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

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Structured Abstract

Aims

To measure demineralisation/remineralisation of sub-surface caries-like lesions placed *in situ* on an orthodontic appliance and treated with Tooth Mousse™ (GC Corporation, Tokyo, Japan).

Objectives

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

Null Hypothesis

This study will test the null hypothesis that there is no difference between the remineralising abilities of Tooth Mousse™ used alongside normal fluoride toothpastes in orthodontic patients.

Design

This was a randomised cross-over *in situ* study.

Ethical Approval

Ethical approval was sought and obtained from the National Research Ethics service (NRES) and the NHS Research and Development Offices. This study was given the REC reference number: 10/H1005/85.

Setting

The Oral Health Research laboratories and The Orthodontic Clinic. Liverpool University Dental Hospital.

Sample Size

The sample size was calculated on the primary outcome of mineral loss (vol%μm). Data were used from a previous study (Benson 2009) using the Transverse Microradiography (TMR) technique, the residual standard deviation was estimated at 189 vol%μm . Four Latin square blocks, with a total of 12 participants, would allow detection of pairwise differences between groups of 300 vol%μm, with 90% power, at the 5% confidence level. To allow for possible dropout, a fifth block was added giving a total of 15 participants.

Participant selection

Inclusion criteria;

a. Age 12 to 16 years.
b. Had adequate space between the lower 2nd premolar region and lower 1st molar region in order to provide space to place the carrier on the archwire.
c. Subjects were in good health.

Exclusion criteria;

a. The subjects were allergic to milk products.
b. The subjects had taken antibiotics in the last 2 months.

Randomisation

Randomisation was generated in blocks of 3, from random number tables by a

statistician who was not involved in the recruitment or allocation of the interventions. The method of randomisation was by envelope concealment. The patients were randomly allocated to one of three possible orders of intervention; A, B, C; B, C, A or C, A, B.

Interventions

Each participant received the three pastes in random order determined by a Latin square design in blocks of 3.

A. A normal fluoride toothpaste (1450ppm).

B. A normal fluoride toothpaste (1450ppm) and topically applied Tooth Mousse™ Plus toothpaste, which incorporates CPP-ACP and fluoride (900ppm) to the carrier only.

C. A normal fluoride toothpaste and topically applied Tooth Mousse™ to be applied to the carrier only.

Blinding

Blinding of the participants was only possible with the Tooth Mousse™ and the Tooth Mousse™ Plus. The tubes were covered in insulation tape. The samples were recoded prior to being sectioned and analysed so the principal investigator was blind to the subject and intervention. Following TMR analysis the coding was revealed.

Samples

Following consent a previously prepared demineralised human premolar enamel sample that had been sterilised was placed in a carrier, which was then attached to the archwire. The carrier was a modified version of a carrier used in previous clinical studies (Benson 2000) and consisted of a stainless steel attachment. Base-line TMR

analysis had been carried out to determine the degree of demineralisation of the enamel samples.

Method

Each participant was provided with the intervention and a toothbrush and was asked to brush their teeth for two minutes twice a day with a pea sized amount for a 4-week period. The participants were asked not to use any other toothpaste or mouth rinses during the 4-week period.

For example; Treatment A paste is given to the subject 1 and instructed to use it for 4 weeks. At the four-week appointment a routine orthodontic appointment was carried out and the carrier with the enamel sample was removed from the mouth and taken to the laboratory for TMR analysis. Subject 1 was then instructed to continue with their normal oral hygiene procedures in the following 4 weeks. This was the washout period. Subject 1 then returned for another routine orthodontic appointment and insertion of a new enamel sample in a carrier. The subject was given treatment B paste to use for 4 weeks. This continued until the subject had used all the treatment pastes. The minimum length of time each participant was involved in the study was 20 weeks, including the washout periods.

Outcome measures

The main outcome measure was the difference in mineral loss/gain between the control specimens and the specimens used in the participants. Analysis of mineral content profiles, three parameters were obtained; mineral loss ΔZ (vol%μm) lesion depth Ld (μm) and lesion width Lw (μm). From these, the data were normalised and a percentage changes in mineral loss, lesion depth and lesion width were calculated. This was

completed by dividing the sample value by the control value and multiplying by 100 (Strang et al 1987).

