An *in situ* study to determine the effects of casein phosphopeptide-amorphous calcium phosphate toothpaste in orthodontic patients

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctorate of Dental Science

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Structured Abstract

Aims

To determine the level of demineralisation or remineralisation of sub-surface carious lesions placed *in situ* on an orthodontic appliance and treated with fluoride toothpaste (1450ppm) or a combination of fluoride toothpaste (1450ppm) and GC Tooth Mousse™ (CPP-ACP).

Objectives

To assess the degree of change in mineralisation of subsurface lesions following the application of fluoride toothpaste (1450ppm) and GC Tooth Mousse™(CPP-ACP) with Transverse Microradiography (TMR) as volume mineral loss (ΔZ), lesion depth and lesion width.

Null Hypothesis

There is no difference between the remineralising potential abilities of GC Tooth Mousse™ and fluoride toothpaste in orthodontic patients.

Design

A randomised cross-over *in situ* study, involving the use of prepared enamel subsurface lesions of previously extracted premolar teeth being placed intra-orally. The interventions of fluoridated toothpaste and fluoridated toothpaste in combination with GC Tooth Mousse™ were given to the orthodontic participants in a randomised order with a mid-treatment wash out phase.

Setting

Department of Health Services Research and the Orthodontic Clinic Liverpool University Dental Hospital (LUDH).

Ethical Approval

Ethical approval was obtained from the National Research Ethics Service (NRES) and the NHS Research and Development Offices. This study was given the REC reference number: 13/NW/0742. The trial was also registered on Current Control Trials http://www.controlled-trials.com/ISRCTN04899524/

Sample Size

The primary outcome variable was percentage mineral Loss ΔZ, calculated by dividing the sample value by the control value, and multiplying by 100. Data were used from a previous study (Bryniarska, 2012) using the Transverse Microradiography (TMR) technique. A total of 12 participants would allow detection between groups of 28% ΔZ with 80% power, at the 5% confidence level.

Participant selection

Inclusion criteria

- Between the ages of 12 to 17 years
- Adequate space in the lower premolar region to allow placement of the enamel carrier
- In a suitably rigid archwire to allow placement of the carrier
- In good general health and oral health

Exclusion Criteria

- Allergic to milk products
- Taking or have taken antibiotics in the last 2 months
- Unable to maintain adequate oral hygiene

Randomisation

Each eligible participant was allocated a subject number as they were recruited to the study. Intervention order was randomised by a statistician who was not involved in the recruitment process using simple computer generated random allocation. The participants were randomly allocated to one of two possible orders of intervention; AB or BA.

Method

The study consisted of 4 distinct 4-week phases.

- Phase 1 - Pre-trial wash out
- Phase 2 - 1st Intervention (A or B)
- Phase 3 - Mid-treatment wash out
- Phase 4 - 2nd Intervention (A or B)

All participants were provided with standard fluoride toothpaste and a toothbrush, and asked to brush their teeth for two minutes twice a day with a pea sized amount for the 4-week phase. Intervention B participants were also provided with GC Tooth Mousse™ and advised to apply directly to the teeth for 5 minutes. The participants were asked not to use any other toothpaste or mouth rinses during this trial. The instructions were provided in written form (Appendix 1a, 1b).

Interventions

Each participant received the pastes in random order: AB or BA

A. Standard fluoride toothpaste (1450ppm)
B. Standard fluoride toothpaste (1450ppm) and topically applied Tooth Mousse™ (GC Corporation, Europe) to be applied directly to the teeth

Blinding

All tubes provided to the participants were covered in insulation tape so the participant was unaware of the paste being used. Once the enamel samples were removed from the carrier following the *in situ* phase, samples were recoded by a research technician not involved in the clinical study. The samples were then sectioned and analysed so the principle investigator was blinded to the participant and intervention during analysis therefore reducing bias. Following TMR analysis the coding was revealed.

Samples

Demineralised enamel samples were created *in vitro* from human premolar teeth. Samples were sectioned, sterilised and placed in a carrier, which was then attached to the orthodontic archwire. The carrier consisted of a custom made stainless steel attachment which was a modified version of a carrier used in previous clinical studies (Benson, 2000; Bryniarska, 2012). Base-line TMR analysis was carried out to allow for a comparison of mineral change following the *in situ* phase of the trial.

Outcome measures

The degree of change in mineralisation of subsurface lesions was assessed from mineral content profiles using Transverse Microradiography (TMR) following the application of fluoride toothpaste (1450ppm) and GC Tooth Mousse™. Three parameters were used; mineral loss ΔZ (vol%μm), lesion

depth Ld (µm) and lesion width Lw (µm). This data were then normalised and a percentage change in mineral loss, lesion depth and lesion width was calculated (Strang et al, 1987).

Statistical analysis

The data from TMR analyses was entered into the Statistical Package for Social Sciences (IBM SPSS Statistics v22.0). Descriptive statistics calculations carried out included mean, standard deviation, and percentage mineral loss. Hypothesis testing was carried out using analysis of covariance (ANCOVA), adjusting for the baseline measurements, participant effect and for the order in which the treatments were received. A Pearson correlation coefficient was used to compare the quantity of Tooth Mousse™ used and the resulting change in mineralisation.

Results

1. 3 samples (12%) were lost during the *in situ* phase or damaged during polishing.

2. TMR analysis showed significant remineralisation in both treatment groups compared with baselines, as demonstrated by reductions in mineral Loss ΔZ, lesion depth and lesion width.

3. Mineral loss ΔZ reduced by 15.4% and 24.6% for fluoride and CPP-ACP groups respectively, with a statistical significant difference between these groups (p=0.023). There was no significant effect for the order in which they received the intervention (p=0.760), participant effect (p=0.138) or for baseline mineral loss (p=0.505). There was no correlation between the amount of mineral loss and the amount of Tooth Mousse™ used (r = -0.488, p=0.152).

4. Lesion depth reduced by 1.6% and 11.1% for fluoride and CPP-ACP groups respectively, with a statistically significant difference between these groups (p=0.037). There was no significant effect for the order in which they received the intervention (p=0.202), participant effect (p=0.970) or for baseline mineral loss (p=0.125). There was no correlation between lesion depth and the amount of Tooth Mousse™ used (r = 0.013, p=0.973).

5. Lesion width reduced by 4.5% and 15.3% for fluoride and CPP-ACP groups respectively, with a statistically significant difference between these groups (p=0.015). There was a statistically significant effect for the order in which they received the intervention (p=0.033). There was no significant effect for baseline lesion width (p=0.155) or participant effect (p=0.947). There was no correlation between lesion width and the amount of Tooth Mousse™ used (r=0.306, p=0.389).

Conclusion

In this study, the null hypothesis that there was no difference between the remineralising potential abilities of GC Tooth Mousse™ and fluoride toothpastes was rejected. Despite a wide range in intra-subject and inter-subject data, an overall trend of remineralisation was observed in both treatment groups as demonstrated by reduction in mineral loss ΔZ, reduction in lesion depth (Ld) and reduction in lesion width (Lw). All baseline lesions, on average remineralised during the *in situ* phase regardless of treatment group allocation, however the fluoride toothpaste combined with GC Tooth Mousse™ (CPP-ACP) group demonstrated an increased remineralising effect with respect to all 3 parameters. A statistically significant reduction in mineral loss ΔZ ($p=0.023$), lesion depth ($p=0.037$) and lesion width ($p=0.015$) was observed in the fluoride toothpaste combined with CPP-ACP group compared to the fluoride toothpaste alone.

Results suggest that the application of GC Tooth Mousse™ in addition to regular fluoride paste does have an increasing remineralisation effect on subsurface enamel lesions in orthodontic patients.

Implications

Enamel demineralisation is a common adverse side effect of orthodontic treatment. The use of toothpastes containing CPP-ACP such as GC Tooth Mousse™ has been shown to reduce this demineralisation process and promote remineralisation. CPP-ACP pastes in combination with regular fluoride toothpaste should be recommended for patients undergoing orthodontic treatment who are at high risk of demineralisation or who have demonstrated early signs of white spot lesion formation. CPP-ACP paste may also be useful for patients after fixed appliances to promote remineralisation of areas where orthodontic demineralisation is evident.

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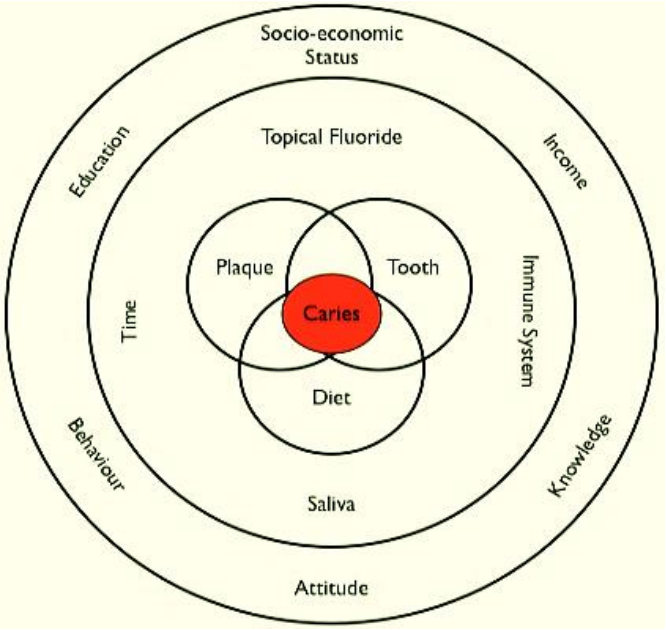
1.0 Background & Rational

1.1 Dental Caries

Definition

Dental caries is the process of localised chemical dissolution affecting hard dental tissue due to metabolic events taking place within the surrounding biofilm. Organic acid production from bacterial fermentation of dietary sugars results in a shift in the dynamic balance of demineralisation-remineralisation with a net loss of tooth substance. This complex process is directly affected by the presence of microbial species, fermentable carbohydrates, fluoride ions, saliva composition and flow rate, buffering capacity and time (Figure 1.1.1). Other contributory secondary factors include education, social class, attitudes and behaviour (Kidd, 2005).

Figure 1.1.1 Factors affecting the carious process (Modified Keyes and Jordan 1963)



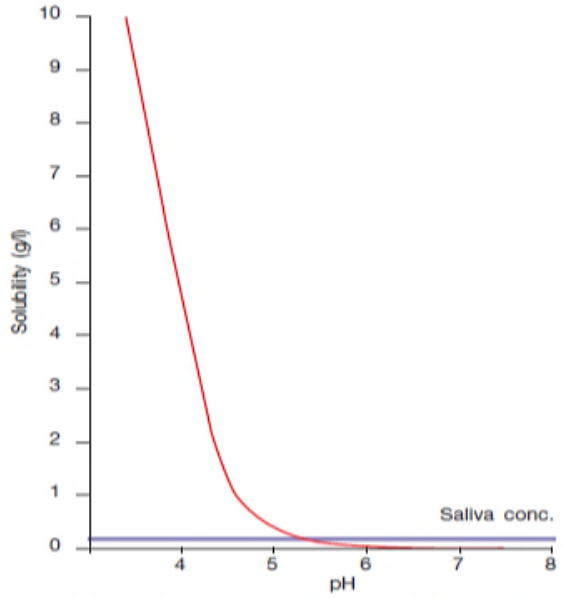
Aetiology of caries

The dental caries process can develop at any tooth site provided the formation and maintenance of a biofilm is achieved. Oral sites which are protected from mechanical cleaning and debris removal are more likely to develop a mature biofilm. Sites such as pits and fissures on occlusal surfaces, and approximal surfaces cervical to contact points are therefore susceptible to stagnation of biofilm and the possibility of localised chemical dissolution. It is within this prerequisite biofilm that the imbalance of tooth mineralisation occurs due to metabolic events and pH fluctuations as a result of microbial activity. This metabolism is dramatically affected by the presence of fermentable carbohydrates (Arens, 1998).

Dental enamel is highly mineralised and acellular with 99% of its dry weight composed of calcium phosphate crystals, and at pH 7.4 hydroxyapatite is the most common form [$Ca_5(PO_4)_3(OH)$]. In normal physiological conditions saliva and oral fluids are supersatured in hydroxyapatite and fluorapatite. This results in mineral deposition on porous areas of teeth seen in developmental enamel maturation and in the remineralisation of carious lesions. This can also give rise to fluorohydroxyapatite and hydroxyapatite formation in calculus deposition. Post dental eruption, tooth surface apatite is continually exposed to chemical modification. A dynamic equilibrium between dissolution and deposition of minerals within the biofilm fluid occurs (Fejerskov and Kidd, 2008).

When pH decreases, the solubility of mineral apatite increases rapidly, with a 10 fold increase in solubility with 1 unit pH reduction (Larson, 1986 Figure 1.1.2). This acid exposure can result in either formation of a carious lesion or an erosion lesion. In pH range 4.0-5.5 the caries lesion develops. This is characterised by partial dissolution of tissue leaving a 20-50 μm intact mineralised outer layer, with a sub-surface demineralised body of the lesion. At this pH range, the oral fluids are undersaturated with respect to hydroxyapatite but remain supersaturated with respect to fluorhydroxyapatite. Therefore subsurface hydroxyapatite is dissolved while fluorhydroxyapatite is formed on the enamel surface layer. It is this fluorhydroxyapatite supersaturation and deposition that maintains the surface layer integrity. In the presence of supersaturated hydroxyapatite and fluorapatite, partially demineralised apatite crystals can remineralise to their original size, and new crystal formation is possible. Although the intact partially demineralised surface layer is accessible to remineralisation, this is not normally possible in the sub surface body lesion due to slower diffusion. The extent of remineralisation is dependent on the presence of fluoride ions, the buffering capacity of saliva and a pH above the critical value. In pH range 2.5-4 oral fluids are undersaturated in both hydroxyapatite and fluorhydroxyapatite, and as a result an erosive lesion develops. This is characterised by complete demineralisation and dissolution of the outer layer, with the underlying remaining enamel unchanged. Once this has occurred remineralisation or replacement of the outer enamel layer is not possible (Silverstone, 1973).

Figure 1.1.2 Curve demonstarting the rapid increase in solubility as pH decresaes (Larson, 1986)

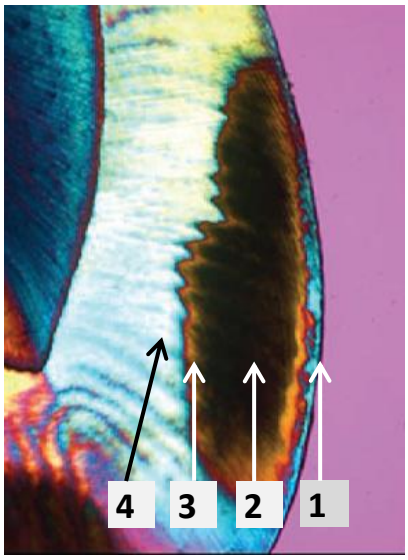


Early Carious Lesion

Early enamel caries lesions are characterised by an intact outer mineral layer with subsurface mineral loss. Although at early stages no macroscopic features are present, microscopically inter-crystalline spaces widen and partial dissolution of the outer enamel surface occurs. A slight increase in enamel porosity is detected at this stage. Lesion progression is associated with increasing inter-crystalline space enlargement and surface porosity. With continued removal of deep subsurface tissue and increasing porosity, a subsurface lesion is formed which is clinically detected as an enamel white spot lesion (Silverstone, 1973).

Histologically this is a wedge shaped lesion with the wider base at the outer enamel surface and the narrow apex toward the amelodentinal junction (Figure 1.1.3). The subsurface lesion can be divided into 4 zones. The surface zone is the intact enamel outer layer, which is well mineralised and is approximately 20-50 µm thick. Below is the body of lesion which is the area of greatest demineralisation with a pore volume of 5% near the periphery and 25% in the centre. This layer makes up the bulk of the lesion. The dark zone is the advancing front of the lesion. It lies deep to the body of the lesion and has a pore volume 2-4% with a reduced level of demineralisation in comparison to the body of lesion. The translucent zone is the deepest layer which is unrecognisable clinically or radiographically and may vary from 5-100 µm in width. It has a slightly increased porosity compared to normal enamel (Fejerskov and Kidd, 2008).

Figure 1.1.3 Polarised light microscopy of an early enamel lesion (Jensen et al , 2000)



1. Surface zone
2. Body of lesion
3. Dark zone
4. Translucent zone

At this stage the white spot lesion is partially reversible and the imbalance between demineralisation and remineralisation can be shifted in favour of mineral deposition. This requires a supersaturated apatite environment, regular fluoride exposure, diet modification and adequate plaque control. Although remineralisation of the outer surface layer is achievable, remineralisation of the body lesion is rarely possible (Silverstone, 1973).

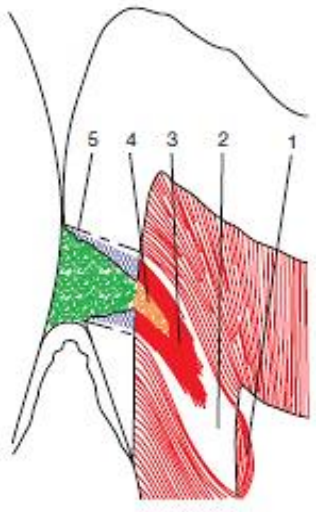
Carious Lesion Progression

Further demineralisation results in progressive advancement of the lesion towards the amelodentinal junction. The first sign of dentine reaction to the enamel lesion is tubular sclerosis at the deepest aspect of the advancing lesion. When the enamel lesion contacts the amelodentinal junction, tubular sclerosis is seen laterally along the junction. Despite previous conventional theories this is unlikely to be lateral spread of dentinal demineralisation and destruction, but merely a pulpodentinal reaction to stimuli (Fejerskov and Kidd, 2008).

As the enamel crystallites progressively dissolve, eventually the outer surface disintegrates and a cavitation is macroscopically present (Figure 1.1.4). At this point the lesion is irreversible. Rapid bacterial invasion occurs with destruction of organic matrix by proteolysis within the most superficial dentine (zone of destruction). Tubular bacterial invasion occurs beneath this zone. As a protective measure by the dentinal tubules, fatty degeneration of the tome's fibres and dentinal sclerosis occurs in an attempt to seal off the tubules. In spite of theses attempts the caries process and destruction continues. Behind this sclerotic layer a narrow zone of decalcification is seen. Initially the tubules have minimal bacterial invasion. With continual decalcification, tubules coalesce and

degrade forming an area of necrotic dentinal debris (Miller's liquefaction foci). Further progression of the carious lesion results in pulpal exposure and subsequent pulpitis (Soames & Southam, 2008).

Figure 1.1.4 Illustration of lesion progression stages



1. Reactive Dentin
2. Sclerotic reaction of translucent zone
3. Demineralisation zone
4. Zone of destruction and bacterial invasion
5. Carious dissolution following peripheral rod direction

Saliva and buffering capacity

The oral environment is continually bathed in saliva. Salivary flow produces effective mechanical cleaning and oral clearance therefore reducing sugar and acid accumulation in the mouth. However saliva's buffering bicarbonate effect also plays an essential protective role. Salivary components; calcium and phospate ions, ensures a supersaturation with respect to hydroxyapatite and offers a protective and reparative environment for the teeth. Calcium and phosphate are present in saliva in a number of different forms. They can be in either its ionised or non-ionised form, or firmly bound to proteins. Non-ionised calcium increases when salivary pH increases as a result of increased saliva flow (Fejerskov and Kidd 2008). In contrast the total phosphate concentration decreases dramatically when salivary pH increases as a result of increased saliva flow.

At pH 5.5 (Critical pH) the ion activity product is equal to the solubility product of hydroxyapatite resulting in a saturated solution, and consequently a balance between remineralisation and demineralisation. However this is a dynamic process. The main determinates of critical pH are the total calcium and phosphate concentrations in the saliva. As this is influenced by salivary flow rates, the critical pH can vary between stimulated and un-stimulated saliva (Fejerskov and Kidd 2008). Antimicrobial salivary constituents include lactoperoxidase, and lysozyme which damage bacterial cell walls and lactoferrin which inhibits bacterial aggregation (Kidd, 2005).

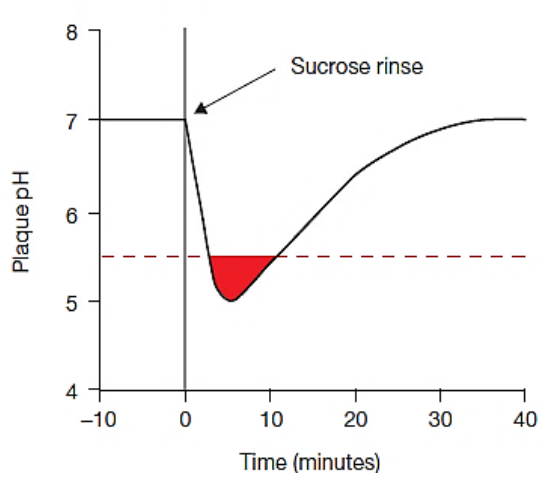
The role of Carbohydrates

There is reliable and conclusive evidence that both the total consumption and frequency of dietary carbohydrate intake is a major cariogenic risk factor. Carbohydrates provide not only the retentive features necessary for bacterial aggregation and colonisation but also act as a substrate for the production of acids. Both the frequency and the duration of carbohydrate consumption are influential factors in this demineralization process (Arens, 1998).

Monosaccharides and disaccharides are small molecular weight carbohydrates, commonly known as sugars. Monosaccharaides such as glucose, fructose and galactose and disaccharides such as sucrose, lactose and maltose are highly cariogenic as they are easily metabolised by bacteria. Complex carbohydrates (starches) are less easily metabolised and have a reduced cariogenic effect. Naturally incorporated intrinsic sugars or milk product sugars are shown to have no adverse effects on dental health (Lingstrom et al 2000, Sheiham 2001).

