experimental period was used, and the reported short-term trends in cytokine or biomarker levels may not reflect the entire nature of OIIRR, which can occur over a longer period of time. Although micro-CT had high sensitivity and specificity for measuring OIIRR craters, measurement occurred only at one time point (day 28), and did not account for any remodeling[36] and repair[67] of resorption craters that may have occurred during the experimental period. There was no evaluation of irreversible apical root resorption. Currently, there are no studies that correlate crater volumes measured by micro-CT on the sides of the root to the degree of eventual post-treatment root shortening: the clinical description for the severity of OIIRR[4]. Although significant trends were identified in the current investigation, it was difficult to differentiate which biological processes contributed to and possibly confounded the levels of cytokines in GCF. Apart from OIIRR, possible contributions may have resulted from the process of OTM[23], baseline bone and connective tissue metabolism[68,69], subclinical or clinical inflammation despite good oral hygiene[70,71], or stress[72].

4.6 Conclusions

Over 28 days, background levels of IL-4 and IL-10, and responding levels of IL-13 collected from GCF, in particular, may appear to have specific roles in the process of OIIRR. There were other weaker but statistically significant trends regarding IL-2, IL-5 and IL-8 levels. These cytokines in GCF may each be a potential biomarker of OIIRR. Despite the functional and morphological similarities between osteoclasts and odontoclasts, the current findings suggested that pro-inflammatory and anti-inflammatory cytokines might have similar (IL-4) or different (IL-2, IL-5, IL-8, IL-10, IL-13) influences on the actions of these two cell types. Further investigation is required to elucidate what seems to be a complex relationship between OIIRR severity, force magnitude and cytokine levels in GCF.

4.7 Acknowledgements

The authors would like to thank the Australian Society of Orthodontists Foundation for Research and Education as a major sponsor of this project, and Dr. Banu Aras for her invaluable help during the analysis of the micro-CT data.

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4.9 List of Figures

Fig 1. Experimental setup: **A**, split-mouth design with cantilever spring; **B**, lower occlusal stops; **C**, collection of GCF using Periopaper™ (Oraflow).

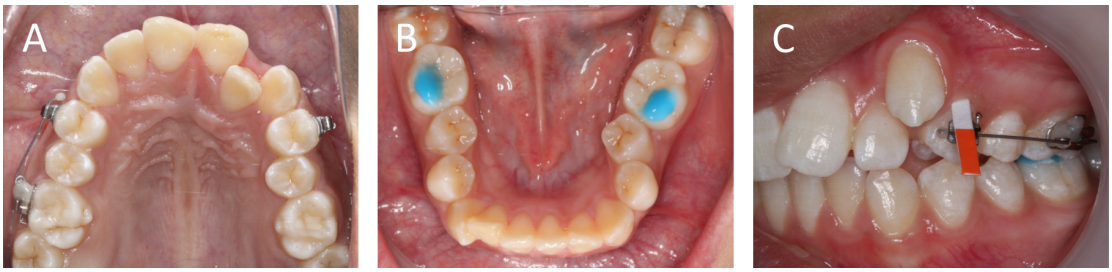


Fig 2. Micro-CT images: **A**, visualization of a maxillary left first premolar; **B**, examination of cross-sections for craters; **C**, cross-section image for measurement of crater volumes.