Statistical analysis

The data from TMR analyses were entered into the Statistical Package for Social Sciences (SPSS v. 20, Chicago, Illinois, USA) and tested for normality using frequency histograms. Hypothesis testing was carried out using a 3-way ANOVA. A Pearson correlation coefficient was calculated for the quantity of Tooth Mousse™ versus the percentage change in mineralisation.

Results

1. TMR analysis revealed that there was no significant effect on the percentage change in mineral loss for the intervention ($p=0.278$), the order in which they received the intervention ($p=0.625$) or for each subject ($p=0.66$). There was no significant effect on the lesion depth for the intervention ($p=0.184$), the order ($p=0.474$) or the subject ($p=0.957$). There was also no significant effect on the lesion depth for the intervention ($p=0.553$), the order ($p=0.582$) or the subject ($p=0.977$).

2. There was large individual variability, with large ranges and no association between intervention and degree of mineral loss.

3. There was no correlation between the amount of mineral loss and the amount of Tooth Mousse™ or Tooth Mousse™ Plus used ($r=0.144$).

4. 22% of the samples were lost.

Conclusion

There were large intra-subject and inter-subject ranges with no association between intervention and degree of mineral loss or gain.

There was no significant difference between the three interventions on mineral gain or loss.

A large number of samples were lost to analysis and this could have had a large effect on the results.

In this study the null hypothesis that there was no difference between the remineralising abilities of Tooth Mousse™ with normal fluoride toothpastes in orthodontic patients could not be rejected.

Based on the results of this study, orthodontic patients are at risk of enamel demineralisation during treatment and attentiveness by the clinician should be maintained at all times.

The aims of this study have been met, however, further improvements of the *in situ* method are required and a movement towards in vivo method is needed in order to advance our knowledge of the remineralising potential of this group of agents.

Implications

Following this study, future research should concentrate on developing the *in situ* model such that it is as representative as far as possible to the *in vivo* method. The work completed in this study should form the basis for subsequent investigation. This *in situ* model is as close a representation as possible to the *in vivo* model, however it cannot fully replicate the *in vivo* model, which could also have had an impact on the results.

In order to assess the full remineralising efficacy of casein phosphopeptide, development of the *in situ* model is recommended. The other possibility is to move to an *in vivo* model, which would require a non-destructive method of measuring demineralisation such as QLF. A randomised controlled clinical trial is an ideal method to answer this question.

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1.0 Literature Review

1.1 Dental Caries

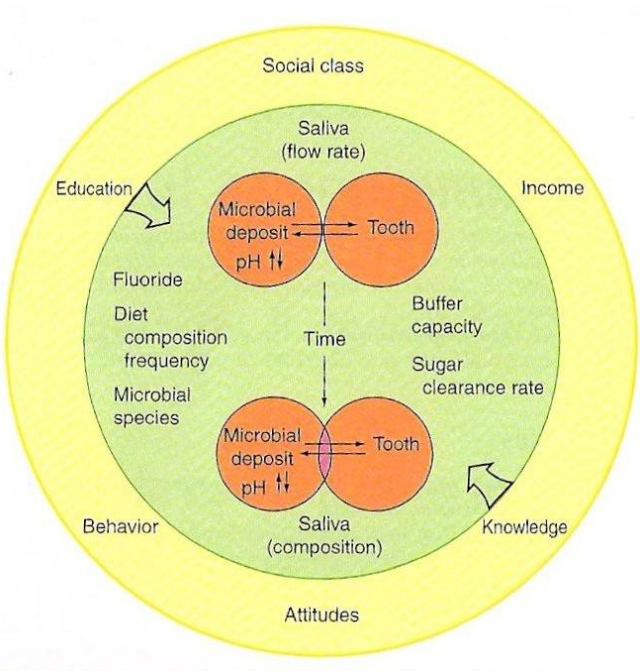
1.1.1 Definition

The term dental caries is applied to the signs and symptoms of a localised chemical dissolution of the tooth surface caused by metabolic events taking place in the bioflm (dental plaque) covering the affected area. The destruction can affect enamel, dentine and cementum (Fejerskov and Kidd 2008).