Sucrose consumption results in a rapid pH reduction below the critical pH within 2-3 minutes (Fig 1.1.5). However it takes the following 40 minutes for the pH to rises gradually and returns to normal. Therefore increased frequency of dietary sugars (especially within 40 minutes) will maintain a reduced pH and a cariogenic environment.

Figure 1.1.5 Stephan curve following sucrose exposure (Modified Stephan and Miller 1943)



Both national and international data have demonstrated a highly significant and strong positive linear correlation between total sugar consumption and caries level (Sreebny, 1982; Miyazaki and Morimoto 1996; COMA report 1989). It has been suggested that 50g of sugar is the maximum daily "safe" limit without increasing caries risk significantly. Similar results have been found in other

studies with a 60g maximum daily limit suggested (Sheiham, 2001). A highly significant and strong positive linear correlation between the frequency of sucrose consumption and caries level has also been clearly demonstrated (Rugg-Gunn, 1993). It has been suggested that a threshold of 4 sugar intakes per day or 3 between meal sugar snacks demonstrated a marked increase in caries activity (Holbrook, 1995).

Although cariology studies clearly demonstrate the association with sugar consumption, it is the low pH environment indirectly generated from dietary sugar consumption which has the more important role in caries development, not the sugar consumption itself (Marsh, 1998).

Microbial species

The microbiology of caries is influenced predominately by the role of *mutans streptococci* within the plaque biofilm, although *Lactobacilli* and *sobrinus streptococci* also play a contributing role (Marsh, 1998). Dietary sugars facilitate the production of insoluble plaque matrix polymers by *mutans streptococci* and *sobrinus streptococci*, which aid initial colonization and retention. The growth of these organisms is dependent on the presence of fermentable monosaccharides and disaccharides which is provided mainly by sucrose and glucose. As *mutans streptococci* are well adapted to low pH conditions, selective pressures caused by acidic conditions causes *mutans streptococci* populations to thrive. Excessive monosaccharides derived from sucrose exposure are metabolised to promote their rapid growth. The result is establishment of *mutans streptococci* colonies on the tooth surface which rapidly metabolise dietary sugars and produce acidic local conditions (Marsh, 1998). Acid production is then rapid, with a minimum pH within 5-10 min after exposure to sugar. As a result of pH falling below the critical level pH 5.5, net loss in surface mineral occurs.

Pellicle layer and biofilm formation

A biofilm is a natural physiological process in which microbial communities colonise, multiply and aggregate with further bacterial species and subsequently embed themselves in a matrix of extracellular polymers. The formation of a pellicle layer is the first stage of biofilm development and therefore an essential pre-requisite for the caries process.

The pellicle layer is a thin bacteria free protein film which forms on tooth surfaces rapidly after mechanical cleaning. Due to its selectively permeable membrane it has an important influence on ion diffusion and therefore influences the demineralisation and remineralisation process. This pellicle layer becomes highly saturated with calcium and phosphate and therefore can act as a

reservoir for remineralisation and inhibit demineralisation (Hay, 1984). Initial microbial colonisation is by bacterial cocci, usually streptococci however as colonisation and development occurs actinomyces species predominate (Law, 2007). The ecological plaque hypothesis suggests that cariogenic bacteria (such as *mutans streptococci*) colonise not only diseased oral sites but also healthy sites. At diseased sites, however the balance is shifted in favour of cariogenic bacteria due to altered environmental conditions (Samaranayake, 2005).

Demineralisation in Orthodontic patients

Demineralisation of enamel adjacent to orthodontic brackets is a widely acknowledged risk of orthodontic treatment. Patients and parents can be disappointed and upset with the appearance of enamel demineralisation following removal of fixed appliances (Benson et al, 2004). The prevalence of enamel demineralisation has been reported as high as 50- 96% of patients (Gorelick et al, 1982; Mitchell, 1992). The most commonly affected areas are the labial surfaces of the maxillary incisors, with the lateral incisor being 3 times more frequently affected than the central incisor (Gorelick et al, 1982). Remineralisation of these lesions is normally expected to occur post debond provided a suitable environment, however Mattousch et al (2007) suggest that adequate remineralisation following fixed appliance removal does not always occur, as demonstrated by the fact that orthodontic participants have a significantly higher incidence of white spot lesions than those who did not undergo orthodontic treatment even 5 years after treatment (Ogaard, 1989). Severe white spot lesions may develop frank cavitation of the enamel surface (Figure 1.1.6) and therefore require restorative intervention (Chang et al, 1997).

This demineralisation process is identical to that of non-orthodontic patients and initially manifests clinically as a white spot lesion due to subsurface tissue loss and increased porosity. However Øgaard and Ten Bosch (1994) suggested that orthodontic white spot lesions may develop and progress more rapidly than conventional smooth surface lesions.

The use of thorough tooth drying allows for early and improved detection of white spot lesions (Gorelick et al, 1982), and after 14 days of undisturbed plaque accumulation enamel changes can be visible. After 3 to 4 weeks, clinical changes can be seen without air drying due to an increased porosity of the outer surface (Fejerskov and Kidd, 2008). The increased susceptibility of enamel to decalcification during orthodontic treatment is due to the irregular surfaces of brackets, bands and wires which increase plaque accumulation and retention. Thorough oral hygiene instruction and dietary analysis and advice are essential to reduce this risk.

Figure 1.1.6 showing severe demineralisation with cavitation (Lovrov, 2007)

White Spot Lesion (WSL) classification

The severity of white-spot lesions can be quantified according to the International Caries Detection and Assessment System II criteria (Pitts, 2004 Figure 1.7). This system was developed to standardise caries quantification in order to allow better comparison among cariology studies.

Fig 1.7 International Caries Detection and Assessment System II (ICDAS II)

Code	Description
0	Sound
1	First visual change in enamel (seen only after prolonged air drying)
2	Distinct visual change in enamel
3	Localized enamel breakdown (without clinical visual signs of dentinal involvement)
4	Underlying dark shadow from dentin
5	Distinct cavity with visible dentin
6	Extensive distinct cavity with visible dentin

Prevention Program

A fundamental role of the dental team is to provide dietary and nutritional advice, as diet is an inextricable factor in oral health and general wellbeing. The information provided by dental professionals should be in accordance with general nutritional recommendations for good health. A six step model for the provision of effective dietary counselling was outlined by Rugg-Gunn and Nunn (1999).

1.2 Caries Prevention

Chlorhexidine

Chlorhexidine (CHX) has been studied for over 30 years as an antimicrobial agent that controls plaque formation and therefore may prevent caries. Chlorhexidine acts bacteriostatically and covers both Gram-negative and Gram-positive bacteria. The efficacy of chlorhexidine is related to its concentration and the frequency of application (Baca and Junco, 2003).

The most common applications of chlorhexidine are mouth rinses, sprays, gels and varnishes. Chlorhexidine varnishes are the most effective at inhibiting *mutans streptococci, followed* by gels and then mouthwashes (Emilson 1994, Law and Seow 2007). Chlorhexidine mouth rinse has demonstrated efficacy in reducing plaque and gingivitis, as well as in decreasing the microbial load in saliva and the gingival sulcus (Escribano et al, 2010).

Clinical trials have demonstrated that chlorhexidine varnish and gels result in a clinically significant reduction in caries of up to 40% (Banting et al, 2002, Huizinga et al, 1990). The addition of fluoride varnish in combination with chlorhexidine varnish has been shown to be a useful, simple, quick and non-invasive method for the control and management of existing root caries lesions (Brailsford et al, 2002). However this has been contradicted by Edwards (2009) who concluded that regular chlorhexidine application does not have a substantial effect on the preservation of sound tooth structure.

A systematic review (Slot et al, 2011) concluded that in the absence of professional cleaning and oral hygiene instruction, chlorhexidine varnish may provide a beneficial effect in patients in need of special care. However the strength of this recommendation was graded as weak.

Ozone

Ozone (O_3) is a powerful oxidising agent which neutralises acids and also disrupts the cell wall of microorganisms (Bocci, 1993). Baysan et al (2000, 2004) reported ozone application for 10-20 seconds was effective in killing 99% of microorganisms in carious lesions, and was also effective in reducing *mutans streptococci* levels in vitro. However more recent in vitro testing showed that ozone had no effect on remineralisation of carious lesions (Zaura et al, 2007). While laboratory studies suggest promising potential for ozone in dentistry, this has not been shown in clinical studies (Rickard, 2004). Ozone should not be considered an alternative to current treatment methods in dental practices (Rickard, 2004). More high quality blinded randomised clinical trials are required to evaluate the possible use of ozone as a treatment modality in dentistry (Azarpazhooh and Limeback, 2008).

1.3 Caries Remineralisation with Fluoride

Non-cavitated carious White Spot Lesions (WSL) can be identified at different developmental stages. When lesions are detected and contained early, interceptive treatment may reduce the need for invasive treatment. Both topical fluoride and Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) have been widely studied to investigate their effectiveness in the remineralisation of enamel subsurface lesions.

Fluoride has an essential role in dental caries prevention and enamel remineralisation. Fluoride is a natural trace element which is present in certain foods and in many water supplies. Fluoridated water at 1ppm concentration provides both a systemic and topical effect and can reduce caries by 50% (Arnold, 1957).

Pre-eruptive effects of fluoride are due to its incorporation into the enamel crystal lattice structure resulting in formation of fluorapatite which has a larger crystal size and improved stability. Fluorapatite has a critical ph 3.5, making it less soluble in comparison to hydroxyapatite. Fluoride incorporation also affects crown morphology by reducing pits and fissures depth and therefore reducing plaque stagnation (Welbury et al, 2005). Post-eruptive effects result in an increased remineralisation of porous and demineralised enamel areas. Fluoride ions are absorbed on to the tooth surface which attracts calcium and phosphate ions required for new mineral deposition.

Fluoride also has a local antibacterial effect on dental plaque bacteria by blocking the enzyme enolase necessary for gycolysis. Bradshaw et al (1990) reported that *mutans streptococci* become increasingly sensitive to fluoride ions as the pH falls. Therefore it is suggested that routine topical application of fluoride may partially inhibit the metabolism of cariogenic bacteria.

The topical intra-oral effect of fluoride in caries prevention is most beneficial at a constant or high frequency low concentration dose as opposed to an irregular high concentration dose (Ogaard, 1990). Therefore the goal of community based public health programmes should be to implement the most appropriate method of maintaining a constant low level of intra-oral fluoride exposure (Peterson, 2003).

Fluoride Toothpaste

The regular use of fluoridated toothpaste has been shown to result in a 25% reduction in caries, compared with non-fluoridated toothpastes (Marinho et al, 2003). Brushing twice a day with fluoride toothpaste compared to once a day has been reported to reduce caries incidence by 20-30% (Chesters et al, 1992). The recommended amount of fluoride in toothpaste for adults and adolescents is 1400ppm (Welbury, 2005). High fluoride dentifrices have been shown to be particularly effective in remineralisation of root caries lesions, due to the increased fluoride requirement for root remineralisation compared with enamel remineralisation (Lynch and Baysan, 2001). A recent randomised controlled trial evaluated the effectiveness of high fluoride toothpaste (5000 ppm) compared with regular concentration fluoride toothpaste (1450ppm) in reducing white spot lesions formation during orthodontic treatment. They concluded that there was a statistically significant benefit of using high fluoride toothpaste resulting in a relative risk reduction of 31% (Sonesson et al, 2014).

Walsh (2010) suggested the benefits of using fluoride toothpaste in preventing caries in children was only significant for fluoride concentrations of 1000 ppm and above. This relative caries preventive effect increases with higher fluoride concentrations. For children under 6 years deciding what fluoride levels to use should be balanced with the risk of fluorosis (Wong, 2010).

Fluoride Varnish

Topical fluoride varnish has been shown in randomised controlled trials to have a statistically significant effective on remineralisation of incipient carious lesions (Autio-Gold, 2001; Fure and Lingström, 2009). Over a 4 year period, fluoride varnish application twice a year resulted in a 40% reduction in caries (Zimmer, 1999). Marinho et al (2013) in a review of the literature concluded that fluoride varnish gel, resulted in a substantial caries reducing effect of approximately 30-40% DMFT. It is generally accepted that for maximum efficiency, fluoride varnish should be applied at least every six months (Weintraub et al, 2006). A placebo double blind RCT (Stecksén-Blicks et al, 2007) assessed the effectiveness of fluoride varnish (0.1% F) application in preventing white spot lesion formation during orthodontic treatment. They concluded that application every 6 weeks resulted in a 70% reduction of white spot lesions formation.

An updated Cochrane review (Benson et al, 2013) established that there is some moderate evidence that fluoride varnish applied every six weeks was effective in reducing demineralised white spot lesion formation in orthodontic patients. But that further adequately powered, double-blind, randomised controlled trials are required to determine the best means of preventing demineralised white spot lesions in patients undergoing orthodontic treatment.

Fluoride Mouthwash

A review of the literature suggested that fluoride mouthwash is effective in caries remineralisation with a DMFT reduction of approximately 25% in children (Marinho et al, 2003). A Cochrane review (Benson et al, 2004) recommended the daily use of 0.05% Fluoride mouth rinse for all patients undergoing orthodontic treatment, although this was based on weak evidence.

Extensive and sometimes conflicting research on the most appropriate topical and systemic methods of fluoride delivery has been carried out. Systematic reviews have concluded that fluoride toothpastes, mouth rinse, varnishes and gels are effective in reducing caries activity regardless of fluoridated water exposure. Fluoridated toothpastes are suggested to be the most practical vehicle for fluoride delivery due to high compliance. However the caries reduction effect of fluoridated toothpaste can be compounded by additional topical fluoride exposures such as mouth rinses and varnishes (Marinho et al, 2008).

The effectiveness of fluoride releasing orthodontic materials has also been demonstrated. A systematic review assessing orthodontic bracket adhesives suggested that fluoride releasing glass ionomers may reduce local decalcification (Benson et al, 2004). Fluorides releasing elastomeric modules have also been suggested as an effective method of reducing orthodontic demineralisation (Banks et al, 2000).

Fluorosis and toxicity

An adverse side effect of excessive fluoride exposure is fluorosis. Fluorosis is a dose related response to excess fluoride ingestion during pre-eruptive tooth development resulting in hypo-mineralisation of enamel and formation of surface and subsurface porosities. The extent can vary from mild enamel translucencies to deep pitting of enamel (Figure 1.3.1). The York Review (McDonagh et al, 2000) found that at 1ppm fluoridation of water, 12.5% of the population developed fluorosis resulting in an aesthetic concern. The critical exposure time for fluorosis is between 1 – 4 years, with a minimal risk after the age of 8 years.

Figure 1.3.1 Demonstrating severe Fluorosis (Yildiz and Celik, 2013)

Fluoride toxicity can occur due to highly excessive systemic ingestion. Adverse gastrointestinal effects can occur at levels of 1mg/kg with symptoms including nausea, vomiting, abdominal pain and diarrhoea. Acute toxicity resulting in hyperkalemia can cause convulsions, cardiac arrest and respiratory failure, with lethal toxic doses at 32-64 mg/kg. This is equivalent to ingestion of 3.3 70g tubes of toothpaste for a 60kg adolescent (Welbury et al, 2005; Baltazar et al, 1980).

1.4 Caries Remineralisation with CPP- ACP

Casein phosphopeptide-amorphous calcium phosphate has been suggested for use in caries prevention since the 1980's and as a mineralising agent since the 1990's (Reynolds, 1998). A group of peptides, Casein Phosphopeptides (CPP) have been shown to stabilise and preserve calcium and phosphate ions as nanoclusters in a stable solution known as Amorphous Calcium Phosphate (ACP). ACP is highly soluble and readily converted to Hydroxyapatite (HA) and is therefore a suitable mineralising agent (Tung and Eichmiller, 1999).

Previous difficulty in using calcium and phosphate ions in remineralisation was due to their low solubility especially in the presence of fluoride. Therefore soluble calcium and phosphate ions are not easily incorporated into dental plaque or localised at the tooth surface at high enough concentrations to promote adequate diffusion required for remineralisation. Subsequently CPP-ACP was developed with CPP stabilising high concentrations of calcium and phosphates together with fluoride by binding to plaque and the pellicle layer at the tooth surface (Reynolds, 2009).

CPP-ACP mechanism of action

Calcium phosphate reservoir

Anticariogenic activity has been attributed to the ability of CCP to localise ACP at the tooth surface thereby increasing calcium phosphate levels in the dental plaque (Reynolds, 1997).

Bacterial adhesion

CPP-ACP may inhibit cariogenic streptococci bacterial adhesion to tooth surface therefore inducing a non-cariogenic plaque formation (Schupbach et al, 1996).

Antibacterial

CPP-ACP competes with calcium for calcium plaque binding sites resulting in a highly extracellular free calcium concentration which may have bactericidal or bacteriostatic effects along with an additional anti-plaque effect (Rose, 2000).

Prevent demineralisation and improved remineralisation

CPP-ACP has been shown to remineralise enamel subsurface lesions at a rate of 1.5 – 3.9 x 10^{-8}mol hydroxyapatite m^{-2} s^{-1} (Reynolds, 1997). CPP-ACP can stabilise 100 times more calcium phosphate than neutral pH solutions. The formation of hydroxyapatite in the lesion produces phosphate and acid, which diffuses out of the lesion. CPP-ACP consumes the acid generated during enamel lesion remineralisation and generates more calcium and phosphate ions required for diffusion into the lesion. CPP-ACP has been shown to inhibit caries activity by a dose related response (Reynolds 1995, 1998). Enamel lesions which have remineralised after topical exposure to CPP-ACP are less susceptible to future acid exposure due to improved crystallinity and lower micro-strain than normal dental enamel (Iijima et al, 2004).

Tooth Erosion

CPP-ACP may prevent tooth erosion by a combination of suppressing demineralisation and enhancing remineralisation. Studies have shown reduced levels of enamel erosion in enamel previously exposed to CPP-ACP (Rees et al, 2007).

CPP-ACP and Fluoride

Plaque enzymes such as phosphatases and peptidases can cause partial degrading of CPP-ACP products. The addition of fluoride to CPP-ACP limits this phosphatase action (Vitorino et al, 2005). Fluoride interacts with CPP-ACP to produce a stable amorphous calcium fluoride phosphate (ACFP) nanocluster at the tooth surface. CPP-ACFP may provide a source of soluble calcium, fluoride and phosphate that is more resistant to pH changes. A CPP molecule can adhere to 25 Calcium ions, 15

phosphate ions and 5 fluoride ions making it an effective delivery system (Cross et al, 2005). When fluoride is incorporated into CPP-ACP paste, it has been shown to further promote remineralisation and enhance caries resistance of root surfaces (Garcia-Godoy et al, 2009).

Cochrane (2008) proposed that at pH levels below 5.5, CPP-ACP solutions containing Fluoride (CPP-ACFP) produced greater ion diffusion activity resulting in an increased remineralisation effect. Reynolds et al (2008) concluded that a dentifrice with 2% CPP-ACP and 1100ppm F was superior to all other CPP-ACP and fluoride combinations. However fluoride has been shown to interact with ACP and may precipitate out as calcium fluoride resulting in both inorganic components being ineffective (Azarpazhooh and Limeback, 2008). Recent studies suggested that CPP-ACP paste containing 900ppm Fluoride has minimal improvements in remineralisation over regular CPP-ACP pastes or regular fluoride pastes alone (Beerens et al, 2010; Vanichvatana et al, 2013).

CPP-ACP evidence

Early *in situ* studies evaluating CPP-ACP for remineralisation demonstrated promising results. Cai et al (2003) carried out a randomised, double blind *in situ* study on remineralisation of enamel subsurface lesions using sugar-free lozenges containing CPP-ACP. CPP-ACP lozenges of over 18.8 mg were effective in promoting remineralisation of early non-cavitated lesions. Shen et al (2001) in a similar *in situ* study concluded that sugar-free chewing gum containing 18.8mg or more resulted in statistically significant increases in enamel remineralisation relative to a control chewing gum. Reynolds (1998) also suggested that exposure to CPP-ACP 1mg solutions twice a day resulted in a reduction in enamel mineral loss of 51% compared to the control.

However, more recent *in vitro* studies evaluating the remineralisation effect of GC Tooth Mousse™ (which incorporates CPP-ACP) concluded that there was no statistically significant difference in remineralisation efficacy of GC Tooth Mousse™ compared to saliva or water (Lovel, 2008; Benson, 2009; Bichu et al, 2013). It was acknowledged however that an *in vitro* model may not be the most appropriate setting and the lack of a biologically active biofilm may be the reason for the limited remineralising ability demonstrated. It was recommended that future *in situ* and *in vivo* randomised control trial may be more appropriate.

A number of QLF (Quantitative Light-Induced Fluorescence) studies assessing *in vivo* post-orthodontic remineralisation of white spot lesions have demonstrated that Tooth Mousse™ has a beneficial and statistically significant remineralising effect (Bailey et al, 2009; Atkin et al, 2012; Vashisht et al, 2013). However a similar study found that although increased remineralisation of

white spot lesions occurred with CPP-ACP cream, this was not significantly more effective than with regular fluoridated toothpaste (Brochner et al, 2011).

A recent TMR *in situ* randomised controlled trial assessed the remineralisation of subsurface lesions in orthodontic patients during treatment using GC Tooth Mousse™ (Bryniarska, 2012). It was concluded that there was no statistically significant difference in remineralisation noted. However a large number of samples were lost in this study and this may have affected any conclusions which could be drawn. Further *in situ* randomised controlled trials were recommended with an increased emphasis on procedural planning to reduce loss of samples during the *in situ* phase and the TMR sectioning and polishing phase. Another *in situ* randomised controlled trial compared the remineralisation abilities of GC Tooth Mousse™, GC Tooth Mousse Plus™, and a range of fluoride concentration solutions. They found that only GC Tooth Mousse™ and GC Tooth Mousse Plus™ provided high concentrations of stabilised, bioavailable calcium, phosphate and fluoride ions in saliva. This enhanced bioavailability produced a statistically significant increase in remineralisation of enamel subsurface lesions *in situ* compared with fluoride solutions (Shen et al, 2011).