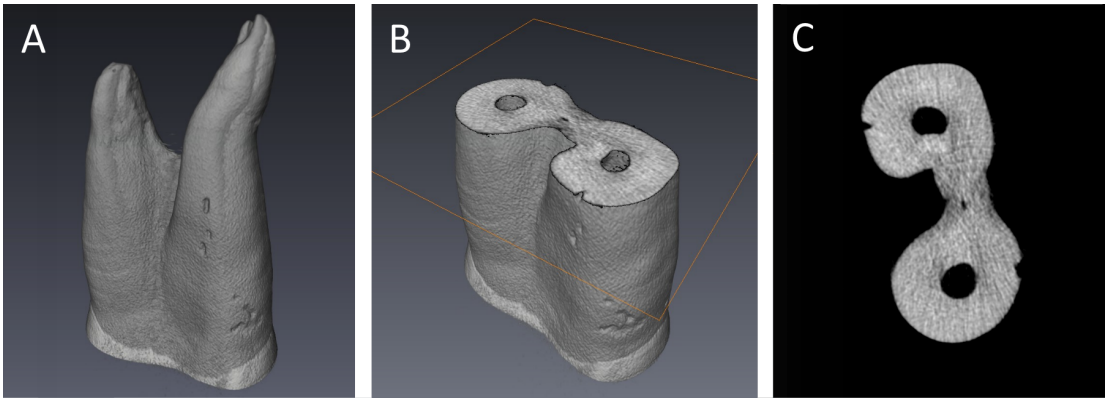


Fig 3. Comparisons of mean R Index values between subgroups using unpaired two-tailed t-tests.

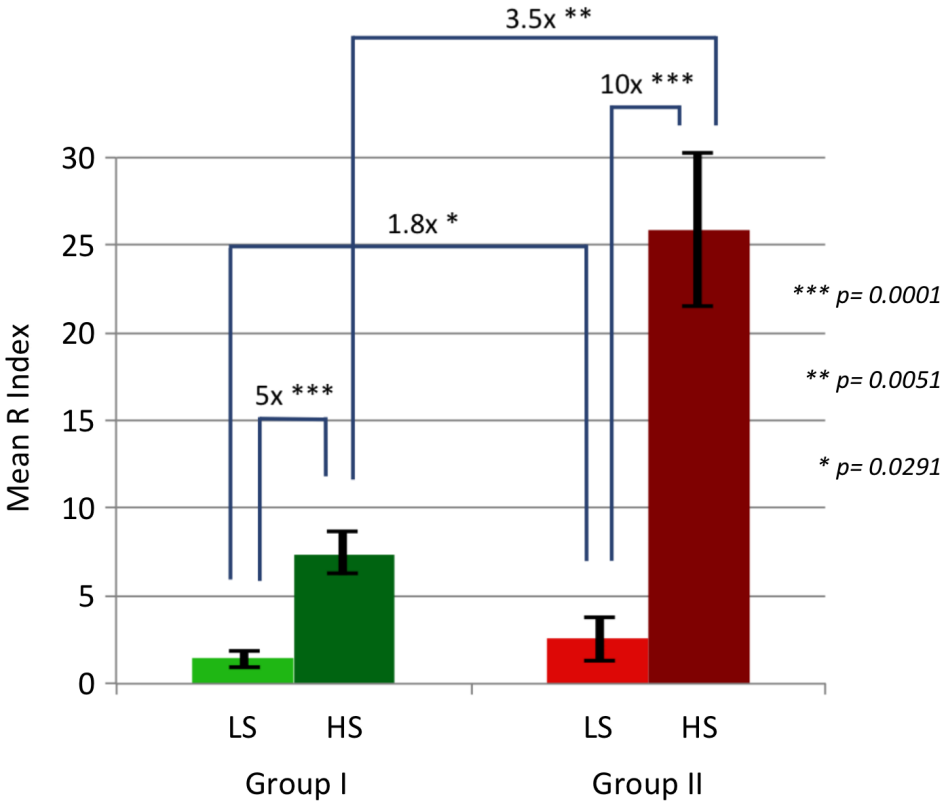


Fig 4. Comparative levels of IL-4 in GCF (control-side data): **A**, concentration; **B**, total protein amount.