1.1.2 Pathogenesis of dental caries

Dental caries lesions occur due to a shift in the ecology and metabolic activity of the biofilm whereby an imbalance in the equilibrium between tooth mineral and biofilm fluid has developed. In the natural state there is a continual process of remineralisation and demineralisation taking place. Whether there is net loss or gain is part of a continuous process and this depends on a number of influences on the metabolic processes, such as composition and thickness of the biofilm, the salivary secretion rate and composition, the diet and fluoride ion concentration in the oral fluids. This is demonstrated in Figure 1.1.1 (Fejerskov and Kidd 2008).

Figure 1.1.1 Illustration of influences of the carious process, taken from Fejerskov and Kidd 2008



The features of an early carious lesion of the enamel is that it is subsurface and most of the mineral loss occurs under an intact mineral layer. This is first seen at the outer enamel surface as partial dissolution of the crystal surface with a commensurate increase in enamel porosity. Further increases in enamel porosity lead to preferential removal of mineral from tissue deep to the outer surface. In this way, an enamel white spot lesion is seen clinically. Histologically the lesion is wedge shaped with the base of the wedge on the enamel surface and the apex pointing towards the amelodentinal junction. These lesions are comprised of four layers, the deepest being the translucent and dark zones, which precede the body of the lesion in which 30 to 50% of mineral has been lost. These lesions have an intact surface layer, which is 20-50μm thick and is well mineralised (Silverstone 1973, Fejerskov and Kidd 2008). This was thought to occur because the hydroxyapatite crystals near the surface have more fluoride and thus are

more resistant to dissolution than the crystals in the interior, however it is now suggested to be due to the result of a remineralisation process which leads to re-deposition of calcium and phosphate, as well as fluoride ions in the partly dissolved surface zone where the caries process is active (Fejerskov et al 1996). Salivary proline-rich proteins and salivary inhibitors such as statherin may also have a protective role as they are present in the pellicle. They prevent direct crystal growth of these salts onto the enamel surfaces and they also tend to prevent demineralisation (Hay et al 1984).

White spot lesions are partially reversible and this can be initiated by redressing the demineralisation – remineralisation balance in favour of mineral gain. This requires exposure to solutions, which are supersaturated with respect to apatite. This can be brought about by effective changes in plaque control, diet or by the appropriate use of fluoride. The body of the lesion is seen as a white scar under a shiny hard surface. Due to slow diffusion, it is not possible to maintain the necessary supersaturation in the lesion fluid and so remineralisation of the lesion body does not occur.

Further demineralisation however, causes the carious lesion to progress to the amelodentinal junction. The demineralisation follows the rods and thus the lesion assumes the shape of a cone towards the amelodentinal junction. Following cavitation, the carious lesion is irreversible. Subsequent caries progression through dentine occurs by demineralisation and then by proteolytic breakdown of the matrix. Despite deposition of reactionary dentine and sealing of dentinal tubules by odontoblasts, further advancement of the carious lesion will cause further dentine destruction with exposure of the pulp and subsequent pulpitis (Soames and Southam 2008).

1.1.3 Determinants of dental caries

Dental caries is a complex, interactive process and a number of factors can influence it.

The traditional list that contributes to the aetiology of dental caries is as follows:

- Diet, composition and frequency
- Microbial species and deposit
- Host susceptibility
- Buffer capacity of saliva
- Time

But other factors such as social class, income, knowledge, attitudes, behavior and education all have a part to play as demonstrated in Figure 1.1.1.

1.1.4 The Role of Carbohydrates

There is overwhelming evidence that frequent consumption of fermentable carbohydrates is associated with a high incidence of dental caries. This was shown in the Vipeholm study (Gustafsson et al 1954), which linked frequency and variety of sugar types with caries. Sugars consumed, which were highly retentive such as toffees exhibited the greatest caries incidence. As well as enabling the synthesis of extracellular polysaccharides to assist aggregation and colonisation, bacteria also utilised dietary carbohydrate as a substrate for acid production. Carbohydrates are not equally cariogenic. The monosaccharides, such as glucose, fructose and galactose and disaccharides, such as sucrose, maltose and lactose, can be readily metabolised by the bacteria in the biofilm tipping the environmental balance to demineralisation. Complex carbohydrates such as starch are not completely digested in the mouth and are therefore less able to diffuse into the plaque to be metabolised into acid, however the cariogenic effect can be increased if the starch is consumed in combination with

increased consumption of sugars. Again an environmental shift can occur towards a caries-inducing plaque (Lingstrom 2000).