A recent systematic review of the literature (Li et al, 2014) concluded that CPP-ACP was clinically proven as a long-term remineralising agent and its enhanced remineralisation effect was comparable to that of fluoride. However the advantages of fluoride as a supplement to CPP-ACP is still unclear and further high quality randomised controlled trials were recommended.

Tooth Mousse™ (GC Corporation, Europe)

Tooth Mousse™ is a water based, sugar free dental topical cream containing Recaldent™ CPP-ACP (10% w/w). The manufacturers of Tooth Mousse™ recommend usage of the product in a number of situations;

- an acidic oral environment,
- active decay, presence of white spot lesions
- sensitive teeth, erosion or tooth wear
- during tooth whitening procedures
- during orthodontic treatment
- dry mouth and dehydration
- suffering from morning sickness during pregnancy
- medically compromised patients

Picture 1.4.1. Tooth Mousse™ (GC Corporation, Europe)

Tooth Mousse™ Application

Direct Application

1. After brushing your teeth, apply a sufficient amount to the tooth surface using a clean dry finger or cotton tip.
2. Leave on the tooth surface undisturbed for a minimum of 3 minutes.
3. Then use your tongue to spread the applied crème throughout the mouth.
4. Hold in the mouth for a further 1-2 minutes. The longer GC Tooth Mousse™ and saliva are maintained in the mouth, the more effective the result.
5. Expectorate thoroughly and if possible avoid rinsing. Any GC Tooth Mousse™ remaining on the tooth surface can be left to gradually dissipate.
6. Do not eat or drink for 30 minutes following application.

Tray Application

1. Before use, clean the custom tray thoroughly under running water.
2. Apply a sufficient amount of cream into the tray and sit the tray in place.
3. Leave the tray undisturbed in the mouth for a minimum of 3 minutes.
4. Remove the tray, and then use your tongue to spread the crème throughout the mouth.
5. Hold in the mouth for a further 1-2 minutes. The longer GC Tooth Mousse™ and saliva are maintained in the mouth, the more effective the result.
6. Expectorate thoroughly and if possible avoid rinsing. Any GC Tooth Mousse™ remaining on the tooth surface can be left to gradually dissipate.
7. Do not eat or drink for 30 minutes following application.

1.5 Methods of study design

Different study models can be used to study the process of enamel demineralisation

- *In vitro*
- *In vivo*
- *In situ*

In vitro

In vitro (Latin for "In Glass") refers to an experiment in which the effects of various biological entities are being performed outside of the normal biological context. This would involve dental samples, whether human or animal, being tested in the laboratory setting.

The advantages of an *in vitro* model are that it may be relatively inexpensive and less time consuming. Due to the laboratory setting, destructive quantification techniques may be utilised such as TMR which would otherwise be prohibited in the *in vivo* setting. Another advantage of an *in vitro* setting is that experimental conditions can be closely controlled and monitored, compared to *in vivo* studies which may suffer from a wide range of confounding environmental variables. Also *in vitro* studies may benefit from less restrictive ethical considerations compared to *in vivo* and *in situ* models (White, 1995).

The main disadvantage however of *in vitro* systems are that they do not accurately replicate the real life biological situation. Caries is a complex process and it is unlikely that a laboratory setting can ever fully replicate the *in vivo* scenario. It is extremely difficult to simulate the microbiology environment and the effect of salivary flow and salivary composition. Demineralisation and remineralisation rates are faster *in vitro* than *in vivo* due to an increased uptake and reactivity of fluoride (White, 1995).

Both bovine and human enamel systems can be utilised to simulate the *in vivo* process of demineralisation. Although bovine enamel is more readily available than human enamel, a major disadvantage is that bovine enamel is softer and more porous than human enamel and therefore demineralises more readily. Human enamel, where applicable should be seen as the gold standard. Human enamel is more representative of the true biological situation and therefore the trial results should be more applicable and relevant (Mellberg, 1992).

In Vivo

In vivo (Latin for "within the living") refers to experiments in which the effects of various biological entities are carried out within the living organism. This would involve assessing the carious process in the patient's normal oral environment on living dental tissue.

Non-destructive techniques are normally utilized such as QLF (Quantitative Light-Induced Fluorescence), unless dental extractions are deemed necessary as part of the orthodontic treatment plan. In such a case an *in vivo* caries model may involve monitoring of the demineralisation-remineralisation process on healthy dental tissue followed by extraction and quantitative analysis.

The key advantage an *in vivo* model is that the teeth are in their most natural state in the natural biological oral environment and therefore the trial results should be more applicable and relevant.

The major disadvantage of destructive *in vivo* techniques is patient recruitment. There is often a limited availability of suitable patients with suitable teeth which require extractions. Also this system is only applicable for the early stages of treatment, as any increased period of observation will result in delayed extractions and therefore increasing the overall treatment time for the patient. Therefore this model is unsuitable to observe enamel changes over a longer duration of time (Mellberg, 1992).

In situ

*In situ (*Latin for "on site") refers to experiments in which biological systems are attempted to be reproduced or simulated in the living organism. This would involve the use of appliances or other devices which create defined conditions being placed in the oral cavity to simulate and analyse the dental caries process (Zero, 1995). This is seen to be an amalgamation between the *in vitro* study and the *in vivo* clinical trial.

The *in situ* caries analysis technique usually involves a specimen of human or bovine enamel being placed in a customised holder which is then attached intra-orally. The use of a baseline control sample derived from the same tooth will allow for comparison with the *in vivo* sample.

The advantage of an *in situ* model is that it takes place in the oral environment and therefore replicates many of the complex biological aspects of the carious process which can not be replicated by *in vitro* studies. As a result we benefit from simulation and analysis of the natural caries process without causing irreversible damage to the patient. With adequate study design it should be possible

to control experimental variables and reduce bias. The possibility of destructive analysis allows for improved scientific analysis with an increased reproducibility and validity.

In situ trials are generally of a shorter duration, thereby reducing the ethical implications and financial restrictions experienced by longer term clinical trials. Usually the *in situ* model can be utilised at various stages of orthodontic treatment so therefore will not hinder or restrict the patients typical orthodontic treatment progress (Zero, 1995).

A major disadvantage however of the *in situ* model is that the techniques involved are clinically very demanding and require skilled analytical expertise. The number of patients is usually restricted due to the extensive resource and time requirements. Patient compliance is crucial and loss due to follow up is a common problem (Zero, 1995).

Randomised Controlled Trials

A randomised controlled trial (RCT) is a prospective interventional study, designed to assess the effects of one or more interventions when compared with a control. This control group can either receive no treatment, a placebo, or the best currently available standard of care. Commonly in cariology studies fluoride is the control remineralisation agent. A major advantage of a RCT design is that they reduce the influence of bias and confounding factors.

Bias

Bias is defined as any factor or process that causes systematic deviation of the trial results or conclusions away from the truth (Jadad, 1998). There are three main types of bias that can occur in clinical trials; selection bias, instrument bias and observer bias. Selection bias is a systemic error in the patient recruitment process which results in a non-representative sample. In randomised controlled trials this is minimized through an effective recruitment and randomisation procedure. Instrument bias is a systematic error in measurement of the outcome or statistical analysis which can reduce the accuracy and reproducibility of the results. In cariology studies this can be minimised by utilising accurate mineral analysis techniques such as Transverse Microradiography.

Randomisation

Randomisation ensures all study participants have an equal chance of being allocated to any group within the study. Random allocation ensures that baseline characteristics of the groups are comparable. It is possible to balance the groups for known factors which affect treatment outcome, however this is not possible for unknown confounding factors**.** A confounding factor has a

relationship with both the potential cause and the outcome but is not involved in the causal pathway (Bruce et al, 2008). These are often unknown and can either under estimate or over exaggerate results of the study. Randomisation ensures that unknown confounding factors that may influence the study are kept to a minimum.

Blinding

Blinding of the participant and/or the investigator reduces the risk of bias in a study. A single blind trial is where the participant is unaware of the treatment allocation. A double blind trial is where the participant and the investigator are both unaware of the treatment allocation. This ensures that the investigators behaviour and approach is not influenced by the knowledge of what treatment the participant is receiving. It is however difficult to blind the investigator, particularly in dental studies where there may be an obvious difference in the treatments provided. It is important in cariology studies to ensure the investigator is blinded during the data analysis stage. This is achieved by Coding of samples so the investigator is unaware of the treatment group allocation. This is sometimes referred to as a triple blind trial.

Cross-over trials

A cross-over trial is a longitudinal study in which participants receive a sequence of different treatments at different time periods. Cross-over trials enable each participant to act as their own control, allowing for inter and intra-participant comparison. Cross over designs are often utilised in *In situ* demineralisation studies as this allows for statistically and clinically significant results with fewer participants (Jadad, 1998). A wash out period between treatments is essential to eliminate any carry-over effects.

1.6 Methods of Mineral Evaluation

The caries process can be diagnosed and monitored by assessing the amount and change of mineral content within the tooth. Quantitative methods of analysis allow for accurate measurement of small changes in individual lesions and assessment of lesion progression (Ten Bosch and Angmar-Mansson, 1991).

Quantitative Light-Induced Fluorescence (QLF)

QLF is a non-destructive diagnostic device used for early detection of demineralisation in enamel and dentine. QLF utilises the differential fluorescence of a low fluorescent white spot lesion in comparison to highly fluorescent healthy enamel. Blue light (370 nm) is directed towards the tooth which excites the enamel and causes it to fluoresce in the yellow-green region. A yellow high-pass filter (540 nm) is used to remove the blue portion of the light. This fluorescence can then be observed using an intraoral camera. If an enamel lesion is present, an increase in light scattering is observed relative to the surrounding enamel. This change in fluorescence between sound enamel and the lesion is measured using a computer program. By using a reconstructed algorithm of fluorescence radiance for healthy enamel and the actual fluorescence radiance of the demineralised lesion, the percentage change can be calculated and therefore the relative mineral loss (De Josselin de Jong, 1995).

QLF has been shown to be sensitive and reproducible. Elton et al (2009) suggested that QLF is highly effective for measuring the subsurface loss of mineralisation but is less effective at measuring lesion depth. QLF ability to measure demineralisation has been shown to be comparable to destructive TMR technique (Lovel, 2008). It has therefore been suggested as an appropriate method for *in vivo* quantification of early smooth surface caries (Pitts, 2009).

QLF imaging has been shown to be an effective *in vivo* method to detect and monitor levels of enamel demineralisation during orthodontic treatment. It has also been suggested for use as an effective visual oral hygiene aid, providing improved analysis and awareness of plaque accumulation levels (Miller, 2014).

Transverse Microradiography (TMR)

TMR is a destructive technique which requires cuts to be made to the specimen and microsamples removed for analysis. TMR is based on the measurement of x-ray absorption by a tooth section compared with absorption by a simultaneously exposed standard (Ten Bosch and Angmar-Mansson, 1991). 80µm sections of the enamel sample are cut perpendicular to the anatomical tooth surface

and placed on high-resolution photographic film along with an aluminium calibration stepwedge and irradiated with monochromatic x-rays. Absorption of X-rays by the tooth sample and stepwedge directly affect the optical density of the developed film. This optical density is used to calculate mineral content. Dedicated software and image analysis systems using a video camera (CCD) are then used to evaluate microradiograph optical densities and to generate mineral content profiles. Three main parameters are obtained from the analysis of mineral content profiles,

- Mineral loss ΔZ (vol%µm)
- Lesion depth L_d (µm)
- Lesion width L_w (µm)

Mineral loss is the difference between the mineral content of sample and that of the sound enamel. Lesion depth and lesion width values are both determined from the mineral distribution. The accuracy of TMR for mineral loss ΔZ is 200 vol%µm and lesion depth (L_d) is approximately 5 µm. (Arends & Ten Bosch, 1992). However incorrect measurements can be created if the lesion is not uniform.

A major disadvantage of the TMR process is that it requires destruction of the sample and therefore limiting its use in the *In vivo* study design (Elton and Cooper, 2008). Technical difficulties include handling and sectioning of small enamel samples and in creation of the required parallel sections. Other limitations include an inability to measure less than 10 µm from the anatomical surface due to the inherent curvature of the samples (White et al, 1992). Despite these limitations TMR is generally accepted as the gold standard in direct quantification of mineral change. A comparison of TMR results with QLF is routinely used for validation.

1.7 Summary

Orthodontic demineralisation is a common adverse side effect of orthodontic treatment. The evidence base for the use of CPP-ACP in enamel remineralisation is limited and conflicting. This study set out to determine the remineralising properties of Casein phosphopeptide amorphous calcium phosphate toothpaste in orthodontic patients using an *in situ* design.

2.0 Trial Aims and Objectives

2.1 Aims

To determine the level of demineralisation or remineralisation of sub-surface carious lesions placed *in situ* on an orthodontic appliance and treated with fluoride toothpaste (1450ppm) or a combination of fluoride toothpaste (1450ppm) and GC Tooth Mousse™.

2.2 Objectives

To assess the degree of change in mineralisation of subsurface lesions following the application of fluoride toothpaste (1450ppm) and GC Tooth Mousse™ with Transverse Microradiography (TMR) as volume mineral loss (ΔZ), lesion depth and lesion width.

2.2 Null Hypothesis

This study will test the null hypothesis that there is no difference between the remineralising potential abilities of GC Tooth Mousse™ and normal fluoride toothpastes in orthodontic patients. Therefore there will be no statistically significant difference between volume mineral loss (ΔZ), lesion depth and lesion width.

3.0 Trial Design

3.1 Ethics

Ethical approval was obtained from the National Research Ethics Service (NRES) and the NHS Research and Development Offices. This study was given the REC reference number: 13/NW/0742. The trial was also registered on Current Control Trials http://www.controlled-trials.com/ISRCTN04899524/.

3.2 Trial Design

The design was a randomised cross-over *in situ* study. This involved the use of prepared enamel lesions of previously extracted premolar teeth being placed *in situ*. The interventions of fluoridated toothpaste and fluoridated toothpaste plus GC Tooth Mousse™ were given to the orthodontic participants in a randomised order with a wash out phase constituting a cross-over study.

3.3 Trial Setting

The trial setting was the Health Services Research Department and the Orthodontic Clinic Liverpool University Dental Hospital (LUDH).

3.4 Sample size calculation

The primary outcome variable was percentage mineral loss ΔZ , calculated by dividing the sample value by the control value, and multiplying by 100. Data were used from a previous study (Bryniarska, 2012) using the Transverse Microradiography (TMR) technique. From this study the standard deviation of the data between the test and control groups was 50. A sample size of 12 participants would allow detection of a difference of 45% ΔZ between the two groups, with 80% power, at the 5% significance level. However this previous study had fundamental procedural difficulties which resulted in incomplete data sets and this may have resulted in more variable data than would be expected. If the standard deviation in this study was reduced to 30, then this would allow detection of a difference of 28% ΔZ between the two groups with 80% power, at the 5% significance level.

4.0 Selection and withdrawal of participants

Participants entering the trial were orthodontic patients taken from the Liverpool Dental Hospital waiting list, which therefore includes only patients assessed and deemed suitable for NHS treatment or for training purposes. This selection bias may unfortunately reduce the representativeness of the sample population compared to the overall population. The selected patients were those who were undergoing fixed appliance orthodontics. All participants were required to provide informed consent both written and verbal.

4.1 Inclusion criteria

The participants were required

- To be between the ages of 12 to 17 years
- To have adequate space in their lower premolar region to allow placement of the carrier
- To be in a suitable rigid archwire to allow placement of the carrier
- To be in good general health and oral health

4.2 Exclusion criteria

The participants were excluded if

- They were allergic to milk products
- They were taking or had taken antibiotics in the last 2 months
- They were unable to maintain adequate oral hygiene

Patients recently taking antibiotics were excluded as this can significantly alter the oral micro-flora, with Streptococcus strains particularly affected (Edlund and Nord, 2000).

4.3 Withdrawal of participants

Participants were removed from the study at any stage if they withdrew consent at their request. If for any reason the patient required their fixed appliance to be removed due to a clinical decision separate to this study, then they would also be removed from the study.

4.4 Consent

At a routine orthodontic appointment, eligible patients were invited to participate in the study. Participant information leaflets were provided to the parents and the children in 'plain English' explaining the purpose of the study (Appendix 2a, 2b). If the participant was under 16 years old they were provided with an assent form and the parent/guardian with a consent form (Appendix 3a, 3b). If the participant was 16 years or over they were provided with a consent form (Appendix 3c).

4.5 Participant compliance

Intervention A and B pastes were weighed before being provided to the participant. The participants were requested to bring the tube of paste/s with them on their review appointment. The tubes were then re-weighed to assess the amount used in the four-week period, and therefore give a suggestion of compliance.

4.6 Randomisation procedure

Each eligible participant was allocated a subject number as they were recruited to the study. Intervention order was randomised by a statistician who was not involved in the recruitment process using simple computer generated random allocation (Table 4.6.1). The participants were randomly allocated to one of two possible orders of intervention; AB or BA.

Table 4.6.1 Participant number and randomised order of interventions

Participant	Intervention	Intervention
1	B	A
2	B	A
3	B	A
4	A	B
5	A	B
6	B	A
7	B	A
8	A	B
9	A	B
10	B	A
11	A	B
12	A	B

5.0 Trial Procedures and Methodology

5.1 Investigational medicinal products

The interventions were as follows;

A. Standard fluoride toothpaste (1450ppm).
B. Standard fluoride toothpaste (1450ppm) and topically applied Tooth Mousse™ (GC Corporation, Europe. 10% w/w CPP-ACP) to be applied directly to the teeth.

'Tesco Toothpaste 75Ml' was chosen as the standard fluoride toothpaste due to the minimal ingredients, and additives present. The ingredients of Tesco Toothpaste Ingredients are listed as : Aqua, Sorbitol, Hydrated Silica, Sodium Lauryl Sulphate, Aroma, Cellulose Gum, PEG-32, Sodium Fluoride, Sodium Saccharin, Hydroxyethylcellulose, Limonene, CI 77891. Contains Sodium Fluoride 1450ppm.

The ingredients of Tooth Mousse™ (GC Corporation, Europe) are listed as: Pure water, Glycerol, CPP-ACP, D-sorbitol, Silicon Dioxide, CMC-Na, Propylene glycol, Titanium dioxide, Xylitol, Phosphoric acid, Guar gum, Zinc Oxide, Sodium Saccharin, Ethyl p-hydroxybenzoate, magnesium oxide, Butyl p-hydroxybenzoate, Propyl p-hydroxybenzoate.

5.2 Visit by visit example

The trial consisted of four distinct 4 week phases, including a pre-trial wash out period and a mid-treatment wash out period. Each participant received both treatment interventions in a randomised order. An example of the sequence is outlined below for allocation AB.

- Visit 1-Pre-trial wash out (4 weeks)

  Routine orthodontic exam

  Participant provided with standard fluoride toothpaste, toothbrush and instructions

- Visit 2-Treatment A (4 weeks)

  Routine orthodontic exam

  Carrier with enamel specimen attached to lower arch wire

  Treatment A paste is given to participant with instructions on its use

- Visit 3-Mid- treatment wash-out (4 weeks)

  Routine orthodontic exam

  Carrier with enamel specimen removed and sent for analysis

  Participant provided with the standard fluoride toothpaste and instructions

- Visit 4-Treatment B (4 weeks)

  Routine orthodontic exam

  New carrier with enamel specimen attached to lower arch wire

  Treatment B pastes are given to participant with instructions on its use

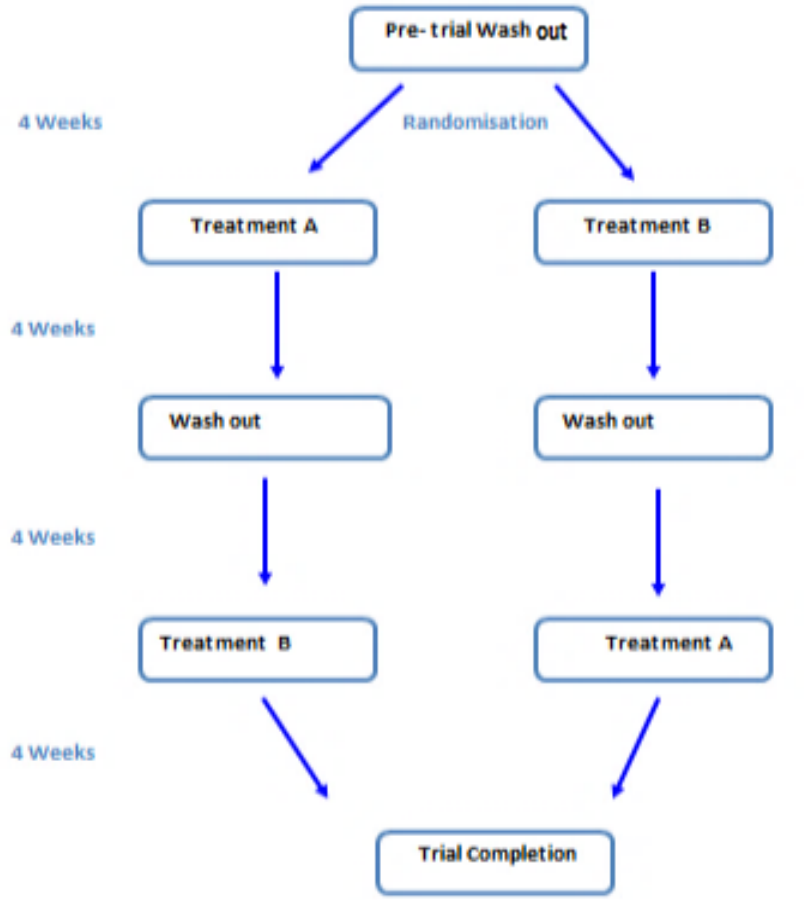
- Visit 5-Trial Competition

  Routine orthodontic exam

  Carrier with enamel specimen removed and sent for analysis

All participants were provided with the standard fluoride toothpaste and a toothbrush and asked to brush their teeth for two minutes twice a day with a pea sized amount for the 4-week phase. Intervention B participants were also provided with Tooth Mousse™ and advised to apply directly to the teeth for 5 minutes. The participants were asked not to use any other toothpaste or mouth rinses during this trial. The instructions were provided in written form (Appendix 1a, 1b).