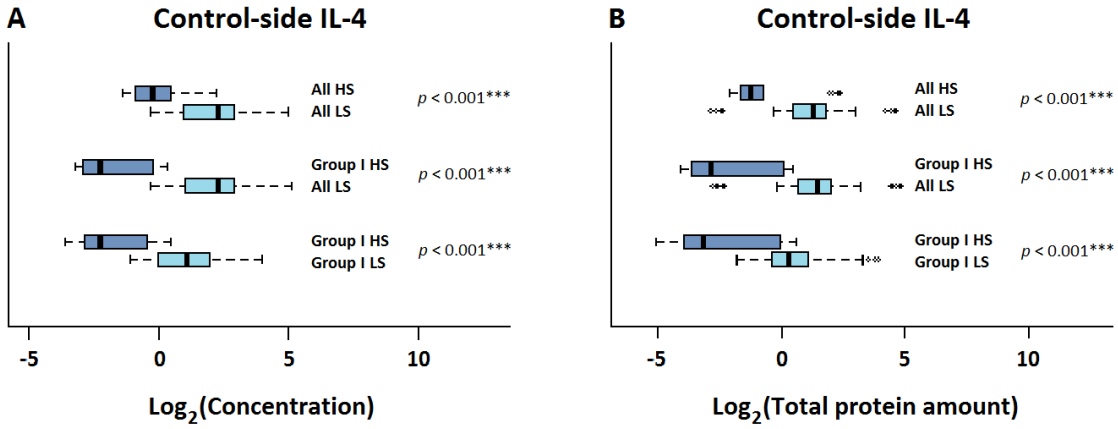


Fig 5. Comparative levels of IL-10 in GCF (control-side data): **A**, concentration; **B**, total protein amount.

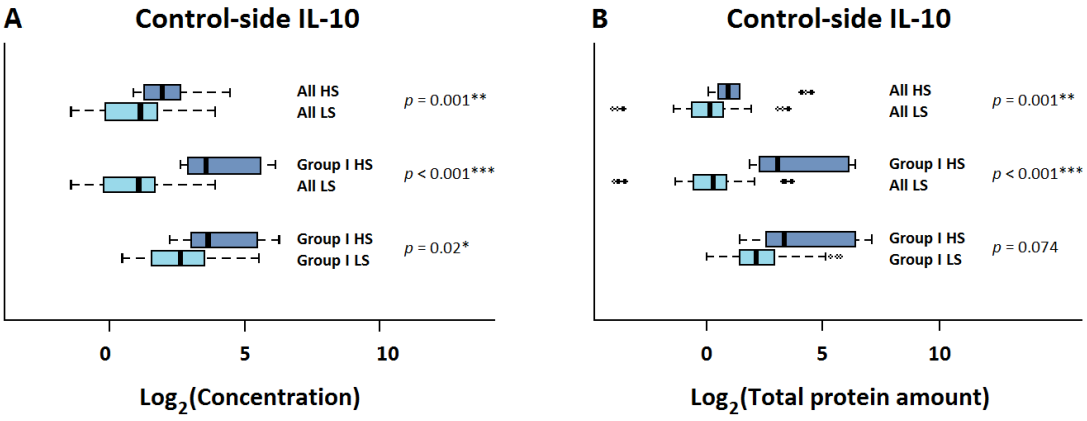


Fig 6. Comparative levels of IL-13 in GCF (force-side/control-side data): **A**, concentration; **B**, total protein amount.

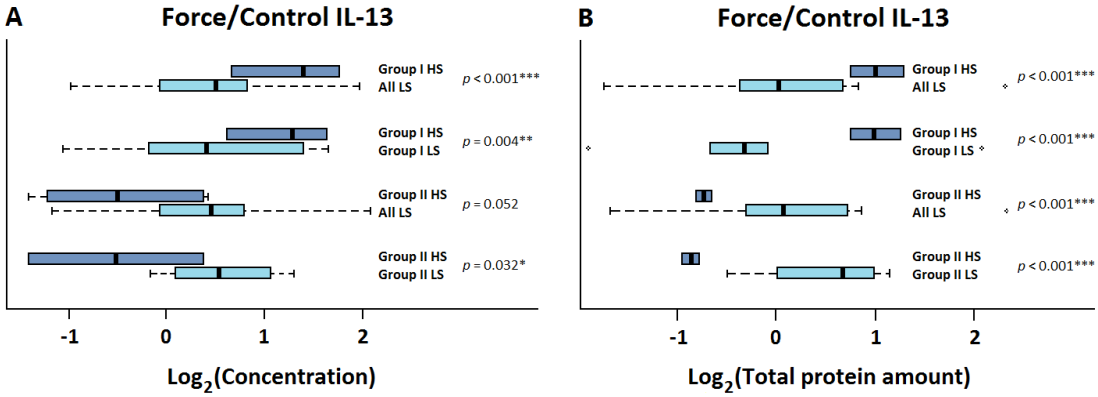


Fig 7. Comparative levels of IL-2 in GCF (force-side/control-side data): **A**, concentration; **B**, total protein amount.