1.1.5 Microbial species and biofilms

The presence of microbial communities on tooth surfaces otherwise known as dental plaque is a prerequisite for caries lesions to develop (Bowen 1972). These microbial communities are known as biofilms. Biofilm formation is a natural, physiological process and represents a community of microorganisms. Biofilms are formed in stages, pellicle formation on the tooth surface being an essential first stage and allowing the bacteria to adhere (Fejerskov and Kidd 2008, Samaranayake 2005).

The role of the pellicle is a protective one. It is a thin, bacteria free, proteinaceous film, which forms on the naked tooth surface within minutes to hours. It is important in caries formation as it has a permeable-selective nature, which restricts the transport of ions in and out of the enamel and thus is integral to the remineralisation/ demineralisation process. In the pellicle, molecular movements due to forces other than diffusion are slow such that this undisturbed layer will reduce the solubility of the enamel surface compared with whole saliva because of its high concentrations of calcium and phosphate. The surface of the enamel is negatively charged as the phosphate groups are arranged such that they are near the surface. Thus calcium which has two positive charges is attracted to the surface forming a hydration layer. Other ions will also be present in this layer. This hydration layer, which is positively charged, will then attract negative charges on salivary macromolecules. Such macromolecules act as receptors for oral bacteria, thus allowing the biofilm to be formed. Thus the pellicle and

biofilm can be seen as a reservoir for calcium and fluoride ions (Hay 1984, Fejerskov and Kidd 2008) or as a barrier to remineralisation (Zero 2006).

Initial tooth surface colonisation is by bacteria of the coccal form, a large proportion of which are streptococci. Further colonisation and bacterial growth on the tooth surface changes the streptococcus-dominated plaque to a plaque dominated by actinomyces (Law 2007). There has been shown to be a strong positive association between increased levels of mutans streptococci and initiation of demineralisation. However the specific plaque hypothesis that only a small number of species are involved in the disease and non-specific plaque hypothesis have now been reconciled in the more recent ecological plaque hypothesis (Marsh 2006). This hypothesis proposes that the organisms associated with caries can be present at sound sites but are in small numbers, whereas in diseased sites the balance of organisms has shifted due to the local environmental conditions (Samaranayake 2005).

1.1.6 Host Susceptibility

Bacterial plaque is an essential to the development of dental caries. Sites in the oral environment in which plaque is able to mature, represent areas in which the ecological shift of the more cariogenic organisms can occur. These stagnation sites include pits and fissures of molars and premolars, approximal enamel smooth surface caries in contact points, margins of restorations, cervical areas adjacent to exposed root and around fixed orthodontic appliances (Mattousch et al 2007).

1.1.7 Buffer capacity of saliva

Saliva continually bathes the teeth under normal circumstances. The saliva is able to keep the oral environment supersaturated with respect to hydroxyapatite and thus confers a protective effect in controlling dental caries. Saliva not only physically removes sugars and acids produced in the mouth but also buffers the acid by keeping the pH near neutral in the saliva and in the plaque. This is by virtue of its bicarbonate content, following bacterial acid formation (Dawes 2008).

The salivary components necessary for maintaining supersaturation are salivary calcium and phosphate ions. Calcium in saliva is found in a number of different forms. 20% of calcium is bound to salivary proteins such as statherin and proline-rich proteins. The remaining 80% is divided equally into either ionised or non-ionised calcium. The non-ionised form is firmly bound to inorganic ions such as phosphate and bicarbonate. The amount of non-ionised calcium increases with increased salivary flow along with increasing the pH and ionic strength increasing (Fejerskov and Kidd 2008).

Phosphate in saliva is also found in a number of forms, some ionised and non-ionised. The lower the pH the lower the concentration of phosphate and higher the concentration of phosphoric acid which reduces the amount of free phosphate. In contrast to calcium the concentration of total phosphate decreases dramatically with increasing saliva flow rates, however the salivary phosphate concentration is determined by pH and will be reduced to very low values by a low saliva pH. Therefore low pH values are more harmful to the teeth than low total phosphate.