5.3 Trial Flow Chart



5.4 Duration of trial

The duration of the clinical trial per participant was 16 weeks. 4 weeks pre-trial wash out, 4 weeks for the 1st intervention (A/B), 4 weeks mid treatment wash out and 4 weeks for the 2nd intervention (B/A). The total duration of the clinical trial was approximately 9 months as this was dependent on participants being at an adequate orthodontic stage to allow carrier placement.

5.5 Blinding

All tubes provided to the participant were covered with opaque insulation tape (Duck Original White Tape 50, ShurTech Brands Avon, UK) so the participant was unaware to the paste being used. Once the enamel samples were removed from the carrier following the *in situ* phase, the samples were recoded by a research technician not directly involved in the study. The samples were then sectioned and analysed so the principal investigator was blinded to the participant and intervention during analysis therefore reducing bias. Following TMR analysis the coding was revealed.

5.6 Creation of artificial subsurface lesions

The creation of artificial carious lesions was similar to that outlined by Benson (2009) and Bryniarska (2012). Non-restored human premolar teeth extracted for orthodontic purposes, were collected and stored in distilled water containing thymol, thereby inhibiting bacterial growth (Sikkema et al, 1995). The teeth were visually examined to confirm the absence of any stains, caries or cracks. The teeth were also examined under QLF imaging for increased detection of surface irregularities. Selected teeth were then lightly abraded with fine 1.200-grit abrasive paper (English abrasives bP320A, English abrasives and Chemicals, UK) to remove the outermost enamel and remnants of the pellicle from the buccal surface.

Teeth were then varnished with acid resistant nail varnish (Max Factor, Nailfinity, Weybridge, UK) except for a window approximately 7mm by 3mm on the buccal surface where the demineralized lesion was to be created. Teeth were then mounted on glass rods using greenstick compound and immersed into an acid buffer demineralising gel at room temperature as described by Edgar (1983) (Figure 5.6.1). This was produced by mixing 0.1M lactic acid (Merck, UK) and 0.1M sodium hydroxide (BDH, UK) until a pH of 4.5 was reached. 6% (w/v) hydroxyethylcellulose (SigmaAldrich.Co.Ltd, UK) was added and mixed with an electric mixer (Kenwood, UK) to give a final gel consistency similar to that of “wallpaper paste”.

Figure 5.6.1 showing placement of mounted teeth into demineralising gel

The teeth were submerged in this demineralising gel for a period of 5-7 days. At this stage they were removed, and visual examination was used to detect if a deminerlalised lesion was present.

QLF imaging was also utilised to confirm the presence of an even demineralised subsurface lesion (Figure 5.6.2) but also to ensure that any lesions of poor quality or uneven distribution were identified and excluded (Figure 5.6.3). If no lesion had developed the teeth were submerged back in the demineralisation gel for another 2-3 days and then re-anlaysed.

Figure 5.6.2 showing evenly demineralised lesion under white light and QLF

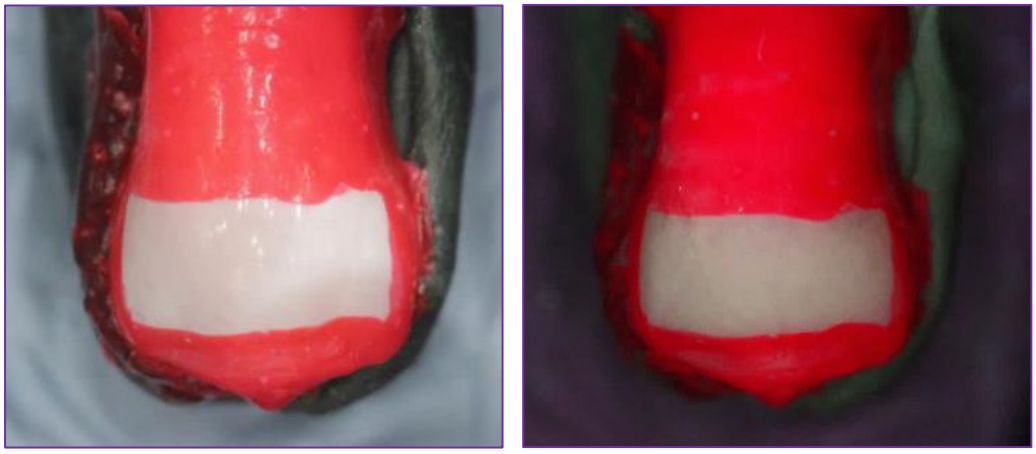
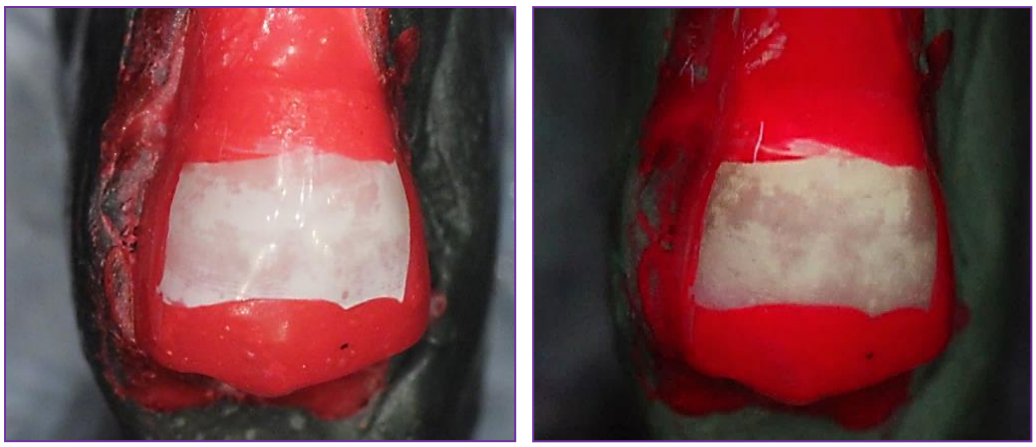


Figure 5.6.3 showing unevenly demineralised lesion under white light and QLF



Adequately demineralised teeth were withdrawn from the gel, rinsed thoroughly with distilled water, gently blotted dry and left to air dry. The block of enamel containing the lesion was then cut from the crown with a margin of sound enamel using a diamond disc (Skilldental, High Wycombe, UK. The lesion was then mounted on green stick and divided into 3 sections; 1 control section and 2 experimental sections using a diamond wire saw (Well, Walter Ebner, Le Locle, Germany),(Figure 5.6.4 + 5.6.5).

Figure 5.6.4 Tooth mounted on green stick ready to be sectioned with the diamond wire saw

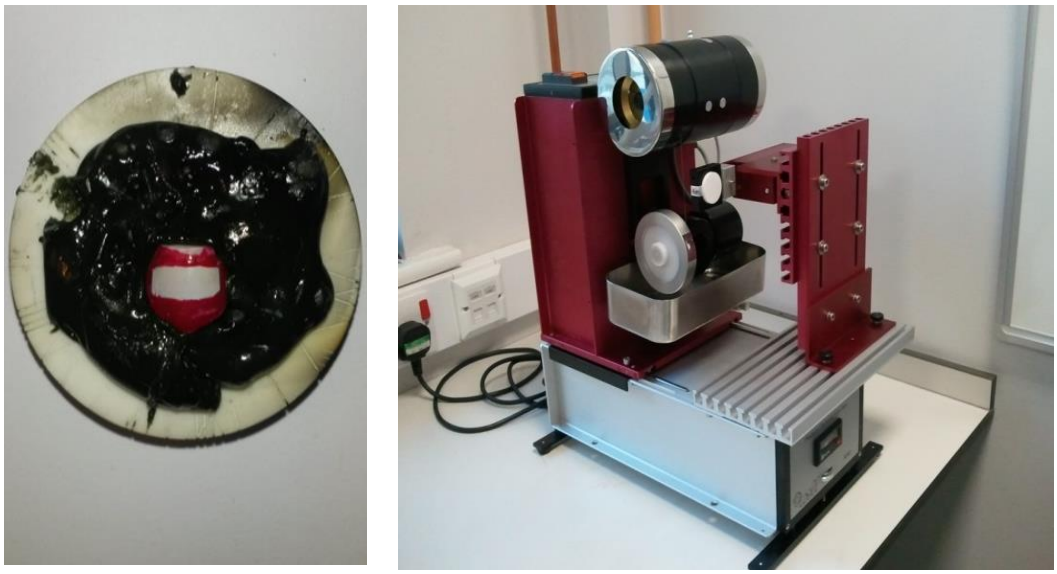
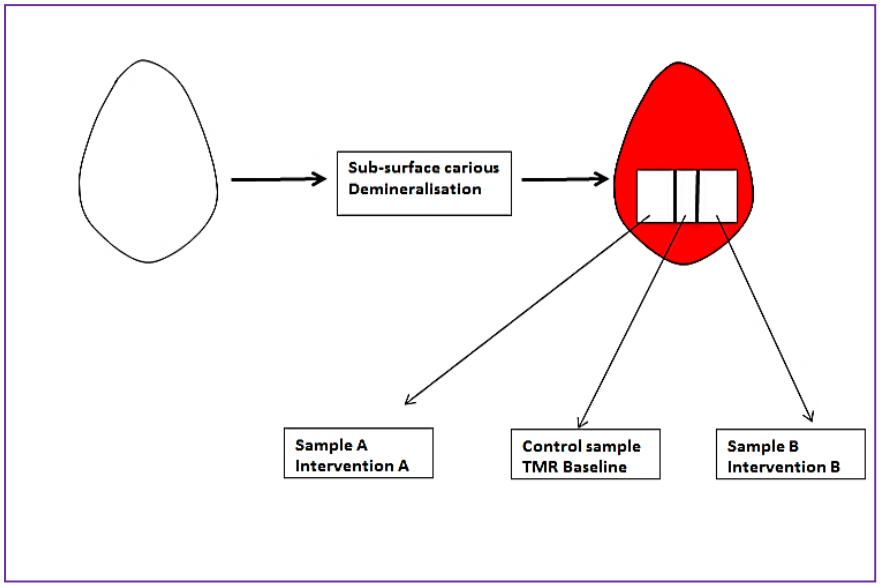


Figure 5.6.5 Diagrammatic representation of Enamel lesion sectioning



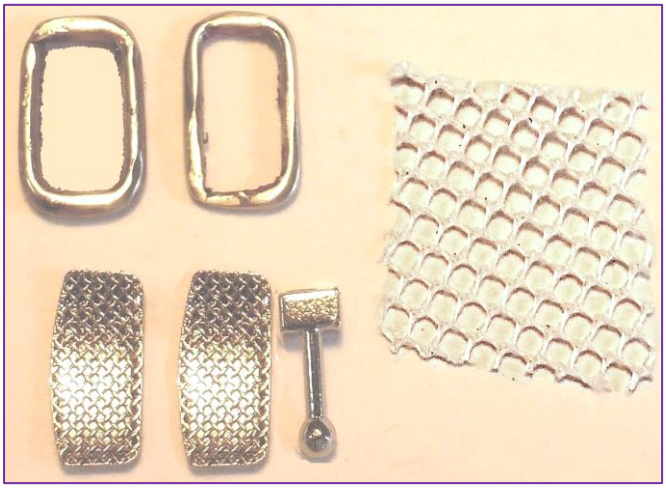
The control section had 2 TMR slices cut of approximately 280µm width, which underwent TMR preparation and polishing (described in section 5.9). Immediate TMR analysis was carried out to assess the extent of mineral loss and therefore suitability of inclusion in the trial. 60 teeth in total were prepared by this method. However, only teeth with a uniform and regular lesion, with mineral loss range of 900-1700 vol%µm were selected. Of the 60 teeth demineralised only 16 were found to have an adequately even demineralisation suitable to be used in the trial.

5.7 Creation of enamel carrier design

The carrier design used was a modified version of that described by Bryniarska (2012). The components required to produce one stainless steel carrier were; (Figure 5.7.1)

- 2 small rectangular rings formed from 0.8mm stainless steel orthodontic wire (DB orthodontics Yorkshire ,UK)
- 2 Stainless steel molar bracket bases (American Orthodontics, Ref 095, 0.225 x 0.120)
- 1 Slide-on surgical ball hook, 0.021" x 0.025". (Orthodontic Technology, USA)
- Dacron gauze (polyester mesh P/N: PETKM3002, USA)

Figure 5.7.1 Components for enamel carrier construction



The 0.8mm stainless steel orthodontic wire was hand bent into a rectangular shaped ring and was spot welded together (Microwelder, Type 95, SLEE, London, UK). This rectangular shaped ring was then spot welded to the molar bracket base (Figure 5.7.2). The joins were polished with a green stone polishing bur (Dura-green Stone PC2, Shofu, Kent, UK) to remove any sharp or rough edges that could cause intra-oral irritation.

Figure 5.7.2 Bracket base and rectangular wire welded together

The slide-on surgical ball hook with internal dimensions 0.021”x 0.025” was then spot-welded to the back of a stainless steel molar bracket base. The ball was positioned in the middle of the bracket base. As this join was deemed to be vulnerable to breakage *in situ*, this was re-inforced with additional solder and then repolished (Figure 5.7.3). Non-fluoride borax flux (Flux Low-Fusing, General Dental Supplies, Dentecon Inc, California, USA) was used to ensure this did not result in fluoride ion release and affect the demineralisation process.

Figure 5.7.3 Ball hook welded to bracket base and polished

The experimental enamel specimens were then reduced in size as required to fit inside the rectangular carrier. The specimen was then adhered to the bracket base using nail varnish (Max Factor, Nailfinity, Weybridge, UK).

Figure 5.7.4 Trimmed enamel specimen prior and post placement in carrier

Dacron gauze was placed over the enamel specimen carrier and another rectangular stainless steel ring positioned above. The two stainless steel rings were then carefully spot welded together to produce the completed carrier (Figure 5.7.5). The manipulation for this procedure was technically difficult and great care was required to avoid enamel sample damage by the spot welder. If the dacron gauze was too dry it became very friable and singed due to the heat of the spot welder, which would result in early detachment from the carrier. If the gauze was slightly moistened and then let to dry at room temperature for 5 minutes, it retained a small amount of moisture to prevent it from being friable or combusting on heat application but not enough moisture that it would affect the spot welding join between the two metals.

Figure 5.7.5 Completed enamel carrier

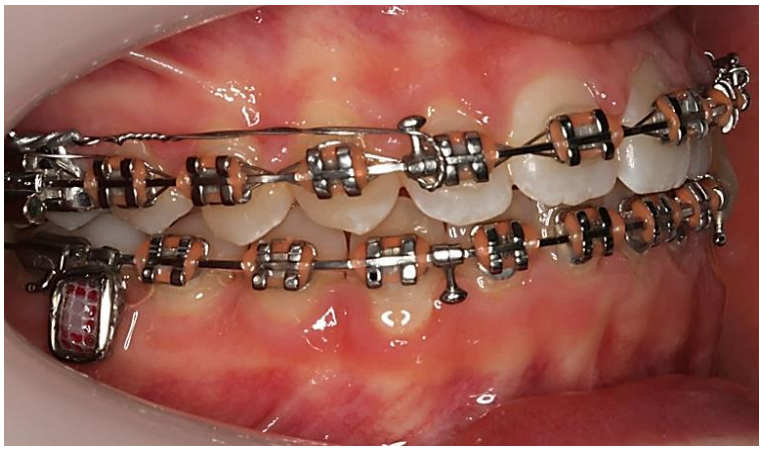
The completed carrier was then placed in a 1.5 ml-capped tube with a small drop of water to maintain moisture content. All experimental sections were sterilized by gamma irradiation with dose 4080 Grays over 3 days under a cobalt source. This effectively sterilises the sample from bacteria without causing any discoloration or mineral content changes (Amaechi et al, 1999). The sterilised enamel carrier was then stored in a refrigerator at approximately 3°-5° until required for the clinical trial.

5.8 Intra-oral carrier placement

At a routine orthodontic appointment the enamel carrier was attached. With the archwire out of the mouth, the enamel carrier was threaded onto the wire posteriorly. It was positioned in the lower premolar region and then the wire was carefully inserted intra-orally. The carrier placement was then adjusted so it was located passively in the premolar embrasure space (Figure 5.8.1). The patient was given time to feel and check that the carrier was comfortable and any required adjustments

were carried out at this stage. The patient would ideally be in a full size rectangular 0.019" x 0.025" wire as this would reduce excess movement of the carrier. The carrier was placed on the side which had the most appropriate embrasure space to ensure a comfortable fit. Benson (1999) found that there was no difference in remineralising effect between placing *in situ* enamel on the dominant or non-dominant brushing side of the mouth.

Figure 5.8.1 Enamel carrier *in situ*



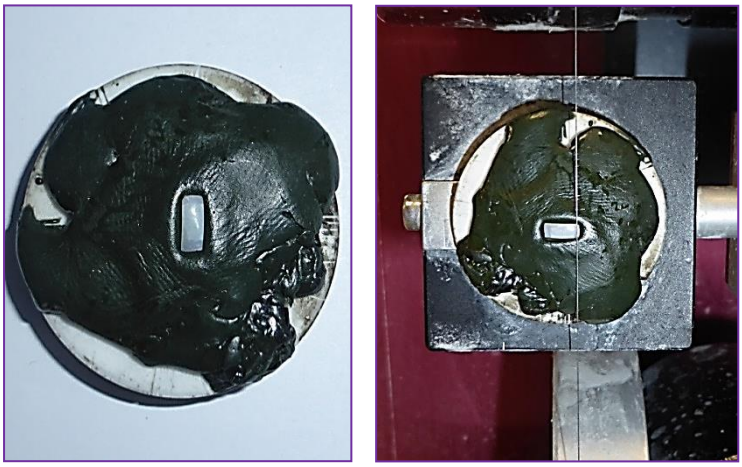
5.9 TMR preparation

Returned enamel carriers were placed in an acetone solution (VWR International Ltd, Poole, England) to dissolve the nail varnish. A surgical scalpel (SKU-0511, Swann Morton, Sheffield, England) was used to carefully remove the Dacron gauge from the enamel carrier. The enamel specimen could then be easily removed from the carrier while minimising any damage. The carrier was then discarded into the sharps bin. The enamel specimen was then re-submerged in acetone solution to ensure all nail varnish residue had been removed (Figure 5.9.1). The sample was thoroughly rinsed with distilled water and stored in a new 1.5ml capped tube ready for sectioning.

Figure 5.9.1 Enamel sample ready for sectioning

When ready for sectioning, the enamel sample was mounted with greenstick compound on the diamond wire saw (Precision Diamond Wire Saw 3242, Well, Walter Ebner, Le Locle, Germany) as shown in Figure 5.9.2.

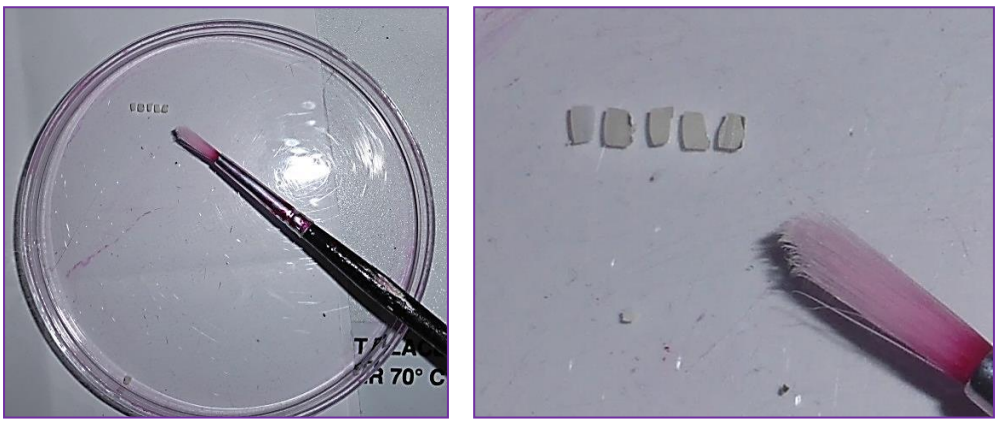
Figure 5.9.2 Mounting of enamel specimen on diamond wire saw



280µm width samples were then cut perpendicular to the anatomical surface of the lesion. The experimental specimen usually had reduced dimensions (approx. 1mm x 3mm) compared to the control section so this stage of treatment was extremely complex. Normally the sections would be cut longitudinally (coronal to apical), however to maximize the number of lesion obtained, the sections were cut transversely (Figure 5.9.3). This resulted in an increased number of samples, however the overall dimensions of the samples were reduced considerably (Figure 5.9.4). This made the subsequent polishing even more difficult. If possible 3-4 sections per sample were obtained, however this was dictated by the size of the sample.