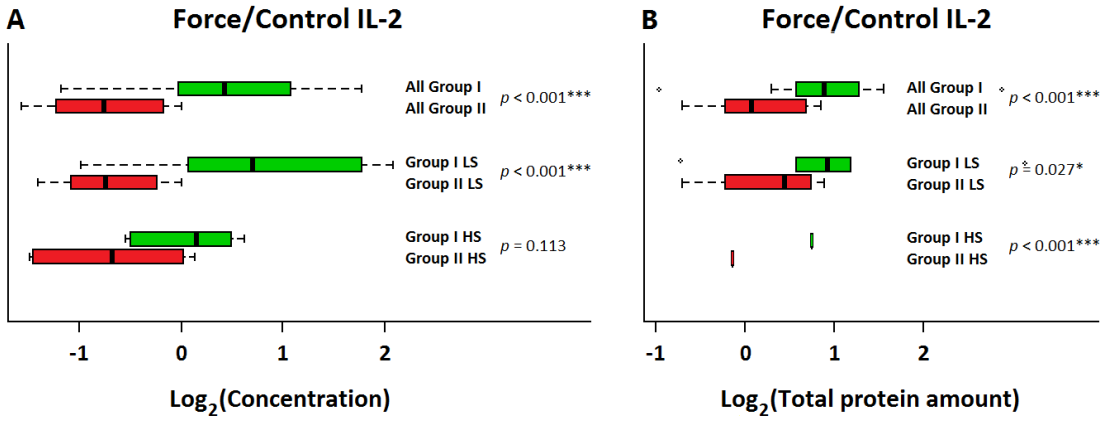
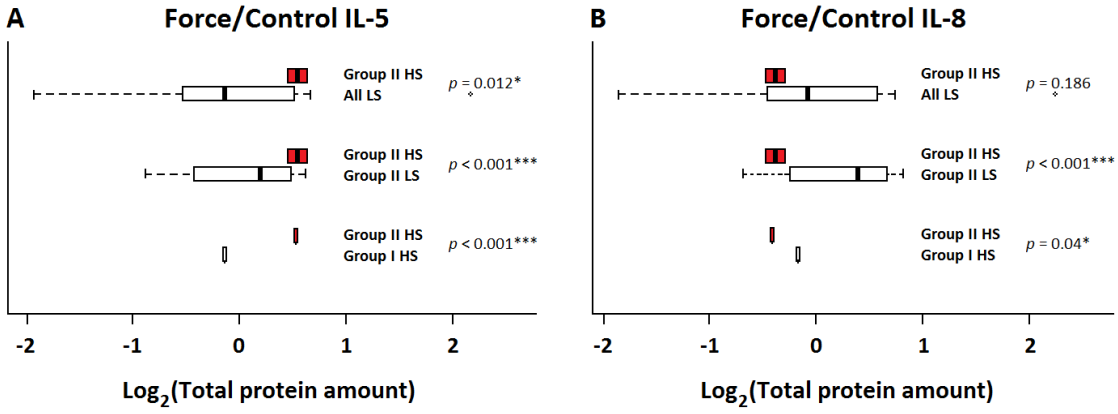


Fig 8. Comparative levels of IL-5 and IL-8 (force-side/control-side data): **A**, IL-5 (total protein amount); **B**, IL-8 (total protein amount).



4.10 List of Tables

Table I. Mean R Index values of each identified subgroup of lower severity and higher severity patients.