When the ion activity product is equal to the solubility product of hydroxyapatite, the solution is saturated and no remineralisation or demineralisation will occur. This is the critical pH and below this mineralised tissue will dissolve. The critical pH is 5.5 for enamel and was determined in human saliva (Schmidt-Nielsen, 1946). The critical pH is a dynamic variable, as it will be affected by the different saliva flow rates; unstimulated saliva will have a lower critical pH than stimulated saliva. Further protective effects are conferred by saliva as it contains lysozyme, lactoferrin, lactoperoxidase and IgA antibodies, which inhibit bacterial aggregation, adhesion and promote bacterial cell death (Van Nieuw Amerongen et al 2004).

1.1.8 Time

Time is required for a carious lesion to progress. The dynamic process of demineralisation to remineralisation is constantly occurring, however a net loss of mineral will favour the conditions for a carious lesion to develop. Frequency of intake of fermentable carbohydrate over time will also favour a cariogenic environment. The Stephan curve demonstrates how, pH reduces to below the critical pH within 2-3 minutes due to a cariogenic challenge. In the following 40 minutes the pH then rises and returns to normal. This is why increased frequency of dietary sugars within 40 minutes will maintain a reduced pH and a cariogenic environment (Lingstrom 2000).

1.1.9 Classification

Dental caries can be classified according to site (Soames and Southam 2008):

- Smooth surface caries: These lesions may start on enamel (enamel caries) or on exposed root cementum and dentin (root caries)

- Pits and Fissure caries

Dental caries can also be

- Cavitated
- Non-Cavitated

Dental caries can also be classified according to their activity

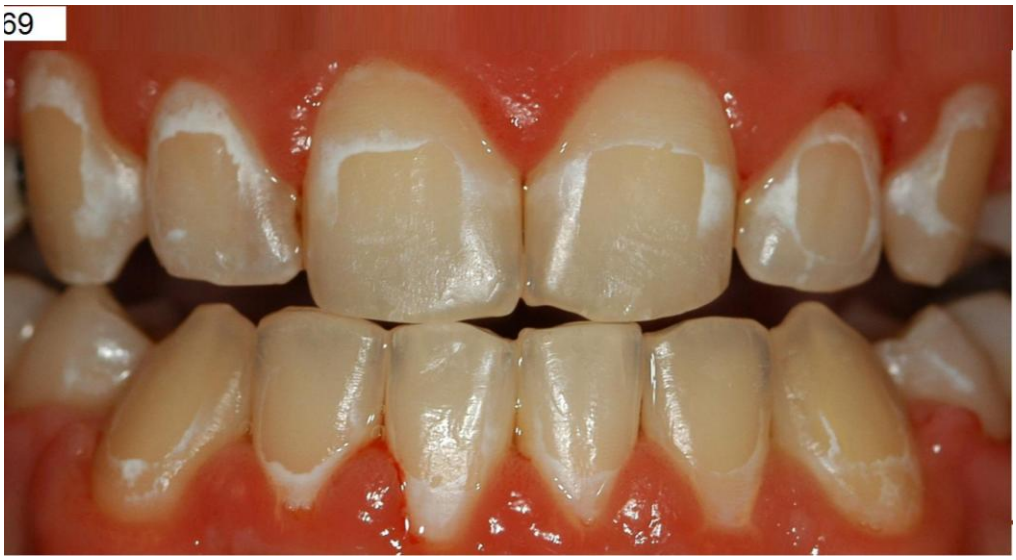
- Active
- Inactive (arrested)

This is an important distinction to make in order to decide how to manage the lesions. The lesions can change dependent on the ecological balance in the biofilm covering the site and the environmental challenge.

1.2 Carious demineralisation in relation to Orthodontic treatment

The occurrence of demineralisation of enamel adjacent to orthodontic brackets placed on the buccal surfaces of teeth is a potential adverse effect of fixed orthodontic treatment. It has been suggested that many of these early lesions will remineralise fully, although some develop into frank cavitation and require restorative intervention (Chang et al 1997). This demineralisation is manifested clinically as a white spot lesion and its appearance is caused by an optical phenomenon owing to subsurface tissue porosity and is exaggerated by thorough drying (Gorelick et al 1982) as demonstrated by the clinical photograph, Figure 1.2.1. After 14 days of completely undisturbed plaque, enamel changes are visible after air-drying. After 3 to 4 weeks the outermost surface exhibits further porosity and clinical changes can be seen without airdrying (Fejerskov and Kidd 2008).