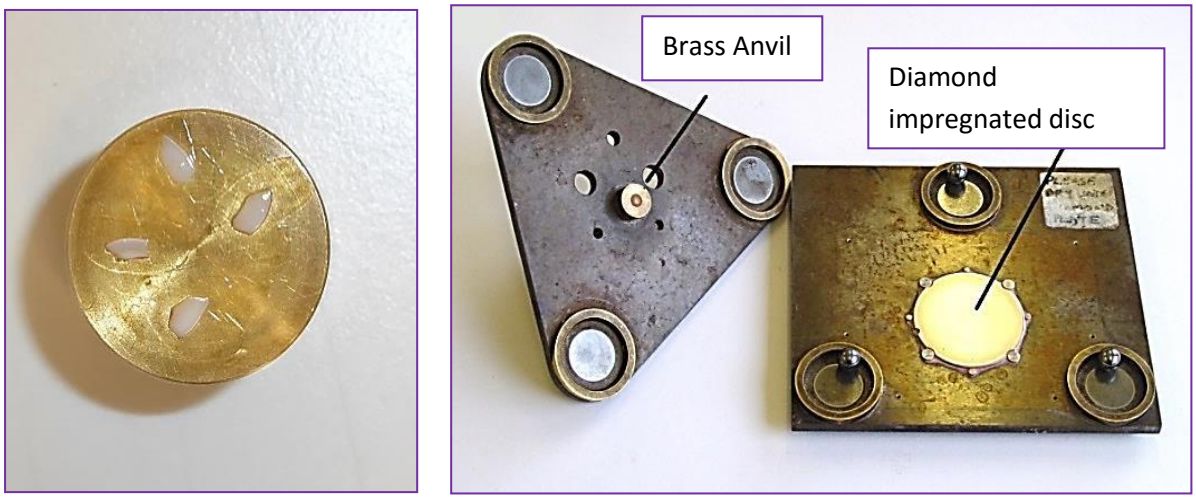
Figure 5.9.3 Sample cut transversely to increase number of sections

Figure 5.9.4 Individual enamel specimens prior to polishing



The sectioned 280 µm slices were then attached to brass anvils (Figure 5.9.5) using nail varnish (Max Factor, Nailfinity, Weybridge, UK) and hand polished to sections of 80±10µm using a diamond-impregnated disc (15µm particles, Buehler, Illinois). This utilised a series of ball bearings in sequence, with a gradual reduction in polishing from 250µm initially, and finishing with 80µm. To ensure sections were plano-parallel the sections were polished on both sides. Polished sections were then carefully removed from the brass anvil using acetone. Finished sections were then submerged in distilled water in microtubes until ready for TMR plate manufacture.

Figure 5.9.5 Brass anvil with mounted enamel sections



5.10 TMR plate creation

80µm polished enamel sections were mounted on a perspex template and covered in double sided Scotch® tape (3M United Kingdom PLC, Berkshire, UK) as shown in Figure 5.10.1. Sections were then placed on a 12 step Aluminium wedge (25µm thickness) on a high resolution radiographic plate (HTA Enterprises, California, USA). Radiographs where then generated by a Philips X-ray set (Philips B. V, Eindhoven, The Netherlands) using a copper target and nickel filter with an exposure time of 35mins at 20kV and 10mA at an anode to film distance of 40cm. This results in production of a stepwedge microradiograph as shown in Figure 5.10.2. Exposure time required to achieve good radiographic contrast was based on a previous study (Lovel, 2008).

Figure 5.10.1 Mounted samples on perspex template

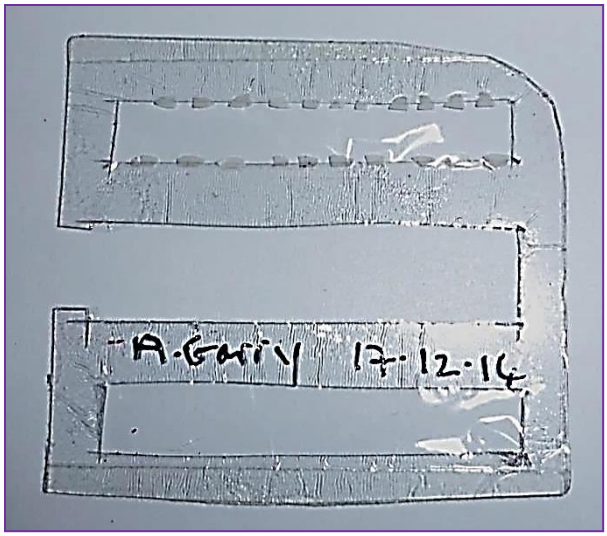
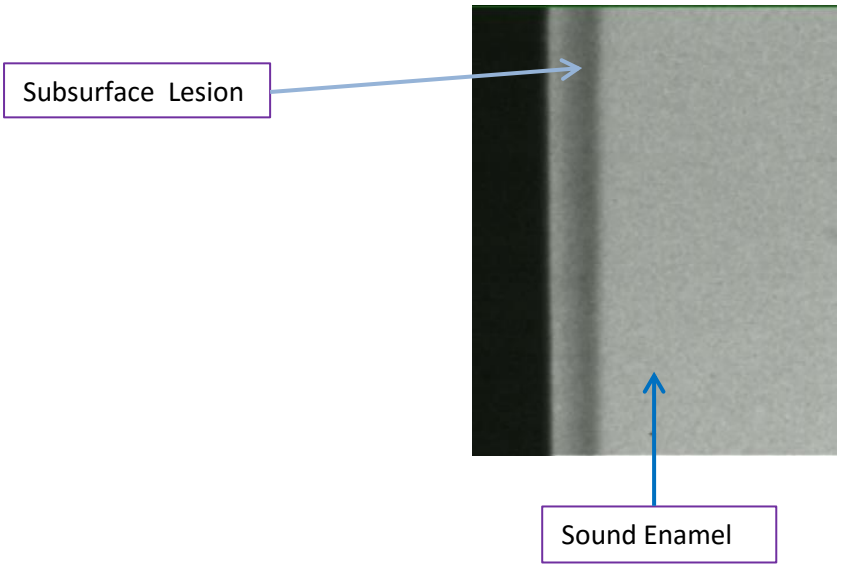


Figure 5.10.2 Stepwedge TMR plate

5.11 TMR Imaging and analysis

The stepwedge microradiographs were then examined using a Leica Leitz DMRB optical microscope (Leica, Wetzlar, Germany). Enamel section images were then captured using a CCD video camera (Sony, Tokyo, Japan), which was connected to a computer (Hewlett Packard Pavilion t3245, USA). Their typical appearance is shown in Figure 5.11.1.

Figure 5.11.1 Transverse microradiograph of a subsurface lesion



Stepwedge calibration was then carried out. Although 0.999 is deemed acceptable, ideally a correlation of 1.00 should be achieved (Figure 5.11.2). Images where then captured using the software TMR 2000 v 2.0.27.16, Inspektor Research System BV, Amersterdam, The Netherlands. The microradiograph images were then analysed to produce a mineral content profile (Figure 5.11.3) using an analysis system (TMR 2006 v 3.0.0.13) using an algorithm developed by De Josselin de Jong (1987)

Figure 5.11.2 Step wedge callibration

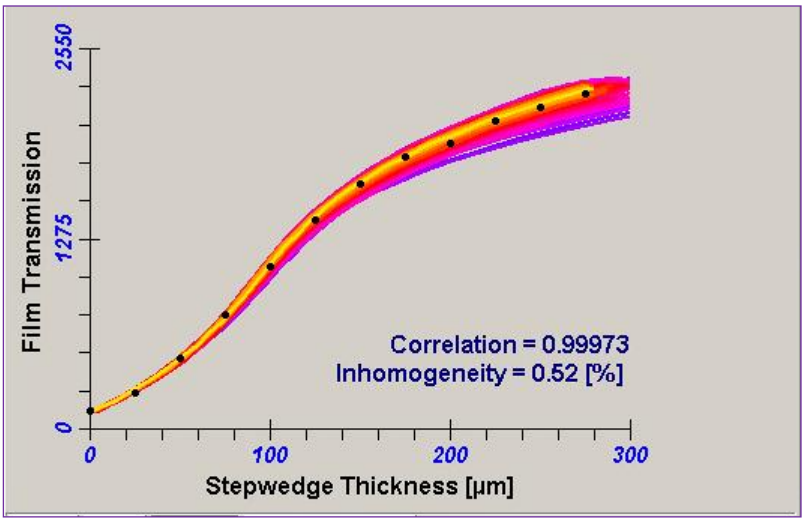
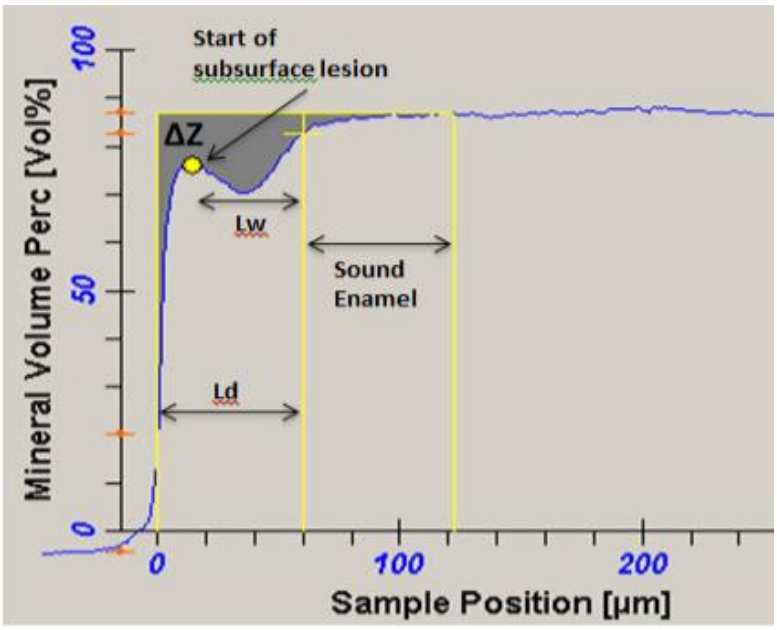


Figure 5.11.3 Typical subsurface lesion mineral content profile



5.12 Measurements

Three main parameters were obtained from the analysis of mineral content profiles;

- Mineral loss ΔZ (vol%µm),
- Lesion depth L_d (µm) and
- Lesion width L_w (µm).

The difference in mineral loss or mineral gain between the control sample and the *in situ* sample was measured. From this, a percentage change in mineral loss, lesion depth and lesion width was calculated. This is achieved by dividing the *in situ* value by the control value and multiplying by 100. A value greater than 100 signifies further mineral loss, a value less than 100 signifies mineral gain and a value of 100 shows no change has occurred. The accuracy of TMR for lesion depth (L_d) is approximately 5 µm and mineral loss ΔZ about 200 vol%µm (Arends and Ten Bosch 1992).

5.13 Statistical Analysis

Statistical support was sought from Dr G. Burnside (Department of Biostatistics, University of Liverpool). Data were entered and analysed using Statistical Package for Social Sciences software (IBM SPSS Statistics v.22). Descriptive statistics calculations carried out included mean, standard deviation and percentage mineral loss group. Hypothesis testing was carried out using analysis of covariance (ANCOVA), adjusting for the baseline measurements, participant effect and for the order

in which the treatments were received. Significance level for the statistical test was set at $p < 0.05$. A Pearson correlation coefficient was used to compare the quantity of Tooth Mousse™ and the resulting change in mineralisation.

Analysis of covariance is a general linear model which blends ANOVA and regression. It is used to analyse if means of a dependent variable are equal for different treatment groups, while statistically controlling for the effects of other continuous variables. Therefore In this study it was used to analyse if the mineral profile means in different treatment groups were equal, whilst statistically controlling for baseline measurements and the order in which the treatments were received.

6.0 Results

6.1 Baseline lesions

Teeth with lesions in the range of mineral loss of 900-1700 vol%µm were selected. At this range shallow enamel subsurface lesions are created, which can demonstrate a significant proportional change in mineralisation over a short period of time. Of the 60 teeth which were demineralised, 16 were suitable and met the criteria for use in this study, of which 15 were used. In 10 participants both the experimental specimens came from the same tooth. In the other 2 participants, specimens from different teeth had to be used due to *in situ* and procedural loss. The parameters of baseline lesions are shown in Table 6.1.1.

Table 6.1.1 Parameters of Baseline lesions selected

Tooth number	Mineral Loss (Vol%µm)	Lesion depth (µm)	Lesion width (µm)
1	1222.5	69.7	59.7
3	1100	56.6	47.2
4	1273.3	58.4	47.2
6	1292.5	68.2	55.4
7	1530	66.1	54.8
19	1213.3	54.4	45
20	1130	55.9	38.8
22	1120	64.85	54.6
25	920	48	41.3
30	1126.6	48.1	40.8
40	1252	63.6	50.3
46	1140	57.3	47.6
47	1176	57.7	44.2
51	1090	50.2	43.8
53	1115	62.5	52.4

6.2 Order of Interventions and specimens

Table 6.2.1 shows the order of intervention and which specimens were used in each intervention. There were no drop outs from the trial. The enamel specimen placed in participant 2 was lost *in situ* during Intervention B, so an additional phase was carried out. Although the base enamel carrier was still in place the outer metal ring and therefore the Dacron gauze retaining the enamel sample had become detached.

The enamel specimen placed in participant 8 for Intervention A was damaged intra-orally during the *in situ* phase. The loss of the enamel layer resulted in an inability to carry out TMR analysis so this phase was also repeated at the end of the trial. The enamel specimen of participant 6 for Intervention A was damaged during TMR polishing, but unfortunately this phase was unable to be repeated as the patient has subsequently had his fixed orthodontic appliances removed by the time this was identified.

Table 6.2.1 Order of Interventions and specimens used in each subject

	Phase 1		Phase 2		Phase 3	
Participant	Intervention	Specimen	Intervention	Specimen	Intervention	Specimen
1	B	4a	A	4b		
2	B	20a	A	20b	B	6a
3	B	3b	A	3a		
4	A	7a	B	30a		
5	A	19a	B	19b		
6	B	1a	A	1b	Unable To redo	
7	B	22a	A	22b		
8	A	25a	B	53a	A	6b
9	A	46a	B	46b		
10	B	40a	A	40b		
11	A	51a	B	51b		
12	A	47a	B	47b		

Key

Lost specimen

Enamel specimen damaged

6.3. Flow Chart of clinical phase

In total 14 orthodontic patients were approached for enrolment in the study, of which 2 patients declined. All 12 participants completed the trial and therefore there were no drop-outs. A Consort flow diagram demonstrating the flow of participants through the trial is shown in Figure 6.3.1.

6.3.1 Consort participant flow diagram

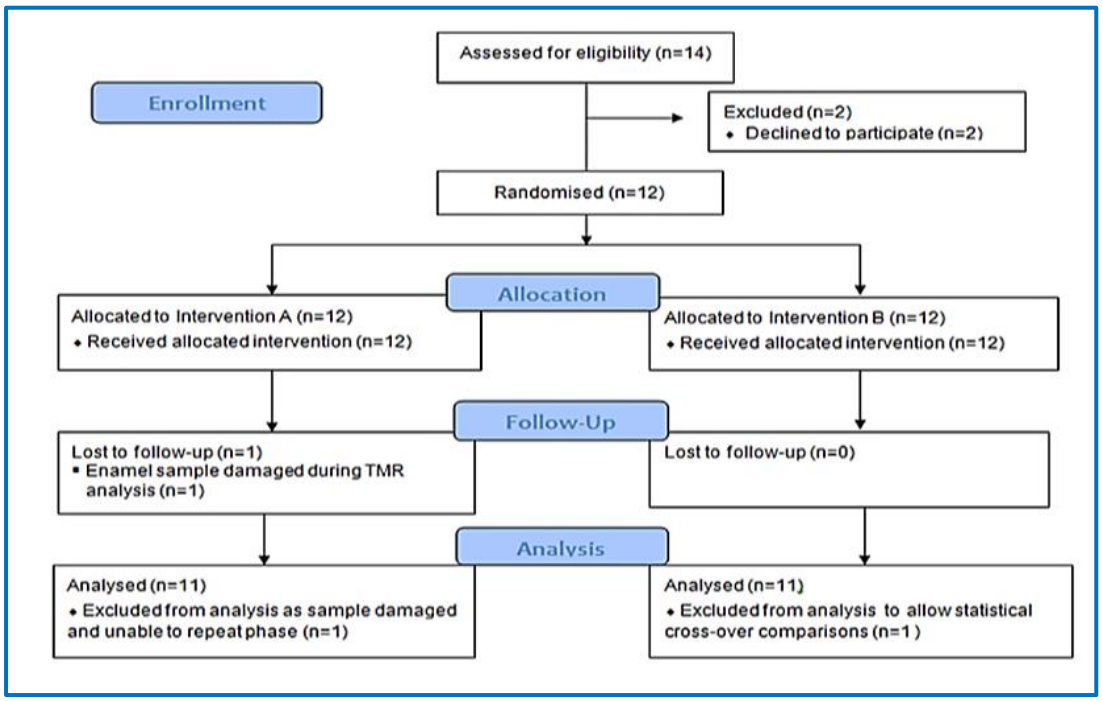
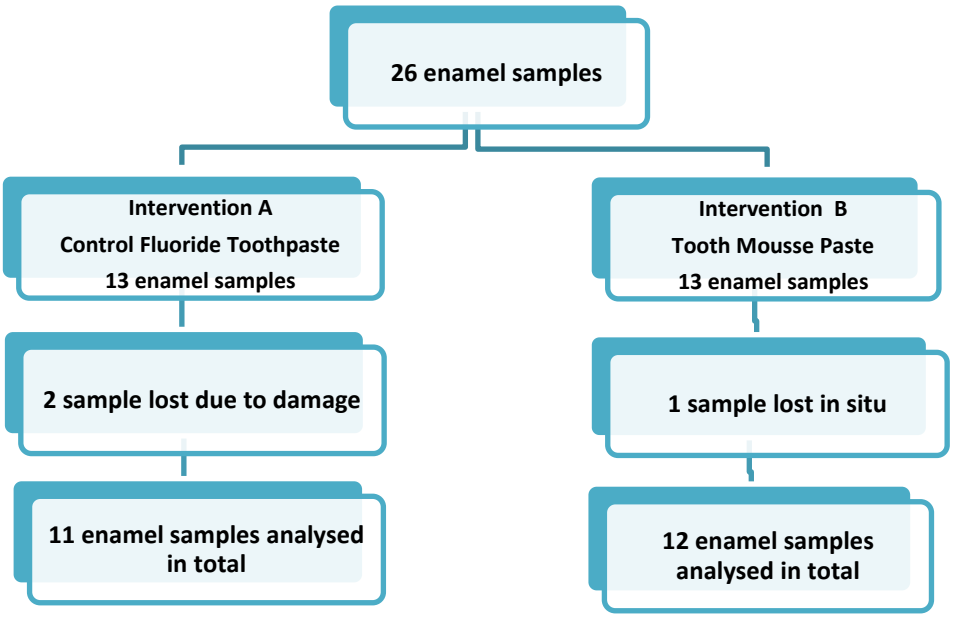


Figure 6.3.2 shows a flow chart of the enamel samples used in the clinical phase. 26 enamel samples were used in total, with 13 in each group. Results were obtained from 11 participants out of a possible 12 in Intervention A with all 12 participants in Intervention B obtaining data results.

Figure 6.3.2 Flow Chart of enamel samples



6.4 Main Results

Raw data for baseline mineral loss, lesion depth and lesion width are shown in Appendices 4a, 4b, 4c. Post treatment data for mineral loss, lesion depth and lesion width are shown in Appendices 5a, 5b, 5c. The measurements were compared using analysis of covariance (ANCOVA), adjusting for the baseline measurements, participant effect and for the order in which the treatments were received.

In order to compare the pre and post treatment mineral loss, lesion depth and lesion width, the data were normalised by dividing the sample value by the control value and multiplying by 100 (Strang, 1987). A value of 100% mineral loss indicates no change has occurred, with values greater than 100 signifying further mineral loss and values less than 100 signifying mineral gain. For lesion depth and lesion width, values below 100 signify a decrease in lesion size and values above 100 signify increase in lesion size (Tables 6.4.1, Table 6.4.2, and Table 6.4.3).

Data was also demonstrated as percentage mineral change to aid identification of trends. Positive percentage mineral change (eg +10%) indicates gain in mineral and negative percentage mineral change (eg -10%) indicating further mineral loss. Similarly a positive percentage change in lesion depth and lesion width (eg +10%) indicates a reduction in lesion size and a negative percentage change in lesion depth and lesion width (eg -10%) indicating an increase in lesion size (Table 6.5.1, Table 6.6.1, Table 6.7.1).

Table 6.4.1 Mineral loss (%) of each sample following the in situ phase

	Phase 1			Phase 2			Phase 3		
Subject	Intervention	specimen	% mineral loss (%Volμm)	Intervention	Specimen	% mineral loss (%Volμm)	Intervention	Specimen	% mineral loss (%Volμm)
1	B	4a	81.8	A	4b	102.9			
2	B	20a	---------	A	20b	74.8	B	6a	71.4
3	B	3b	94.9	A	3a	101.4			
4	A	7a	68.8	B	30a	102.5			
5	A	19a	72.5	B	19b	67.6			
6	B	1a	61.3	A	1b	-------			
7	B	22a	75.2	A	22b	81.5			
8	A	25a	--------	B	53a	74.4	A	6b	78.1
9	A	46a	91.4	B	46b	62.9			
10	B	40a	69.9	A	40b	78.0			
11	A	51a	95.6	B	51b	80.5			
12	A	47a	85.6	B	47b	61.7			

Table 6.4.2 Lesion depth (%) of each sample following the in situ phase

	Phase 1			Phase 2			Phase 3		
Subject	Intervention	Specimen	Lesion Depth (%)	Intervention	Specimen	Lesion Depth (%)	Intervention	Specimen	Lesion Depth (%)
1	B	4a	92.5	A	4b	104.5			
2	B	20a		A	20b	88.2	B	6a	88.7
3	B	3b	82.3	A	3a	119.8			
4	A	7a	89.5	B	30a	108.5			
5	A	19a	94.9	B	19b	97.8			
6	B	1a	70.4	A	1b				
7	B	22a	80.0	A	22b	91.8			
8	A	25a		B	53a	86.1	A	6b	87.7
9	A	46a	97.7	B	46b	91.3			
10	B	40a	83.5	A	40b	88.8			
11	A	51a	114.9	B	51b	101.8			
12	A	47a	104.5	B	47b	84.1			

Table 6.4.3 Lesion width (%) of each sample following the in situ phase

	Phase 1			Phase 2			Phase 3		
Subject	Intervention	Specimen	Lesion width (%)	Intervention	Specimen	Lesion width (%)	Intervention	Specimen	Lesion width (%)
1	B	4a	85.4	A	4b	106.4			
2	B	20a		A	20b	90.2	B	6a	81.8
3	B	3b	74.6	A	3a	120.3			
4	A	7a	76.9	B	30a	100.5			
5	A	19a	91.6	B	19b	98.9			
6	B	1a	62.6	A	1b				
7	B	22a	72.0	A	22b	91.8			
8	A	25a		B	53a	75.8	A	6b	83.4
9	A	46a	99.8	B	46b	84.5			
10	B	40a	81.1	A	40b	93.4			
11	A	51a	105.5	B	51b	86.8			
12	A	47a	91.0	B	47b	90.5			

Key

Lost specimen

Enamel specimen damaged

6.5 Mineral loss data

Pre and post-treatment mineral loss ΔZ means and standard deviations are demonstrated in Table 6.5.1. Mean mineral loss ΔZ (vol%µm) reduced by 193 and 277 for treatment groups A and B respectively, demonstrating an increase in mineral gain and therefore remineralisation. Treatment A had a mean percentage mineral loss of 84.6%, therefore a + 15.4% mineral gain, with Treatment B having a mean percentage mineral loss of 75.4% and therefore a +24.6% mineral gain.