Group/severity (n)	Mean R Index	SD	P*
Group I LS (6)	1.47	0.37	0.0263
Group I HS (3)	7.35	0.96	0.0075
Group II LS (6)	2.59	1.01	0.0125
Group II HS (2)	25.86	4.55	0.0819

* Comparisons to a hypothetical R Index value of 1.0 using one-sample t-tests.

5 Future Directions

The present randomized prospective clinical trial suggested that levels of certain cytokines in GCF were significantly related to the severity of OIIRR. Other cytokines in GCF appeared to be related to force magnitude. These cytokines may have specific roles to play in the histopathology of OIIRR and may influence the function of odontoclasts during root resorption. However, the shortcomings of this study cannot adequately reveal the complex relationship between cytokine levels, force magnitude and OIIRR. Therefore, the following recommendations are proposed for future research:

- A larger sample of patients should be recruited in a more powerful study to validate the current findings.
- Immunoassay of GCF samples may be primarily targeted at interleukins IL-4, IL-10 and IL-13. This may reduce the financial cost of proprietary assay kits when fewer cytokines are multiplexed, allowing more patient samples to be analyzed. Interleukins IL-2, IL-5 and IL-8 may be of secondary interest.
- A different split mouth design may be used to directly investigate the effect of force magnitude on cytokine levels in GCF. A light force (25g) should be applied to one premolar, whilst the contralateral premolar should receive a heavy force (225g).
- A short 28-day experimental period as used in the present study may not be adequate to reflect the full event of OIIRR, which occurs over many months or a complete course of orthodontic treatment. A retrospective case-control study could be designed to investigate differences in the resting cytokine levels in GCF between treated patients who experienced severe OIIRR, compared to treated patients without OIIRR as detected from finished OPGs.

- Different constituents in GCF may be targeted for immunoassay, such as local dentine breakdown products. This will avoid the confounding factors contributed by other biological processes that may influence the cytokine composition of GCF.

Pursuit of this research may eventually lead to the development of a rapid chair side test to spot specific characteristic biomarkers that predict patients' susceptibility to OIIRR, and monitor its risk during treatment. More ambitiously, knowledge of GCF biomarkers for OIIRR may also lead to the development of therapeutic measures that can halt the active process of root resorption.

6 Appendices

6.1 Appendix 1: Information for parents / guardians

"Markers of orthodontic root resorption in blood, saliva and GCF and genetic prediction of orthodontic root resorption"

INFORMATION FOR PARENTS / GUARDIANS

Introduction

You are invited to allow your child to take part in a clinical research study that will investigate the changes in chemical composition of blood, saliva and gingival crevicular fluid (GCF) (fluid from the gums) associated with root resorption (a type of damage to tooth roots) when heavy orthodontic forces are placed on teeth. This study is part of an ongoing series of projects on root resorption co-ordinated by the Department of Orthodontics at the Sydney Dental Hospital. The findings from this study will be compared to other related studies performed previously. By following these chemical changes over a few weeks, we hope to develop a simple test to predict whether some patients will experience root resorption more readily than others when they receive orthodontic treatment. This will increase our quality of care and help prevent this damage to patients' teeth during treatment.

The study is being conducted by:

Professor M. Ali Darendeliler, Head, Discipline of Orthodontics, Sydney Dental Hospital

Dr Jenkin Chiu, Orthodontic Registrar, Discipline of Orthodontics, Sydney Dental Hospital

Dr Rajiv Ahuja, Orthodontic Registrar, Discipline of Orthodontics, Sydney Dental Hospital

Mr Torren Carter, Dental Assistant Manager, Department of Periodontics, Sydney Dental Hospital

Study Procedures

Your child is eligible to participate in this study because their orthodontic treatment plan includes the extraction of both of their upper first premolars.

If you agree to allow your child to participate in this study, you will be asked to sign the Parent / Guardian Consent Form. Your child will then be asked to undergo the following procedure before their orthodontic treatment plan begins:

Step 1: Collection of Blood and Saliva

Please do not allow your child to drink tea on the day before their appointment.