Figure 1.2.1 Example of demineralization related to orthodontic treatment



1.2.1 The prevalence of white spot lesions in orthodontic patients

The prevalence of white spot lesions that has been reported in the literature is varied but has been reported as high as 96% (Mitchell 1992) with the labial surfaces of the maxillary incisors most commonly affected (Gorelick et al 1982; Mizrahi 1983; Geiger et al 1988). Even 5 years after orthodontic treatment white spots lesions were still present on a treated group and present an aesthetic problem (Øgaard 1989).

1.2.2 Aetiology of Orthodontic Demineralisation

The increased prevalence of enamel demineralisation during fixed appliance therapy is due to the irregular surfaces of brackets, bands and wires creating stagnation areas for plaque accumulation. Ideally this is prevented through fluoride use, oral hygiene and diet advice.

1.3 Remineralisation Interventions

Natural regression of these white spot lesions following appliance removal is expected however Mattousch et al (2007) and Øgaard (1989) showed that only a very small

minority of lesions will regress with only some aesthetic improvement of the majority of lesions. Interventions such as fluoride mouth rinses have been recommended in order to prevent the occurrence of these lesions during treatment and following debonding to help remineralisation. However there has been some concern that using high fluoride mouthrinses can lead to a hypermineralised surface layer, which can prevent ions from entering the enamel and remineralising the lesion. In a systematic review conducted by Benson et al (2004), and updated in 2008, found some evidence that the use of topical fluoride or fluoride-containing bonding materials during orthodontic treatment reduces the occurrence and severity of white spot lesions, however there is little evidence as to which method or combination of methods to deliver the fluoride is the most effective. Based on current best practice patients are recommended to rinse daily with 0.05% sodium fluoride mouthrinse. A differing approach to remineralisation has been with the use calcium based technology toothpastes such as Tooth Mousse™, which is a topical remineralising cream containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) (10% w/v).

1.3.1 Methods of remineralisation using Fluoride

Fluoride is important in the prevention of dental decay and enamel demineralisation (Benson et al, 2004). Fluoride is a trace element, which is present naturally in water supplies and food, such as fish and tealeaves. Fluoridated water provides both a systemic and topical effect and at a concentration of 1ppm it can reduce caries rate by 50% (Arnold et al 1953,1962). This, in conjunction with the fact that there seemed to be little if any fluorosis, resulting in the widespread adoption of 1-1.2 ppm at the optimal level in drinking water (Hodge 1950).

Fluoride has a number of methods of action, the most important of which is the topical effect. Fluoride is incorporated into the crystal lattice of the mineral of enamel and, resulting in the formation of fluorapatite, which is less soluble than hydroxyapatite, the critical pH of fluorapatite being 3.5. The resulting crystals are larger and more stable. Fluoride also inhibits dental plaque bacteria by blocking the enzyme enolase during glycolysis, however this is only at high concentrations (ten Cate 2001, Marsh, 1994). It also affects crown morphology by making the pits and fissures shallower and less likely to create stagnation areas (Welbury et al 2005).

The topical effect of fluoride is by far the most significant. The degree of fluoride concentration at the outermost surface of the enamel is dependent on posteruptive changes. Due to the porosity, it has been suggested that the surface of the enamel undergoes a period in which ions and minerals diffuse into the surface layer. Fluorhydroxyapatites are formed when the fluoride concentration in solution is low, less than 50ppm and in an acidic environment. Therefore under neutral conditions, the formation of fluorhydroxyapatite is slow and so in areas brushed regularly the fluoride content will be reduced. In areas where plaque stagnates, fluoride concentration of the surface of the tooth will increase over the years (Aasenden 1975). The constant supply of fluoride delivered by toothpastes or other topical agents on a daily basis maintains the reservoir of fluoride in the oral environment, allowing the equilibrium to tip towards supersaturation and remineralisation (Øgaard 1990).

Calcium fluoride is produced deposited on the tooth surface as spherical globules in conditions of high fluoride concentration and acidic conditions. This is the basis of treatment with acidified fluoride solutions. These globules precipitate in plaque, in

pellicle, in enamel porosities and in other inaccessible stagnation areas. They are thus a temporary storage of fluoride from which the ion can be released (Øgaard 1990).