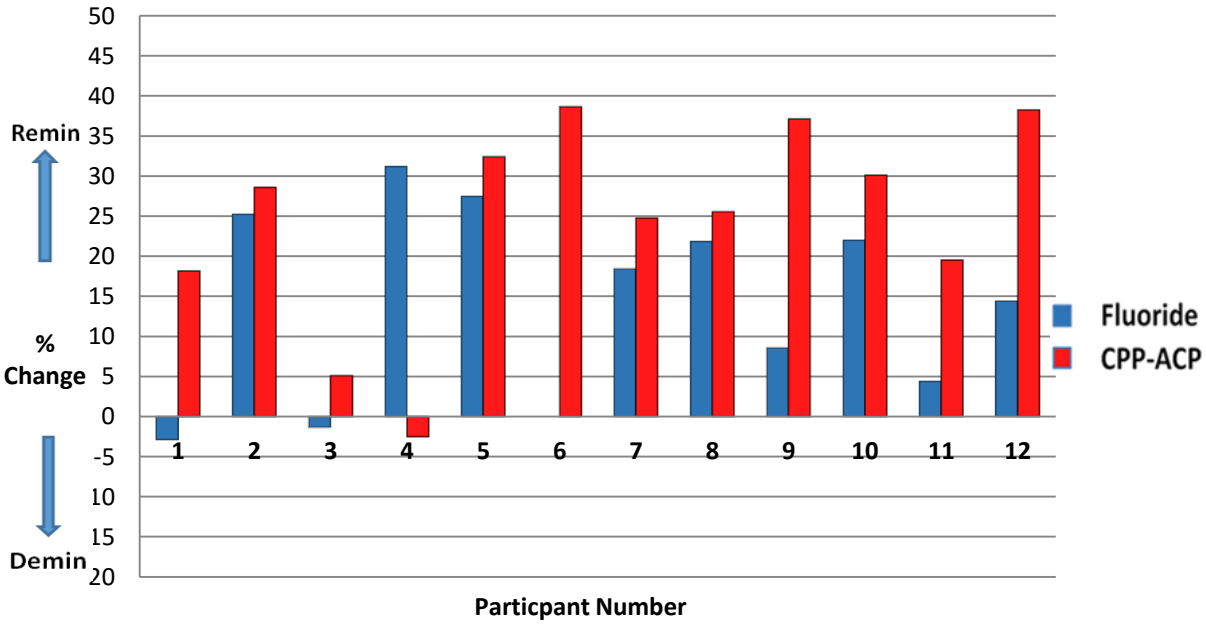
Table 6.5.1. Mineral loss results

Mineral loss ΔZ (vol%µm)	Treatment A (Fluoride)	Treatment B (CPP-ACP)
Baseline mean (s.d)	1210.6 (127.3)	1172.6 (73.4)
Post-treatment mean (s.d)	1017.6 (126)	895.6 (136.3)
Post-treatment mean Adjusted (95% CI) *	1014.8 (927.3, 1102.4)	898.3 (810.0,985.9)
p- value between groups	p = 0.023	
Mineral loss % (s.d)	84.6 (11.7)	75.4 (12.7)
Mineral change % (s.d)	+15.4 (11.7)	+24.6 (12.7)

* Adjusted for order of treatment, participant effect and mineral loss at baseline

Figure 6.5.2 demonstrates the percentage mineral change in both treatment groups for each individual participant. This demonstrates a generalised trend of remineralisation across all participants in both treatment groups, with 9 of 11 participants receiving treatment A (Fluoride) and 11 of 12 participants receiving treatment B (CPP-ACP) having positive changes in mineral gain.

Figure 6.5.2 Graph of Mineral change (%) per participant



Null Hypothesis

The following Null Hypotheses (Ho) were tested for mineral loss ΔZ using ANCOVA (Table 6.5.3)

1. Mineral loss ΔZ was independent of treatment

   p=0.023 ➔ Ho is rejected

2. Mineral loss ΔZ was independent of order

   p=0.760 ➔ Ho is not rejected

3. Mineral loss ΔZ was independent of baseline mineral loss

   p=0.505 ➔ Ho is not rejected

4. Mineral loss ΔZ was independent of participant effect

   p=0.138 ➔ Ho is not rejected

Therefore there was no significant effect on mineral loss ΔZ for the order in which they received the intervention (p=0.760), participant effect (p=0.138) or for baseline mineral loss (p=0.505). However there was a significant effect of the treatment allocation (p=0.023).

Table 6.5.3 ANCOVA table for mineral loss

Variable	Sum of squares	Degrees of freedom	Mean square	F	p-value
Treatment	88491.01	1	88491.01	7.89	0.023
Order	1123.18	1	1123.18	0.10	0.760
Baseline mineral loss	5461.81	1	5461.81	0.49	0.505
Participant effect	246756.19	10	24675.62	2.20	0.138
Error	89681.75	8	11210.22		

6.6 Lesion depth data

Pre and post-treatment lesion depth (Ld) means and standard deviations are demonstrated in Table 6.6.1. Mean lesion depth reduced by 1.4µm and 5.9µm for treatment groups A and B respectively. This reduction in lesion depth suggests a minimal remineralising effect with treatment A (Fluoride) and an increased remineralisation taking place with treatment B (CPP-ACP). Treatment A had a mean percentage lesion depth of 98.4%, therefore a + 1.6% reduction in depth, with treatment B having a mean percentage lesion depth 88.9 % and therefore an +11.1% reduction in lesion depth.

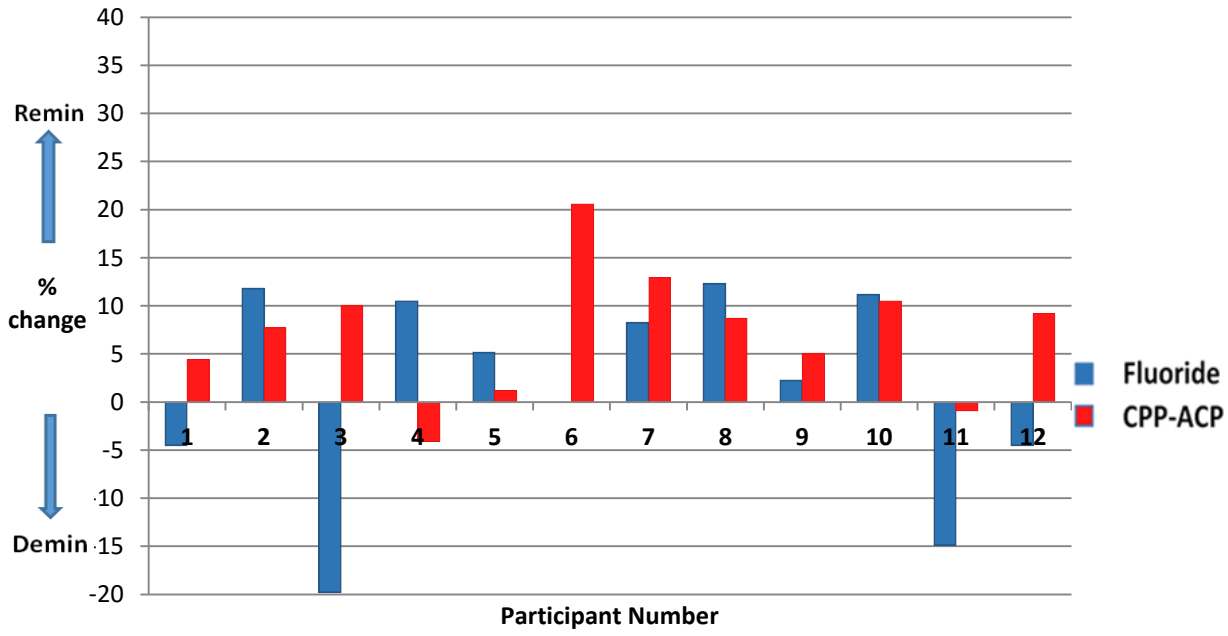
Table 6.6.1. Lesion depth results

Lesion depth Ld. (µm)	Treatment A (Fluoride)	Treatment B (CPP-ACP)
Baseline mean (s.d)	59.7 (6.0)	58.3 (6.2)
Post-treatment mean (s.d)	58.3 (5.1)	52.4 (3.5)
Post-treatment mean Adjusted (95% CI) *	58.1 (54.6, 61.7)	52.7 (49.1, 56.2)
p- value between groups	p = 0.037	
Lesion depth % (s.d)	98.4% (10.7)	88.9% (10.3)
Lesion depth change % (s.d)	+1.6% (10.7)	+11.1% (10.3)

* Adjusted for order of treatment, participant effect and baseline lesion depth

Figure 6.6.2 demonstrates the lesion depth change (%) in both treatment groups for each individual participant. An overall generalised trend of remineralisation was seen across most patients in both treatment groups; however this appears to be more pronounced in participants receiving treatment B (CPP-ACP). A wide intra-subject and inter subject variation in lesion depth change was observed. Only 7 of 11 participants receiving treatment A had a reduction in lesion depth, whereas 10 of 12 participants receiving treatment B had a reduction in lesion depth and therefore a remineralising effect.

Figure 6.6.2 Graph of lesion depth change (%) per participant



Null Hypothesis

The following Null Hypotheses (Ho) were tested for lesion depth (Ld) using ANCOVA (Table 6.6.3)

1. Lesion depth was independent of treatment

   p=0.037 ➔ Ho is rejected

2. Lesion depth was independent of order

   p=0.202 ➔Ho is not rejected

3. Lesion depth was independent of baseline lesion depth

   p=0.125 ➔Ho is not rejected

4. Lesion depth was independent of participant effect

   p=0.970 ➔ Ho is not rejected

Therefore there was no significant effect on lesion depth (Ld) for the order in which they received the intervention (p=0.202), participant effect (p=0.970) or for the baseline lesion depth (p=0.125). However, the intervention which they received did have a significant effect on the lesion depth (p=0.037).

Table 6.6.3 ANCOVA table for lesion depth (Ld)

Variable	Sum of squares	Degrees of freedom	Mean square	F	p-value
Treatment	158.54	1	158.54	6.22	0.037
Order	49.32	1	49.32	1.93	0.202
Baseline lesion depth	74.78	1	74.78	2.93	0.125
Participant effect	70.05	10	7.01	0.28	0.970
Error	204.00	8	25.50		

6.7 Lesion width data

Pre and post-treatment lesion width (Lw) means and standard deviations are demonstrated in Table 6.7.1. Mean lesion width reduced by 2.5µm and 7.6µm for treatment groups A and B respectively. This reduction in lesion width suggests a small remineralising effect with treatment A (Fluoride), and an increased remineralisation taking place with treatment B (CPP-ACP). Treatment A had a mean percentage lesion width of 95.5%, therefore a +4.5% reduction in width, with treatment B having a mean percentage lesion width 84.7% and therefore a +15.3% reduction in lesion width.

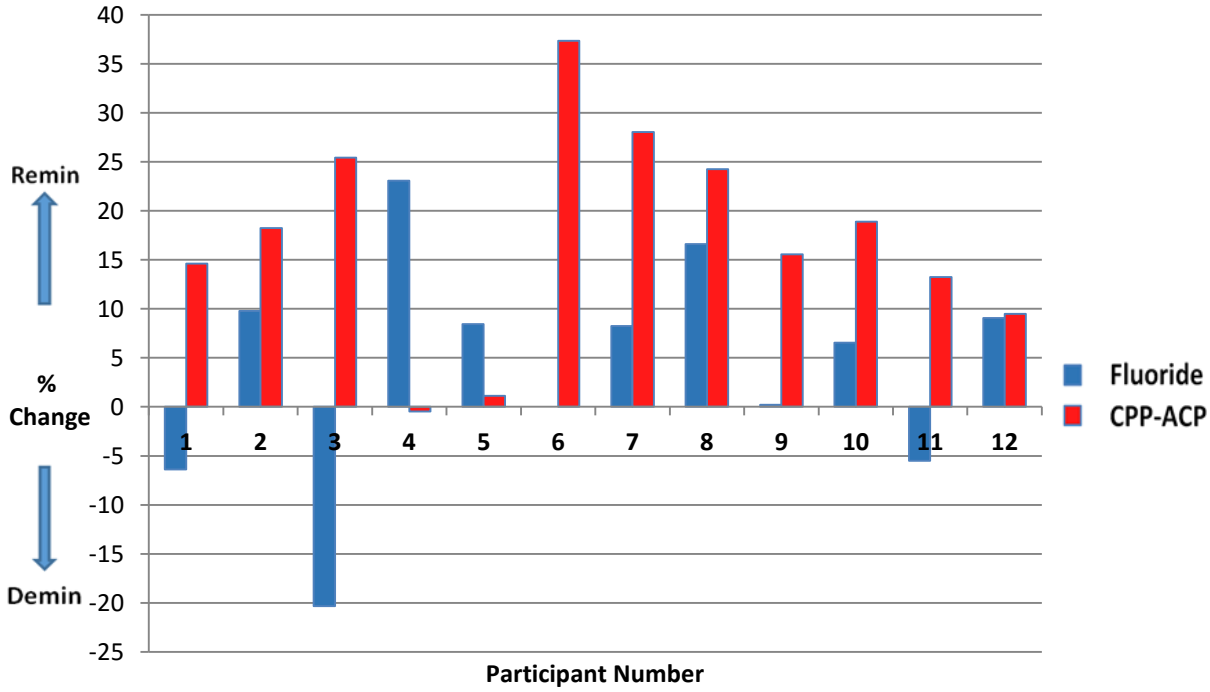
Table 6.7.1. Lesion width results

Lesion width Lw (µm)	Treatment A (Fluoride)	Treatment B (CPP-ACP)
Baseline mean (s.d)	48.5 (6.1)	48.0 (4.7)
Post-treatment mean (s.d)	46.0 (5.8)	40.4 (2.8)
Post-treatment mean Adjusted (95% CI) *	46.2 (43.1, 49.3)	40.3 (37.2, 43.4)
p- value between groups	p = 0.015	
Lesion width % (s.d)	95.5% (11.4)	84.7% (8.8)
Lesion width change % (s.d)	+4.5% (11.4)	+15.3% (8.8)

* Adjusted for order of treatment, participant effect and baseline lesion width

Figure 6.7.2 demonstrates the lesion width change (%) in both treatment groups for each individual participant. An overall generalised trend of remineralisation was seen across most participants in both treatment groups; however this appears to be greater in participants receiving treatment B (CPP-ACP). In treatment A, 7 of 11 participants had a reduction in lesion width, whereas 10 of 12 participants receiving treatment B had a reduction in lesion depth and therefore demonstrating a remineralising effect.

Figure 6.7.2 Graph of lesion width change (%) per participant



Null Hypothesis

Null Hypothesis

The following Null Hypotheses (Ho) were tested for lesion width (Lw) using ANCOVA (Table 6.7.3)

1. Lesion width was independent of treatment

   p= 0.015 ➔ Ho is rejected

2. Lesion width was independent of order

   p= 0.033 ➔ Ho is rejected

3. Lesion width was independent of baseline lesion width

   p= 0.155 ➔ Ho is not rejected

4. Lesion width was independent of participant effect

   p= 0.947 ➔ Ho is not rejected

Table 6.7.3 ANCOVA table for Lesion width (Lw) between subject effects

Variable	Sum of squares	Degrees of freedom	Mean square	F	p-value
Treatment	188.91	1	188.91	9.62	0.015
Order	129.32	1	129.32	6.59	0.033
Baseline lesion width	48.48	1	48.48	2.47	0.155
Participant effect	65.25	10	6.53	0.33	0.947
Error	157.05	8	19.63		

Therefore there was a significant effect on lesion width (Lw) for the intervention they received (p=0.015) and for the order in which they received the intervention (p=0.033). There was no significant effect on lesion width (Lw) for baseline lesion width (p=0.155) or for participant effect (p=0.947).

Treatment order

ANCOVA suggested that lesion width was not independent of treatment order. Participants in the 1st phase of treatment had a mean post treatment lesion width of 41.0µm (sd. 4.1) compared with 45.4µm (sd.5.6) in the 2nd phase of treatment. A hypothesis for this increased remineralisation effect in the 1st phase of treatment could be that participants were more compliant with the prescribed instructions and toothpaste usage earlier in the study. However there was no statistically significant difference in the quantities of fluoride toothpaste used (p=0.890) or Tooth Mousse™ used (p=0.678).

6.8 Quantity of Toothpaste and Tooth Mousse™ used

Table 6.8.1 shows the quantity of fluoride toothpaste and Tooth Mousse™ used by each participant over the 4 week period. The quantity of toothpaste used ranged from 33.9g - 91.1g with an average mean of 74.1g used over the 4 week period. The median quantity used was 77.7 grams with an interquartile range of 13.1g (Figure 6.8.2).

The mean quantity of Tooth Mousse™ used was 24.2g with a range of 18.2 g – 29.3g over the 4 week period. The median quantity used was 25.50g with an interquartile range of 4.0g. However there were 11 incomplete data sets due to failure of the participant to return the tubes, with only 73% of toothpaste and 83% of Tooth Mousse™ tubes being returned for analysis

Table 6.8.1 Quantity of Toothpaste and Tooth Mousse™ used (g)

	Phase 1				Phase 2				Phase 3			
Subject	Intervention	Specimen	TP (g)	TM (g)	Intervention	Specimen	TP (g)	TM (g)	Intervention	Specimen	TP (g)	TM (g)
1	B	4a	74.1	22	A	4b	70.9	-				
2	B	20a	UK	UK	A	20b	UK	-	B	6a	25.5	16.5
3	B	3b	33.9	18.2	A	3a	43.3	-				
4	A	7a	70.9	-	B	30a	64	25.6				
5	A	19a	UK	-	B	19b	85	25.8				
6	B	1a	78.4	26.1	A	1b	83	-				
7	B	22a	87.6	25.5	A	22b	91.1	-				
8	A	25a	UK	-	B	53a	76.5	19.2	A	6b	84.4	
9	A	46a	80.8	-	B	46b	UK	UK				
10	B	40a	87.4	26	A	40b	UK	-				
11	A	51a	UK	-	B	51b	UK	UK				
12	A	47a	75.7	-	B	47b	82.1	29.3				

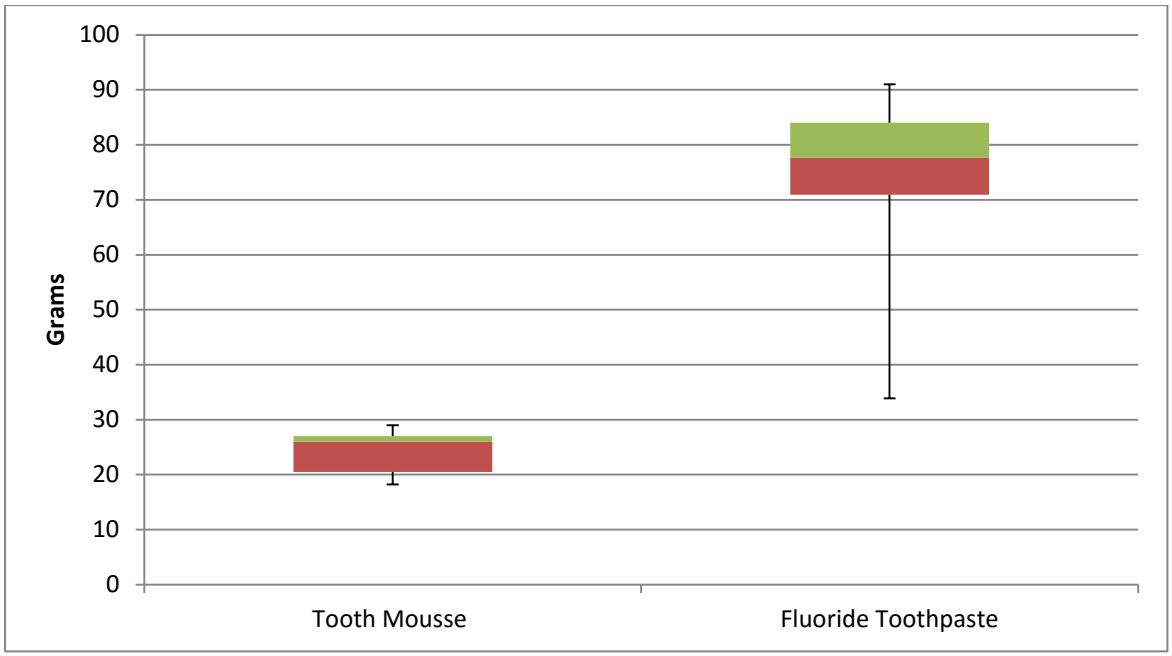
Key

UK Unknown as participant did not return toothpaste

Lost specimen In situ

Enamel specimen damaged

Figure 6.8.2. Box and whisker plot of quantity of Tooth Mousse™ and fluoride toothpaste used demonstrating upper and lower values, median and interquartile ranges.