Your child will also need to fast (i.e. nothing to eat or drink other than water for 8 hours) prior to their appointment. However, for your convenience, their appointment will be scheduled in the early morning. If they haven't fasted, we will reschedule the appointment.

Your child will have a 33 mL blood sample and a 10 mL saliva sample collected.

Mr Torren Carter will take the blood sample from a vein in your child's arm. Blood collection involves some discomfort at the site from which the blood is taken. There is also a risk of some minor bruising at the site, which may last one to two days.

Step 2: Scaling and Oral hygiene instruction

Prior to beginning the study procedures, it is essential to establish good oral hygiene. Your child's teeth will be cleaned and you and your child will be given oral hygiene instructions. If your child's oral hygiene is not good, they will be excluded from the study and will be returned to the waiting list for Orthodontic treatment.

Step 3: Collection of gingival crevicular fluid

For this, your child's teeth will be rinsed with distilled water, gently air-dried with an air syringe and the collection area will be isolated using a cotton roll. The fluid that is secreted within the gum will be collected from the first premolar on the right and left in the top jaw. This will be done by gently inserting a *thin paper strip* called 'Periopaper' into the gum crevice of the first premolars, up to 1mm and holding it there for 30 seconds and then removing it. A second strip will be placed in the same site for another 30 seconds.

Step 4: Fitting of braces

Braces will then be fitted between the first premolar and first molar, on the left or right side of the top jaw. The opposite side will have braces fitted but without the wire. The braces will be left in place for 4 weeks. A force will be applied to either the upper right or the upper left first premolar tooth. Your child may feel some soreness in that tooth. You will be asked to bring your child to the clinic again *punctually* at the following times after the braces are attached:

3 hours

24 hours

3 days

7 days

4 weeks

At each of these appointments, we will collect GCF and saliva samples, as described above. Additionally, at the 4-week appointment, the braces will be removed and the upper right and left first premolars will be extracted.

The samples collected from your child will be subjected to laboratory analysis to study several factors related to root resorption (root damage). The gingival crevicular fluid will be subject to biochemical analysis to study its constituents. The extracted teeth will be subjected to microscopic radiographic analysis.

Finally, the researchers would like to have access to your child's dental record to obtain information relevant to this study.

From this point onwards, routine orthodontic procedures will take effect.

During the time the appliance is in place, if you or your child notice any changes, such as the braces loosening or coming off, you are asked to contact Dr Jenkin Chiu immediately by telephone. We will fix an early appointment and the braces will be re-attached as soon as possible.

Benefits

While we intend that this research study furthers medical knowledge and may improve treatment of orthodontic patients in the future, it will not be of direct benefit to your child. However, your child will receive comprehensive treatment promptly after their first premolars are extracted.

Costs

Participation in this study will not cost you anything, nor will you or your child be paid.

Voluntary Participation

Participation in this study is entirely voluntary. If your child does take part, you can withdraw them at any time without having to give a reason. Whatever your decision, please be assured that it will not affect your child's medical treatment or your and your child's relationship with the staff who are caring for them. Of the people treating your child, only Professor Darendeliler, Dr Jenkin Chiu, Dr

Rajiv Ahuja and their dental assistants will be aware of your child's participation or non-participation.

Confidentiality

All the information collected from your child for the study will be treated confidentially, and only the researchers named above, Professor Darendeliler, Dr Jenkin Chiu, Dr Rajiv Ahuja and Mr Carter will have access to it. The study results may be presented at a conference or in a scientific publication, but individual participants will not be identifiable in such a presentation.

Further Information

When you have read this information, Professor Darendeliler, Dr Jenkin Chiu or Dr Rajiv Ahuja will discuss it with you and your child further and answer any questions you and your child may have. If you would like to know more at any stage, please feel free to contact them on 02 9293 3388.

This information sheet is for you to keep.

Ethics Approval

This study has been approved by the Ethics Review Committee (RPAH Zone) of the Sydney Local Health District. Any person with concerns or complaints about the conduct of this study should contact the Executive Officer on 02 9515 6766 and quote Protocol No X11-0028.