In summary it is the topical activity of the fluoride ion in the oral fluid that is of greater importance in reducing enamel solubility than having a high content of fluoride in the surface enamel and so a constant supply of low level of intraoral fluoride is of most benefit in preventing dental caries (Øgaard 1990). Fluoride is most effective in dental caries prevention when a low level of fluoride is constantly maintained in the oral cavity such as in the topical application of a fluoride toothpaste. The World Oral health report recommends that the goal of community-based public health programmes, therefore, should be to implement the most appropriate means of maintaining a constant low level (Peterson 2003).

There are various methods of delivering fluoride in children and adolescents, some topical and others systemic. A number of systematic reviews have been completed in this subject. Marinho (2008) has summarised seven previous Cochrane reviews on this subject and the research shows that fluoride toothpastes, mouthrinses, gels and varnishes can reduce dental caries, regardless of water fluoridation or other sources of fluoride exposure. Toothpaste was the method that young people are most likely to persist with, than using fluoride mouthrinses, or having gels or varnishes applied. The evidence also shows that children using an additional form of topical fluoride will experience additional reductions in dental caries, compared with children using fluoride toothpaste only.

Other methods of delivering fluoride related to orthodontics are the use of fluoride-releasing bonding agents. In a systematic review (Benson et al 2004) there was some evidence found that glass ionomer cement for attaching brackets reduced the number and severity of white spot lesions in comparison to composite. Incorporating fluoride into elastomeric modules has been investigated (Banks et al 2000) as a method of reducing the incidence of demineralization, however a systematic review carried out by Benson et al (2005) shows this study to have a high risk of bias.

Fluorosis occurs when there has been an increased concentration of fluoride within the microenvironment of ameloblasts during enamel formation. This causes the enamel to be hypomineralised, with pitting and staining seen clinically. There are various severities depending on exposure. The critical period of exposure is between 1 to 4 years of age and by the age of 8 the child is no longer at risk if exposed to too much fluoride. For adolescents and adults the recommended amount of fluoride in a toothpaste should be 1400ppm. The lethal dose of fluoride for most adult humans is estimated at 5mg/Kg. A dose that may lead to adverse health effects is estimated at 1 mg/kg. This is the equivalent for a 60kg adolescent ingesting 3.3 tubes of a 70g toothpaste for a potentially lethal fatal dose and 0.3 of a 70g tube of toothpaste for a safely tolerated dose. If adding Tooth Mousse™ Plus, which contains 900ppm of fluoride, to a standard 1400ppm toothpaste, a resultant amount of 2300ppm of fluoride is delivered. This would reduce the required amount of toothpaste ingested for a toxic dose by 40%. Symptoms of fluoride toxicity include nausea, vomiting, hypersalivation, abdominal pain, and diarrhoea, subsequently depression of calcium levels, results in convulsions, cardiac and respiratory failure (Welbury 2005).

1.3.2 Methods of remineralisation using CPP-ACP

There are several challenges to establishing the clinical effectiveness of remineralisation agents. They must rapidly precipitate on partially demineralised tooth structure and transform into a stable apatite that is resistant to subsequent bacterial acid and erosive challenges; provide a benefit in an environment where a highly effective remineralisation agent is widely available ie fluoride. It also needs to provide a remineralising benefit in addition to the natural remineralising properties of saliva and diffuse through the biofilm-pellicle-covered enamel surface and into the subsurface lesion area (Zero 2009).

Calcium and phosphate ion solutions have been shown to have a limited effect on remineralising as calcium and phosphate salts have limited solubility and so are not suitable for remineralisation (Reynolds 1997). Fluoride ions can also promote the remineralisation of previously demineralised enamel if salivary or plaque fluoride is applied. For every 2 fluoride ions, 10 calcium ions and 6 phosphate ions are required to form one unit cell of fluorapatite [Ca10(PO4)6F2]. Hence on topical application of fluoride ions, the availability of calcium and phosphate ions can be limiting for net enamel remineralisation to occur, which is exacerbated in xerostomic conditions (Reynolds et al 2008).