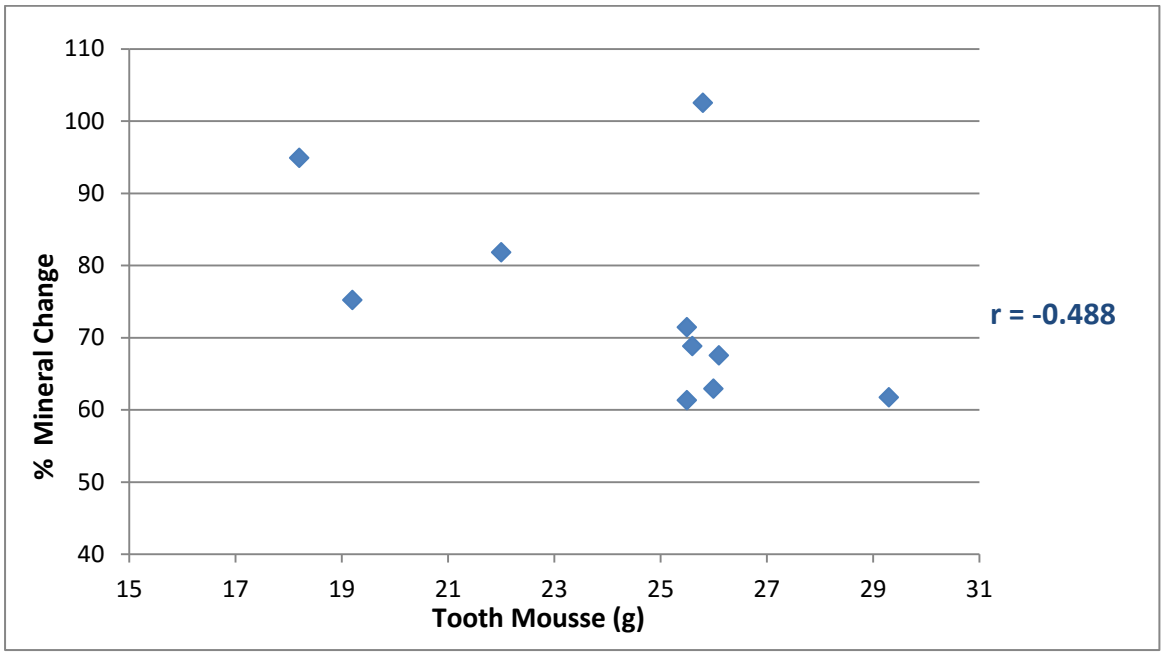
6.9 Correlation testing for quantity of Tooth Mousse™ used

Correlation Testing: Mineral loss vs quantity of Tooth Mousse™

A Pearson correlation coefficient can be used for parametric data to measure the strength and direction of a linear relationship for two continuous variables. Values range from -1.0 (a perfect negative relationship) to +1.0 (a perfect positive relationship) with 0 indicating no linear relationship.

The correlation coefficient for percentage mineral loss and quantity of Tooth Mousse™ used was r = - 0.488, indicating a negative correlation, however this was not statistically significant (p=0.152) (Figure 6.9.1).

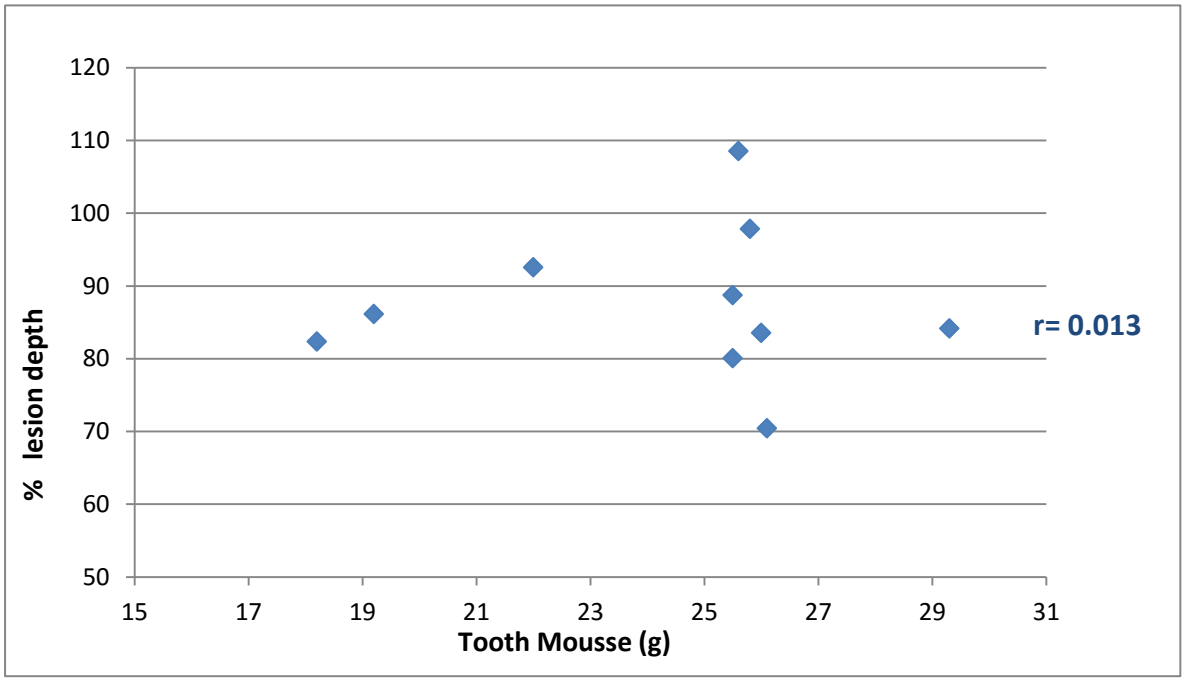
Figure 6.9.1 Scatter plot of % mineral change vs quantity of Tooth Mousse™ used



Correlation Testing: Lesion depth vs quantity of Tooth Mousse™

The correlation of coefficient for percentage lesion depth and quantity of Tooth Mousse™ used was r = 0.013 (p=0.973), indicating that there is no correlation between the quantity of Tooth Mousse™ used and percentage lesion depth (Figure 6.9.2).

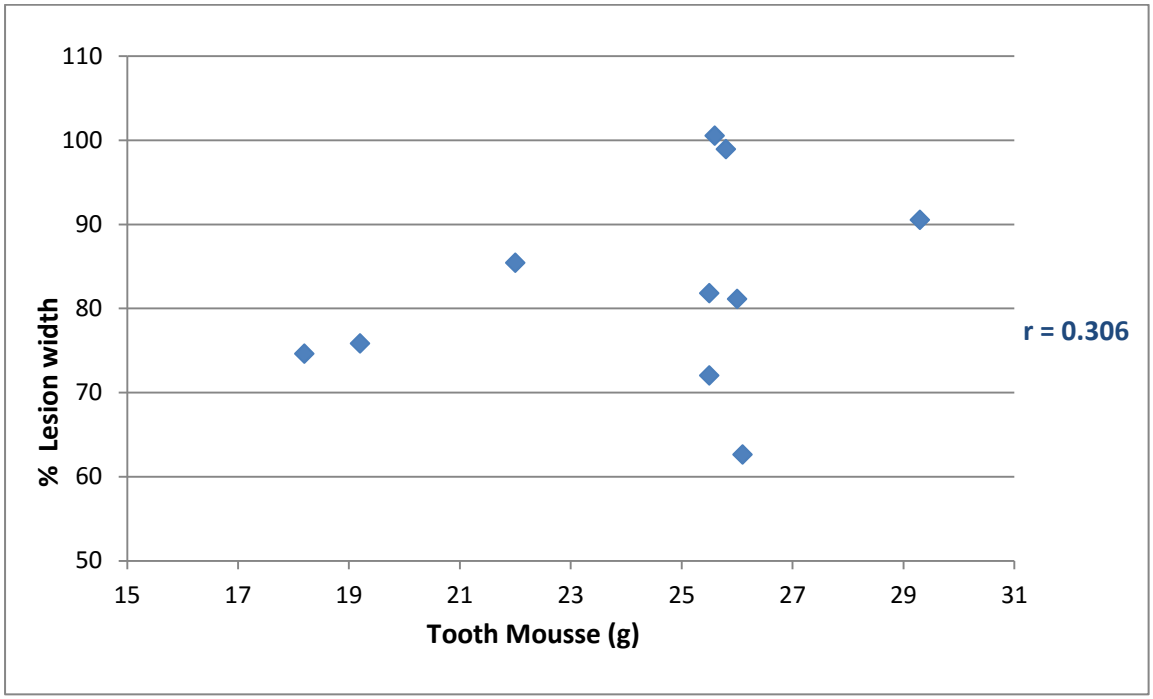
Figure 6.9.2 Scatter plot of lesion depth vs quantity of Tooth Mousse™ used



Correlation Testing: Lesion width vs quantity of Tooth Mousse™

The correlation of coefficient for percentage lesion width and quantity of Tooth Mousse™ used was r = 0.306 (p=0.389), indicating that there is no correlation between the quantity of Tooth Mousse™ used and percentage lesion width.

Figure 6.9.3 Scatter plot of lesion depth vs quantity of Tooth Mousse™ used



7.0. Summary

The summarised mean, standard deviation and significance levels for the 3 main parameters for both treatment groups are shown in Table 7.1.1. During the *in situ* phase the following changes were observed;

- An overall trend of mineral gain ΔZ, reduction in lesion depth and reduction in lesion width was observed in both treatment groups therefore demonstrating a remineralisation effect. All baseline lesions, on average remineralised during the *in situ* phase regardless of treatment group allocation, however the fluoride toothpaste combined with GC Tooth Mousse™ (CPP-ACP) group demonstrated an increased remineralising effect. A statistically significant reduction in mineral loss ΔZ (p=0.023), lesion depth (p=0.037) and lesion width (p=0.015) was observed in the fluoride toothpaste combined with CPP-ACP group compared with the fluoride toothpaste alone.

- Mineral loss ΔZ reduction occurred in both treatment groups, with a 15.4% and 24.6% mineral gain in the fluoride and fluoride combined with CPP-ACP group respectively. The difference between these groups was statistically significant (p = 0.023). There was no significant effect on mineral loss ΔZ for the order in which they received the intervention (p=0.760), participant effect (p=0.138) or for baseline mineral loss (p=0.505).

- Lesion depth (Ld) reduction occurred in both treatment groups, with a 1.6% and 11.1% reduction in the fluoride and fluoride combined with CPP-ACP group respectively. The difference between treatment groups was statistically significant (p = 0.037). There was no significant effect on lesion depth (Ld) for the order in which they received the intervention (p=0.202), participant effect (p=0.970) or for the baseline lesion depth (p=0.125).

- Lesion width (Lw) reduction occurred in both treatment groups, with a 4.5% and 15.3% reduction in the fluoride and fluoride combined with CPP-ACP group respectively. This difference between treatment groups was statistically significant (p = 0.015). There was a significant effect on lesion width for the order in which they received the intervention (p=0.033). There was no significant effect on lesion width for baseline lesion width (p=0.155) or for participant effect (p=0.947).

- A wide range of intra and inter subject data variation was noted. Mineral loss ΔZ ranged from 2.9% mineral loss to 38.3% mineral gain. Lesion depth ranged from a 19.8% increase to a 29.6%

reduction in lesion depth. Lesion width ranged from 20.3% increase to a 37.4% lesion width reduction.

- There was a large range in the quantity of fluoride toothpaste and GC Tooth Mousse™ used per participant over the 4 week period. The mean quantity of fluoride toothpaste used was 74.1g, with the lowest and highest quantities being 33.9g and 91.1g respectively. The mean quantity of GC Tooth Mousse™ used was 24.2g although the lowest and highest quantities used were 18.2g and 29.3g respectively. However there was no statistically significant correlations between the quantity of GC Tooth Mousse™ used and changes in mineral loss ΔZ (p=0.152), lesion depth (p=0.973) or lesion width (p=0.389).

- *In vitro* subsurface lesion creation is often an imprecise and wasteful process. In total 60 human premolar teeth required demineralisation in order to produce 16 adequately demineralised lesions required for the study (28%).

- The *In situ* phase of treatment was relatively successful with only 1 enamel carrier being lost, and 2 enamel samples being damaged during the TMR preparation process (12% total). There were no drop outs from the study. A total of 11 enamel samples out of a possible 12 were successfully analysed for the fluoride group and a complete set of data was obtained for the fluoride and Tooth Mousse™ group.

Table 7.1.1 Summarised mean data for both treatment groups

>100 = Mineral Loss <100 = Mineral Gain	**Fluoride paste**	**Fluoride paste + CPP-ACP**	**Statistical Significance between groups**
Mineral loss ΔZ % (sd)	84.6% (11.7)	75.4% (12.7)	P = 0.023*
Lesion depth % (sd)	98.4% (10.7)	88.9% (10.3)	P = 0.037 *
Lesion width % (sd)	95.5% (11.4)	84.7% (8.8)	P = 0.015 *

8.0 Discussion

8.1 Discussion

Results demonstrated an increased remineralisation effect in both treatment groups compared with baseline values. In most circumstances it would be expected that remineralisation would occur intra-orally. Artificial subsurface lesions are created *in vitro* with a high strength demineralising gel. Removal from this extreme environment and placement intra-orally with a high bioavailability of calcium and phosphate ions is likely to cease further demineralisation and promote remineralisation, even in the absence of a specialised calcium and phosphate delivery system such as CPP-ACP. Lovel (2008) suggested that remineralisation may even take place in a distilled water solution alone. This *in vitro* study concluded that there was no statistically significant difference in remineralisation efficacy of GC Tooth Mousse™ compared with saliva or water. Therefore, when assessing the effectiveness of the addition of CPP-ACP as a remineralising agent it is important to view the results in comparison with the *in situ* fluoride group and not with the baseline parameters.

The addition of CPP-ACP does seem to suggest an improved remineralisation effect compared with fluoride paste alone with statistically significant reductions in mineral loss ΔZ ($p=0.023$), lesion depth ($p=0.037$) and lesion width ($p=0.015$). The addition of CPP-ACP paste resulted in a 9.2% reduction in mineral loss ΔZ, 9.5% reduction in lesion depth and a 10.8% reduction in lesion width compared with fluoride toothpaste alone. Shen et al, (2011) in a similarly designed *in situ* randomised controlled trial found comparable subsurface remineralisation results. Tooth Mousse™ (CPP-ACP) solutions demonstrated a statistically significant increase in remineralisation with a 24.2% percentage mineral gain compared to 7.9% and 16.3% for 1000ppm and 5000ppm fluoride solutions respectively. It was suggested that CPP–ACP containing products enhance the level of stabilised, bioavailable calcium and phosphate ions in saliva by a factor of 6.5 times. However Vanichvatana et al (2013) in another *in situ* study suggested that fluoride toothpaste and fluoride toothpaste combined with CPP-ACP yielded a 15.5% and 16.8% reduction in lesion area respectively. They concluded that the addition of CPP-ACP paste did not produce a statistically significant increase in remineralisation. However with an *in situ* period of only 14 days, it is possible that with an increased observation time a significant difference may be achieved. A recent systematic review of the literature suggested more high quality randomised control trials are required to establish the supplementary benefit of CPP-ACP application in addition to fluoride toothpaste (Li et al, 2014).

In this study a large intra-participant and inter-participant variation in post-treatment changes was recorded. This was particularly noted for lesion depth and lesion width changes. An explanation for this may be due to the fact that subsurface lesions often remineralise in an uneven or irregular

distribution. To minimise this, additional consideration was taken in the assessment of suitable subsurface lesions using QLF to ensure they appeared to be as regularly distributed at the surface as possible. Remineralisation traditionally occurs from the base of the lesion with continued mineral deposition gradually reducing the lesion depth; however this is a complex and dynamic process. Due to opposing fluxes of ions both mineral loss and deposition can be occurring simultaneously within the depth of the lesion (Ten Cate, 1990). TMR sections only assess a small area of the total lesion which can be at various stages of remineralisation and or demineralisation. Sectioning of the enamel samples after the *in situ* phase was extremely delicate and difficult. Any inaccuracy in producing the required parallel sections will alter the cross-sectional dimensions of the lesion and may alter the resulting mineral profile distribution.

Another explanation of the large inter participant variation is the varied environmental conditions in which the *in situ* carriers were placed. Despite regular oral hygiene and dietary advice many orthodontic patients still have an oral environment which may support demineralisation. Increased quantity and frequency of cariogenic food and drinks along with poor plaque control can predispose *in vivo* enamel and also *in situ* enamel samples to prolonged mineral loss. Another important intra-oral factor influencing the demineralisation process is the composition and quantity of saliva flow which can vary considerably, depending on the individual. In an attempt to standardise oral conditions, participants were given written oral hygiene instructions, detailed information of how to use the treatment pastes and asked not to use any additional hygiene products. Also they were instructed not to rinse after brushing as this can affect the retention of fluoride in the saliva and in the oral cavity. Despite these instructions it is possible that some participants will not have accurately followed the prescribed regime. The wide range in fluoride used (33.9 - 91.1g) and GC Tooth Mousse™ (18.2 - 29.3g) is suggestive that some participants complied with the prescribed instructions to various degrees. However this is unlikely to have impacted the results obtained as no statistically significant correlations were obtained between the quantity of GC Tooth Mousse™ used and mineral loss, lesion depth or lesion width.

8.2 Limitations of study

It was not possible for the position of the enamel carriers intra-orally in the premolar region to be completely standardised. Not only was their placement dependent on adequate space being available, but also the adjacent dental and alveolar anatomy had to be taken into account to allow for a passive and comfortable fit. In theory it could be suggested that the side of the mouth the carrier was placed may affect the remineralisation as right-handed brushers on average tend to spend less time brushing the right side of their mouth and therefore have reduced plaque control and fluoride exposure. However, Benson (1999) found no difference in remineralisation between in *situ* enamel placed on the dominant or non-dominant side of the mouth.

This study was blinded through the use of opaque insulation tape on both the regular fluoride toothpaste and GC Tooth Mousse™. However as these tubes were a different size and shape, this could have affected the standardisation required for the blinding process. Although insulation tape was carefully and securely placed, it could have been possible for participants to remove the tape at a later stage. Ideally both intervention pastes should have been in identical size and shape containers which were professionally concealed to ensure complete confidentiality.

A major limitation of *in situ* studies is procedural loss. In this study 3 out of 26 enamel samples were lost or damaged (12%). One enamel carrier (participant 2) was lost during the *in situ* phase due to an inadequate join between the outer and inner stainless steel ring. Although the base enamel carrier remained in place, the outer metal ring and therefore the Dacron gauze retaining the enamel sample became detached. This is a delicate procedure as over welding of this join can damage the Dacron gauze resulting in early detachment. There was an increased risk of this happening if the Dacron gauze was too dry during welding as it became very friable. Slightly moistening and then letting dry at room temperature for 5 minutes aided retention of a small amount of moisture to prevent it from being friable but not enough moisture that it would affect the spot welding join between the two metals. 2 enamel samples (8%) were irreversibly damaged during the TMR preparation process. The sectioning and polishing of enamel samples is very intricate and delicate and as a result damage can easily occur. Often it is difficult to assess if the sample is damaged until TMR image capture has been completed. Enamel samples post treatment were sectioned transversely instead of longitudinally (as described in section 5.9) as this increased the number of sections obtained. However due to their reduced size this made handling incredibly difficult and increased the risk of damage. The 8% loss due to TMR sectioning and processing in this study is favourable compared with other similar studies ranging from 13-22% loss (Benson, 2009; Bryniarska, 2012).

An additional clinical phase was carried out when samples were lost or damaged, however this was not possible in one case (participant 6) as the participant had already had their fixed appliances removed. This resulted in one incomplete set of data for treatment A (Fluoride group). Although a complete set of data was obtained for treatment B (CPP-ACP group), the corresponding sample from participant 6 was excluded from statistical probability testing to ensure appropriate levels of certainty. Therefore only 11 of the 12 participants were used for the final statistical analysis, which may have affected the power of the study. Ideally more participants could have been recruited to allow for drop out and for procedural loss. However this has to be carefully balanced with justifying the exposure of additional orthodontic patients to the risks of an interventional trial.

As the accuracy of TMR for mineral loss ΔZ is 200 vol%µm and lesion depth (L_d) is approximately 5µm (Arends and ten Bosch 1992), it could be suggested that lesions of increased mineral loss and lesion dimensions would have a reduced percentage error and therefore improved precision. Other *in situ* TMR studies have suggested baseline subsurface lesions of mineral loss ΔZ range 2000 – 3000 vol%µm may be more suitable in accurately quantifying remineralisation (Shen et al, 2011, Cochrane et al, 2008). However this may require increased *in situ* time to achieve an adequate change to demonstrate a statistically significant remineralisation effect.

In this study the mean baseline parameters were mineral loss ΔZ 1180 (vol%µm), lesion depth 58.7µm and lesion width 48.2µm. Baseline lesions in this study were of smaller dimensions and less mineral loss than ideally would be used, but this was limited by the difficulty in production of a large number of evenly demineralised high quality subsurface lesions. This was compounded by an inability to quantitatively analyse the mineral content profile until after destructive TMR sectioning took place.