Version No.: 4

Date: 24.07.2012

6.2 Appendix 2: Information for participants

"Markers of orthodontic root resorption in blood, saliva and GCF and genetic prediction of orthodontic root resorption"

INFORMATION FOR PARTICIPANTS

Introduction

You are invited to take part in a clinical research study that will investigate the changes in chemical composition of blood, saliva and gingival crevicular fluid (GCF) (fluid from the gums) associated with root resorption (a type of damage to tooth roots) when heavy orthodontic forces are placed on teeth. This study is part of an ongoing series of projects on root resorption co-ordinated by the Department of Orthodontics at the Sydney Dental Hospital. The findings from this study will be compared to other related studies performed previously. By following these chemical changes over a few weeks, we hope to develop a simple test to predict whether some patients will experience root resorption more readily than others when they receive orthodontic treatment. This will increase our quality of care and help prevent this damage to patients' teeth during treatment.

The study is being conducted by:

Professor M. Ali Darendeliler, Head, Discipline of Orthodontics, Sydney Dental Hospital

Dr Jenkin Chiu, Orthodontic Registrar, Discipline of Orthodontics, Sydney Dental Hospital

Dr Rajiv Ahuja, Orthodontic Registrar, Discipline of Orthodontics, Sydney Dental Hospital

Mr Torren Carter, Dental Assistant Manager, Department of Periodontics, Sydney Dental Hospital

Study Procedures

You are eligible to participate in this study because your orthodontic treatment plan includes the extraction of both of your upper first premolars.

If you agree to participate in this study, you will be asked to sign the Participant Consent Form. You will then be asked to undergo the following procedure before your orthodontic treatment plan begins:

Step 1: Collection of Blood and Saliva

Please avoid drinking tea on the day before your appointment.

You will also need to fast (i.e. nothing to eat or drink other than water for 8 hours) prior to your appointment. However, for your convenience, your appointment will be scheduled in the early morning. If you haven't fasted, we will reschedule your appointment.

You will have a 33 mL blood sample and a 10 mL saliva sample collected.

Mr Torren Carter will take the blood sample from a vein in your arm. Blood collection involves some discomfort at the site from which the blood is taken. There is also a risk of some minor bruising at the site, which may last one to two days.

Step 2: Scaling and Oral hygiene instruction

Prior to beginning the study procedures, it is essential to establish good oral hygiene. Your teeth will be cleaned and you will be given oral hygiene instructions. If your oral hygiene is not good, you will be excluded from the study and will be returned to the waiting list for Orthodontic treatment.

Step 3: Collection of Gingival Crevicular Fluid

For this, your teeth will be rinsed with distilled water, gently air-dried with an air syringe and the collection area will be isolated using a cotton roll. The fluid that is secreted within the gum will be collected from the first premolar on the right and left in the top jaw. This will be done by gently inserting a *thin paper strip* called 'Periopaper' into the gum crevice of the first premolars, up to 1mm and holding it there for 30 seconds and then removing it. A second strip will be placed in the same site for another 30 seconds.

Step 4: Fitting of braces

Braces will then be fitted between the first premolar and first molar, on the left or right side of the top jaw. The opposite side will have braces fitted but without the wire. The braces will be left in place for 4 weeks. A force will be applied to either the upper right or the upper left first premolar tooth. You may feel some soreness in that tooth. You will be asked to attend the clinic again *punctually* at the following times after the braces are attached:

3 hours

24 hours

3 days

7 days

4 weeks

At each of these appointments, we will collect GCF and saliva samples, as described above. Additionally, at the 4-week appointment, the braces will be removed and the upper right and left first premolars will be extracted.

The samples collected from you will be subjected to laboratory analysis to study several factors related to root resorption (root damage). The gingival crevicular fluid will be subject to biochemical

analysis to study its constituents. The extracted teeth will be subjected to microscopic radiographic analysis.

Finally, the researchers would like to have access to your dental record to obtain information relevant to this study.

From this point onwards, routine orthodontic procedures will take effect.

During the time the appliance is in place, if you notice any changes, such as the braces loosening or coming off, you are asked to contact Dr Jenkin Chiu immediately by telephone. We will fix an early appointment and the braces will be re-attached as soon as possible.

Benefits

While we intend that this research study furthers medical knowledge and may improve treatment of orthodontic patients in the future, it will not be of direct benefit to you. However, you will receive your comprehensive treatment promptly after your first premolars are extracted.

Costs

Participation in this study will not cost you anything, nor will you be paid.

Voluntary Participation

Participation in this study is entirely voluntary. If you do take part, you can withdraw at any time without having to give a reason. Whatever your decision, please be assured that it will not affect your medical treatment or your relationship with the staff who are caring for you. Of the people treating you, only Professor Darendeliler, Dr Jenkin Chiu, Dr Rajiv Ahuja and their dental assistants will be aware of your participation or non-participation. However, your withdrawal from this study will result in your return to the orthodontic waiting list.

Confidentiality

All the information collected from you for the study will be treated confidentially, and only the researchers named above, Professor Darendeliler, Dr Jenkin Chiu, Dr Rajiv Ahuja and Mr Carter will have access to it. The study results may be presented at a conference or in a scientific publication, but individual participants will not be identifiable in such a presentation.

Further Information

When you have read this information, Professor Darendeliler, Dr Jenkin Chiu or Dr Rajiv Ahuja will discuss it with you further and answer any questions you may have. If you would like to know more at any stage, please feel free to contact them on 02 9293 3388.

This information sheet is for you to keep.

Ethics Approval

This study has been approved by the Ethics Review Committee (RPAH Zone) of the Sydney Local Health District. Any person with concerns or complaints about the conduct of this study should contact the Executive Officer on 02 9515 6766 and quote Protocol No X11-0028.

Version No.: 8

Date: 24.07.2012

6.3 Appendix 3: Parent / Guardian Consent Form

"Markers of orthodontic root resorption in blood, saliva and GCF and genetic prediction of orthodontic root resorption"

PARENT / GUARDIAN CONSENT FORM

I, ...[*name of parent/guardian*]

of .....[*address*]

parent/guardian of …….. [*name of child*]

have read and understood the Information for Parent/Guardian on the above named research study

and have discussed the study with .....

I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.

I understand that participation in this study will allow the researchers and others, as described in the Information for Parents / Guardians, to have access to my child's medical and dental records, and I agree to this.

I freely choose to allow my child to participate in this study and understand that I can withdraw him/her at any time.

I also understand that the research study is strictly confidential.

I hereby agree to my child's participation this research study.

NAME OF PARENT/GUARDIAN: .....

SIGNATURE: .....

DATE: .....

NAME OF WITNESS: .....

SIGNATURE OF WITNESS: .....

MASTER Parent-Guardian Consent Form, Version 4, 24/07/2012
SITE SPECIFIC Parent-Guardian Consent Form, Version #, DD/MM/YYYY

6.4 Appendix 4: Participant consent form

"Markers of orthodontic root resorption in blood, saliva and GCF and genetic prediction of orthodontic root resorption"

PARTICIPANT CONSENT FORM

I, ...[*name*]

of .....[*address*]

have read and understood the Information for Participants on the above named research study and

have discussed the study with.....

I have been made aware of the procedures involved in the study, including any known or expected inconvenience, risk, discomfort or potential side effect and of their implications as far as they are currently known by the researchers.

I understand that my participation in this study will allow the researchers to have access to my and dental medical records, and I agree to this.

I freely choose to participate in this study and understand that I can withdraw at any time, in which case I will return to the regular waiting list for orthodontic treatment.

I also understand that the research study is strictly confidential.

I hereby agree to participate in this research study.

NAME: .....

SIGNATURE: .....

DATE: .....

NAME OF WITNESS: .....

SIGNATURE OF WITNESS: .....

MASTER Information for Participants, Version 4, 24/07/2012
SITE SPECIFIC Information for Participants, Version #, DD/MM/YYYY