Every effort was taken to try to reproduce the *in vivo* clinical scenario of orthodontic demineralisation by using human premolar enamel, placed *in situ* in the premolar area, in participants receiving active orthodontic treatment. Standardisation of baseline lesions was possible due to the *in vitro* subsurface lesion creation process. Destructive TMR analysis allowed for detailed and accurate evaluation of the mineral changes occurring. However despite these considerations it is never possible in an *in situ* model to fully replicate the complex natural biological process of enamel demineralisation occurring in health living *in vivo* tissue.

An *in vivo* model investigates enamel demineralisation in the natural biological oral environment and therefore can potentially produce highly applicable and relevant findings. However a major

limitation includes the inability to standardise the baseline *in vivo* lesion parameters and the location of their placement. Also the inability to utilise destructive quantitative mineral analysis techniques may reduce the accuracy of results obtained.

8.3 Future areas for research

The *in situ* study model is an effective method of quantitatively analysing the demineralisation and remineralisation process. Further *in situ* research should utilise baseline lesion of larger dimensions and increased mineral loss ΔZ 1500-2000 vol%µm as this may minimise the relative effect of measurement error. Recent research demonstrating the effectiveness of high concentration fluoride toothpastes (Sonesson et al 2014) would propose that future trials should involve the comparison of 3 intervention arms; regular fluoride (1450ppm), high concentration fluoride (5000ppm) and CPP-ACP paste.

More *in vivo* research may also be beneficial as this can accurately replicate the complex and dynamic demineralisation process. As non-destructive quantitative analysis is required for such studies, QLF is suggested as the most appropriate method of identifying, analysing and monitoring the demineralisation process. QLF could be used to identify and quantify patients with white spot lesion prior to and during orthodontic treatment. Those with white spot lesions could be randomly allocated to one of the remineralising treatment groups, to allow for *in vivo* comparison of remineralising effectiveness.

9.0 Conclusions

In this study the null hypothesis that there was no difference between the remineralising potential abilities of GC Tooth Mousse™ and fluoride toothpaste in orthodontic patients was rejected. Despite a wide range in intra-participant and inter-participant data, an overall trend of remineralisation was observed in both treatment groups as demonstrated by reduction in mineral loss ΔZ, reduction in lesion depth and reduction in lesion width. On average, all baseline lesions remineralised during the *in-situ* phase regardless of treatment group allocation, however the fluoride toothpaste combined with GC Tooth Mousse™ (CPP-ACP) group demonstrated an increased remineralising effect. A statistically significant reduction in mineral loss ΔZ ($p=0.023$), lesion depth ($p=0.037$) and lesion width ($p=0.015$) was observed in the fluoride toothpaste combined with CPP-ACP group compared to the fluoride toothpaste alone.

This study suggests that the application of GC Tooth Mousse™ in addition to regular fluoride paste does have an increasing remineralisation effect on subsurface enamel lesions in orthodontic patients.

9.1 Implications

Enamel demineralisation is a common adverse side effect of orthodontic treatment. The use of toothpastes containing CPP-ACP such as GC Tooth Mousse™ has been shown to reduce this demineralisation process and promote remineralisation. CPP-ACP pastes in combination with regular fluoride toothpaste should be recommended for patients undergoing orthodontic treatment who are at high risk of demineralisation or who have demonstrated early signs of white spot lesion formation. CPP-ACP paste may also be useful for patients after fixed appliances to promote remineralisation of areas where orthodontic demineralisation is evident.

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11.0 Appendices

Appendix 1. Participant instruction sheets

Appendix 1a

Participant Instruction Sheets

Title of Research Project:

An in situ study to determine the effects of calcium based toothpastes in orthodontic patients

Instructions for Toothpaste A

Brush your teeth with the toothbrush and toothpaste A provided.

Use a pea-sized amount of toothpaste and brush for two minutes.

Do not rinse your mouth, spit out the excess if you wish.

Brush your teeth twice a day.

Do not use any other mouth rinses or toothpastes.

Appendix 1b

UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

Participant Instruction Sheets

Title of Research Project:

An in situ study to determine the effects of calcium based toothpastes in orthodontic patients

Instructions for Toothpaste A + Paste B

Brush your teeth with the toothbrush and toothpaste A provided.

Use a pea-sized amount of toothpaste and brush for two minutes.

Do not rinse your mouth, spit out the excess if you wish.

Take a pea-sized amount of paste B

Wipe this across your teeth, including the carrier.

Leave on the tooth surface undisturbed for a minimum of 3 minutes

Then use your tongue to spread the paste throughout the mouth.

Hold in the mouth for a further 1-2 minutes

Spit out thoroughly but avoid rinsing

Clean your teeth in this way twice a day.

Do not use any other mouth rinses or toothpastes.

Appendix 2. Participant information sheets

Appendix 2a

Department of clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place L3 5PS

PARENT/ LEGAL GUARDIAN INFORMATION SHEET

An in situ study to determine the effects of calcium based toothpastes in orthodontic patients

What is the purpose of the study?
During fixed brace treatment teeth are susceptible to loss of mineral which may appear as white or brown marks on the teeth. This is due to plaque accumulating on the teeth around the brace where effective tooth brushing is more difficult. Fluoride toothpastes are recommended to prevent the occurrence of these white spots. Calcium based toothpastes are now available and have been shown in some studies to be more effective at preventing this tooth damage. The purpose of this study is to find out if calcium based toothpastes in combination with fluoride toothpastes are more effective than regular fluoride toothpastes alone.

An in situ study is a study where the participants are the carrier for a specimen of enamel which has previously been treated to mimic a white spot on a tooth. This allows us to study the effectiveness of toothpastes on a particular specimen of enamel that has previously been sterilised.

A Cross-over study is where each participant has the opportunity to use both toothpastes in turn. There is a rest period between treatments so that one toothpaste can not affect the result of the other toothpaste being tested.

Has this study been approved?
Yes. Liverpool Local Research Ethics Committee has given approval for this study.

Who is paying for the study?
The School of Dental Sciences is paying for the study. The Royal Liverpool and Broadgreen University Hospital Trust and the University of Liverpool are co-sponsoring the study.

Who will be conducting the study?
The study is being run by Prof. Susan Higham (Professor in Oral Microbiology), Dr Norah Flannigan (Senior Clinical Lecturer in Orthodontic) and Mr Andrew Garry (Specialist Registrar in Orthodontics).

Why has my child been asked to take part?
Your child had been asked to take part because they are undergoing orthodontic treatment with fixed braces on their teeth

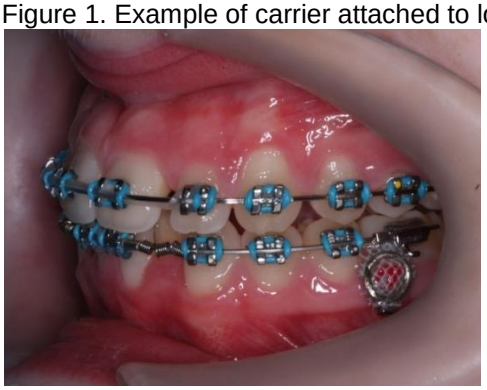
What will I have to do?
During a routine brace appointment a small carrier containing a piece of sterilised human enamel will be attached to the wire part of the brace, positioned towards the back part of the lower

brace (Figure 1). This will not affect how the brace works. Your child will be given a toothbrush and either one or two toothpastes and given instructions on their use.. 4 weeks later at the next orthodontic appointment the carrier will be removed from the brace and sent to the laboratory for analysis. The brace will be adjusted as usual. Your child will then be instructed to use a standard fluoride toothpaste for the next 4 weeks with no carrier on the brace. At the next 4 week appointment a new carrier will be attached to the brace and your child will be given another paste/pastes to use. At the next orthodontic appointment in 4 weeks the second carrier will be removed and the trial will be finished

Your child will have in total 2 carriers attached to your brace with 2 different types of toothpastes to use. One of the regimes will involve brushing with a pea-sized amount of fluoride toothpaste for 2 minutes twice a day. The other regime will involve brushing with a pea-sized amount of fluoride toothpaste for 2 minutes twice a day in addition to a calcium-based toothpaste being applied to the teeth for 5 minutes twice a day.

Your child will be asked not to use any other type of toothpaste or mouth rinse during the trial.

Figure 1. Example of carrier attached to lower brace



How long will the study last?

The study will last for approximately 4 months of your child's treatment.

What if I don't want to take part?

Your child's treatment will continue as normal. You should not feel obliged to take part and you do not have to give a reason if you don't want to. If you do take part, but decide later that you don't want to continue you can withdraw at any time without giving a reason.

What if you have a question or there is a problem on the study?

If you have a concern about any aspect of this study, you should speak to the researchers who will do their best to answer any questions on 0151 706 5210. If you remain unhappy and wish to complain formally, you can do this through the Patient Advice Liaison service on 0151 706 3216 or by emailing; complaints@rlbuht.nhs.uk. Details can be obtained from http://www.rlbuht.nhs.uk/for_patients/Complaints_FAQs.asp

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How will my child's data be collected and managed?

All information about your child will be anonymous. As soon as we have collected the necessary data all information which identifies your child will be removed and replaced by a code number. The data will be processed and analysed by the research staff of the study. The person responsible for security and access to your data is Dr Norah Flannigan, chief investigator of the study. Data will be stored for 10 years in compliance with hospital policy.

What do I do if I would like my child to take part?

If you would like your child to take part, please sign the relevant sections of the consent form that you have been provided with.

Thank you for taking the time to read this

Appendix 2b

UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

Department of clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place L3 5PS

CHILD INFORMATION SHEET

An in situ study to determine the effects of calcium based toothpastes in orthodontic patients

What is the purpose of the study?

During fixed brace treatment teeth are susceptible to tooth damage which may appear as white or brown marks on the teeth. Fluoride toothpastes are recommended to prevent the occurrence of these white spots. Calcium based toothpastes are now available and have been shown in some studies to be more effective at preventing this tooth damage. The purpose of this study is to find out if calcium based toothpastes in combination with fluoride toothpastes are more effective than regular fluoride toothpastes alone.

In this study a small section of sterilised tooth is attached to the brace to study the effectiveness of different toothpastes. Both toothpastes will be used in turn, with a rest period between treatments so that one toothpaste can not affect the result of the other toothpaste being tested.

Has this study been approved?

Yes. Liverpool Local Research Ethics Committee has given approval for this study.

Who is paying for the study?

The School of Dental Sciences is paying for the study. The Royal Liverpool and Broadgreen University Hospital Trust and the University of Liverpool are co-sponsoring the study

Who will be conducting the study?

The study is being run by Prof. Susan Higham (Professor in Oral Microbiology), Dr Norah Flannigan (Senior Clinical Lecturer in Orthodontics) and Mr Andrew Garry (Specialist Registrar in Orthodontics).

Why have I been asked to take part?

You have been asked to take part because you are undergoing orthodontic treatment with fixed braces on your teeth.

What will I have to do?

During your routine brace appointment a small carrier containing a piece of sterilised human tooth will be attached to the wire part of your brace, positioned towards the back part of your lower brace (Figure 1). This will not affect how your brace works. You will be given a toothbrush and either one or two toothpastes and given instructions on their use. 4 weeks later at your next orthodontic appointment the carrier will be removed from your brace and sent to the laboratory for analysis. Your brace will be adjusted as usual. You will then be instructed to use a standard fluoride toothpaste for the next 4 weeks with no carrier on your brace. At your next 4 week appointment a new carrier will be

attached to the brace and you will be given another paste/pastes to use. At the next orthodontic appointment in 4 weeks the second carrier will be removed and the trial will be finished.

You will have in total 2 carriers attached to your brace with 2 different types of toothpastes to use. One of the regimes will involve brushing with a pea-sized amount of fluoride toothpaste for 2 minutes twice a day. The other regime will involve brushing with a pea-sized amount of fluoride toothpaste for 2 minutes twice a day in addition to a calcium-based toothpaste being applied to the teeth for 5 minutes twice a day.

You will be asked not to use any other type of toothpaste or mouth rinse during the trial.

Fig.1. Example of carrier attached to lower brace

How long will the study last?

The study will last for approximately 4 months of your treatment

What if I don't want to take part?

Your treatment will continue as normal. You should not feel obliged to take part and you do not have to give a reason if you don't want to. If you do take part, but decide later that you don't want to continue you can withdraw at any time without giving a reason.

What if you have a question or there is a problem on the study?

If you have a concern about any aspect of this study, you should speak to the researchers who will do their best to answer any questions on 0151 706 5210. If you remain unhappy and wish to complain formally, you can do this through the Patient Advice Liaison service on 0151 706 3216 or by emailing; complaints@rlbuht.nhs.uk. Details can be obtained from http://www.rlbuht.nhs.uk/for_patients/Complaints_FAQs.asp

How will my data be collected and managed?

All information about you will be anonymous. As soon as we have collected the necessary data all information which identifies you will be removed and replaced by a code number. The data will be processed and analysed by the research staff of the study. The person responsible for security and access to your data is Dr Norah Flannigan, chief investigator of the study. Data will be stored for 10 years in compliance with hospital policy.

What do I do if I would like to take part?

If you would like to take part, please sign the relevant sections of the consent form that you have been provided with.

Thank you for taking the time to read this

Appendix 3. Consent forms

Appendix 3a

UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

CHILD ASSENT FORM

Centre Number:

Study Number:

Participant Identification Number:

Title of Research Project:
An in situ study to determine the effects of calcium based toothpastes in orthodontic patients.

Researcher: Andrew Garry

	Please initial box
1. I confirm that I have read and have understood the information sheet dated 16th September 2013 (Version 1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my rights being affected.	☐
3. I understand that my medical records may be looked at by regulatory authorities or by persons from the Trust where it is relevant to my taking part in this research. I give permission for these individuals to access this information	☐
4. I understand that the data collected during the study will be analysed by the study investigators. I give permission for these individulas to have access to my records.	☐
5. I agree to take part in the above study.	☐

Name of Participant	Date	Signature
Name of Person taking consent	Date	Signature

The contact details of lead Researcher are:
Dr Norah Flannigan
Orthodontic Department, Liverpool Dental Hospital,
0151 706 5210
nlf@liverpool.ac.uk

Appendix 3b

UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

PARENT/ LEGAL GUARDIAN CONSENT FORM

Centre Number:

Study Number:

Participant Identification Number:

Title of Research Project:
An in situ study to determine the effects of calcium based toothpastes in orthodontic patients.

Researcher : Andrew Garry

	Please initial box
1. I confirm that I have read and have understood the information sheet dated 16th December 2013 (Version 1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
2. I understand that my child's participation is voluntary and that I am free to withdraw my child at any time without giving any reason, without my rights being affected.	☐
3. I understand that my child's medical records may be looked at by regulatory authorities or by persons from the Trust where it is relevant to my child taking part in this research. I give permission for these individuals to access this information	☐
4. I understand that the data collected during the study will be analysed by the study investigators. I give permission for these individulas to have access to my child records.	☐
5. I agree for my child to take part in the above study.	☐

Parent/ Legal Guardian	Date	Signature
Name of Person taking consent	Date	Signature

The contact details of lead Researcher are:
Dr Norah Flannigan
Orthodontic Department, Liverpool Dental Hospital,
01517065210
nlf@liverpool.ac.uk

Appendix 3c

UNIVERSITY OF LIVERPOOL

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

PARENT/ LEGAL GUARDIAN CONSENT FORM

Centre Number:

Study Number:

Participant Identification Number:

Title of Research Project:
An in situ study to determine the effects of calcium based toothpastes in orthodontic patients.

Researcher : Andrew Garry

		Please initial box
6.	I confirm that I have read and have understood the information sheet dated 16th December 2013 (Version 1.2) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	☐
7.	I understand that my child's participation is voluntary and that I am free to withdraw my child at any time without giving any reason, without my rights being affected.	☐
8.	I understand that my child's medical records may be looked at by regulatory authorities or by persons from the Trust where it is relevant to my child taking part in this research. I give permission for these individuals to access this information	☐
9.	I understand that the data collected during the study will be analysed by the study investigators. I give permission for these individulas to have access to my child records.	☐
10.	I agree for my child to take part in the above study.	☐

Parent/ Legal Guardian	Date	Signature
Name of Person taking consent	Date	Signature

The contact details of lead Researcher are:
Dr Norah Flannigan
Orthodontic Department, Liverpool Dental Hospital,
01517065210
nlf@liverpool.ac.uk

Appendix 4. Baseline raw data

Appendix 4a

Raw data for baseline mineral loss (%Volμm)

	Phase 1			Phase 2			Phase 3		
Subject	Intervention	specimen	Baseline mineral loss (%Volμm)	Intervention	Specimen	Baseline mineral loss (%Volμm)	Intervention	Specimen	Baeline mineral loss (%Volμm)
1	B	4a	1273	A	4b	1273.0			
2	B	20a		A	20b	1130.0	B	6a	1292.0
3	B	3b	1100.0	A	3a	1100.0			
4	A	7a	1530.0	B	30a	1126.0			
5	A	19a	1213.0	B	19b	1213.0			
6	B	1a	1223.0	A	1b				
7	B	22a	1120.0	A	22b	1120.0			
8	A	25a		B	53a	1115.0	A	6b	1293.0
9	A	46a	1140.0	B	46b	1140.0			
10	B	40a	1252.0	A	40b	1252.0			
11	A	51a	1090.0	B	51b	1090.0			
12	A	47a	1176.0	B	47b	1176.0			

Appendix 4b

Raw data for baseline lesion depth (μm)

	Phase 1			Phase 2			Phase 3		
Subject	Intervention	Specimen	Baseline Lesion Depth (μm)	Intervention	Specimen	Baseline Lesion Depth (μm)	Intervention	Specimen	Baseline Lesion Depth (μm)
1	B	4a	58.4	A	4b	58.4			
2	B	20a		A	20b	55.9	B	6a	68.2
3	B	3b	56.6	A	3a	56.6			
4	A	7a	69.5	B	30a	48.0			
5	A	19a	54.4	B	19b	54.4			
6	B	1a	69.7	A	1b				
7	B	22a	64.9	A	22b	64.9			
8	A	25a		B	53a	62.5	A	6b	68.2
9	A	46a	57.3	B	46b	57.3			
10	B	40a	63.6	A	40b	63.6			
11	A	51a	56.2	B	51b	56.2			
12	A	47a	57.7	B	47b	57.7			

Appendix 4c

Raw data for baseline lesion width (µm)

	Phase 1			Phase 2			Phase 3		
Subject	Intervention	Specimen	Baseline Lesion Width (µm)	Intervention	Specimen	Baseline Lesion Width (µm)	Intervention	Specimen	Baseline Lesion Width (µm)
1	B	4a	47.2	A	4b	47.2			
2	B	20a		A	20b	38.8	B	6a	55.4
3	B	3b	47.2	A	3a	47.2			
4	A	7a	59.8	B	30a	40.8			
5	A	19a	45.0	B	19b	45.0			
6	B	1a	59.7	A	1b				
7	B	22a	54.6	A	22b	54.6			
8	A	25a		B	53a	52.4	A	6b	55.4
9	A	46a	47.6	B	46b	47.6			
10	B	40a	50.3	A	40b	50.3			
11	A	51a	43.8	B	51b	43.8			
12	A	47a	44.2	B	47b	44.2			

Appendix 5. Post treatment raw data

Appendix 5a

Raw data for post treatment mineral loss (%Volµm)

	Phase 1			Phase 2			Phase 3		
Subject	Intervention	specimen	End mineral loss (%Volµm)	Intervention	Specimen	End mineral loss (%Volµm)	Intervention	Specimen	End mineral loss (%Volµm)
1	B	4a	1042	A	4b	1310.0			
2	B	20a		A	20b	845.0	B	6a	923.0
3	B	3b	1044.0	A	3a	1115.0			
4	A	7a	1053.0	B	30a	1155.0			
5	A	19a	880.0	B	19b	820.0			
6	B	1a	750.0	A	1b				
7	B	22a	843.0	A	22b	913.0			
8	A	25a		B	53a	830.0	A	6b	1010.0
9	A	46a	1042.0	B	46b	716.0			
10	B	40a	875.0	A	40b	976.6			
11	A	51a	104.2	B	51b	877.0			
12	A	47a	1006.6	B	47b	726.0			

Appendix 5b

Raw data for post treatment lesion depth (µm)

	Phase 1			Phase 2			Phase 3		
Subject	Intervention	Specimen	End Lesion Depth (µm)	Intervention	Specimen	End Lesion Depth (µm)	Intervention	Specimen	End Lesion Depth (µm)
1	B	4a	54.0	A	4b	61.0			
2	B	20a		A	20b	49.3	B	6a	60.5
3	B	3b	46.6	A	3a	67.8			
4	A	7a	62.2	B	30a	52.2			
5	A	19a	51.6	B	19b	53.2			
6	B	1a	49.1	A	1b				
7	B	22a	51.9	A	22b	59.5			
8	A	25a		B	53a	53.8	A	6b	59.8
9	A	46a	56.0	B	46b	52.3			
10	B	40a	53.1	A	40b	56.5			
11	A	51a	57.7	B	51b	51.1			
12	A	47a	60.3	B	47b	48.5			

Appendix 5c

Raw data for post treatment lesion width (µm)

	Phase 1			Phase 2			Phase 3		
Subject	Intervention	Specimen	End Lesion Width (µm)	Intervention	Specimen	End Lesion Width (µm)	Intervention	Specimen	End Lesion Width (µm)
1	B	4a	40.3	A	4b	50.2			
2	B	20a		A	20b	35.0	B	6a	45.3
3	B	3b	35.2	A	3a	56.8			
4	A	7a	46.0	B	30a	41.0			
5	A	19a	41.2	B	19b	44.5			
6	B	1a	37.4	A	1b				
7	B	22a	39.3	A	22b	50.1			
8	A	25a		B	53a	39.7	A	6b	46.2
9	A	46a	47.5	B	46b	40.2			
10	B	40a	40.8	A	40b	47.0			
11	A	51a	46.2	B	51b	38.0			
12	A	47a	40.2	B	47b	40.0			