The side effects of fluoride such as fluorosis has highlighted the need for a development of a non-toxic, anticariogenic agent that could be added to toothpaste mouthwash and food in an approach to lower caries experience (Reynolds 1998). Dairy products, such as milk, milk concentrates and cheeses have been shown to be anticariogenic in animal and human *in situ* caries models. The investigators concluded that the protective effect was

attributed to the phosphoprotein casein and calcium phosphate contents of the dairy products (Reynolds 1987). Unfortunately a level of caseinate, which significantly reduces the cariogenicity of chocolate confection, was considered too unpalatable and therefore it is unable to be added to food (Reynolds 1998). By combining tryptic peptides with casein, the amount of casein needed to prevent enamel subsurface demineralisation, was reduced. The tryptic peptide responsible were the calcium-phosphate-stabilising casein phophopeptides (CPP). CPP is incorporated into dental plaque and can stabilise amorphous calcium phosphate (ACP) through the formation of casein-phosphopeptide amorphous calcium phosphate nanocomplexes (CPP-ACP). Nanocomplexes of CPP-ACP are composed of a cluster of calcium and phosphate ions (1.3nm in diameter) surrounded and protected phosphopeptides. When the phosphopeptides bind to a tooth surface the calcium and phosphate ions are released and buffered, maintaining a state of supersaturation (Reynolds et al 2003). CPP-ACP possesses the characteristics of continuously supplying calcium phosphate ions to the enamel without precipitating even under its supersaturated condition, by localising in plaque and binding on specific tooth surfaces. Thus enamel demineralisation is depressed and remineralisation is enhanced (Reynolds 1998).

An additional benefit of CPP–ACP is that these products can work effectively as a remineralising agent under acidic pH levels as well as in the neutral and alkaline range. It has been suggested that, surface etching with or without pumicing of white spot lesions before remineralisation with these products will lead to the removal of the surface enamel (up to 30μm) allowing neutral ion species to gain access to the subsurface lesion through the porous enamel surface (Peariasamy et al 2001).

Reynolds et al (1995) assessed the potential of CPP-ACP in an animal model, using specific pathogen-free rats inoculated with *streptococcus sobrinus* on a cariogenic diet. The rats were exposed to various combinations twice daily, including CPP-ACP and 500ppm fluoride. They found that CPP-ACP significantly reduced both smooth surface and fissure caries in a dose-response fashion with CPP-ACP 1.0% solution reduced smooth surface caries by 55% and fissure caries by 46%. The synergistic effect of calcium and fluoride can be attributed to the formation of CPP-stabilised amorphous calcium fluoride phosphate, which results in increased incorporation of fluoride ions into plaque, together with increased concentrations of bioavailable calcium and phosphate ions (Reynolds et al 2008).

The anticariogenicity of CPP-ACP in human *in situ* studies has been demonstrated by Reynolds (1998). Samples were exposed to 1% CPP solution twice daily and this produced a reduction of 51% in enamel mineral loss relative to the control, with a 144% increase of plaque calcium and 160% plaque phosphate increase. A clinical trial of 3% CPP-ACP mouthwash showed an increase in plaque calcium content. This led to proposition that the CPP-ACP would act as a reservoir in plaque buffering free calcium and phosphate ion activities, maintaining a super-saturation state with respect to enamel. Importantly, the binding of ACP to CPP is pH dependent, with binding decreasing as the pH falls (Reynolds 1998).

An *in vitro* study carried out by Lovel (2008) found no remineralising benefit of GC Tooth Mousse™. This study looked to determine the remineralisation of subsurface caries-like lesions in bovine enamel by water, saliva, GC Tooth Mousse™ and fluoridated toothpaste. The mineral content of the lesions was measured longitudinally, over the 12

week experimental period, with QLF, and at the end of the study with TMR. They found no significant difference in the remineralisation efficacy of GC Tooth Mousse™ compared to a water control, however did find that fluoridated toothpaste significantly increased mineral gain.

More recently Benson (2009) carried out an *in vitro* study, in order to determine the remineralising potential of GC Tooth Mousse™ on subsurface caries-like lesions of varying severity in bovine enamel. No difference was found in remineralising capability of GC Tooth Mousse™ compared with artificial saliva. However it was proposed that the *in vitro* model may not be the ideal setting for testing of GC Tooth Mousse™ and recommended that *in situ* and *in vivo* testing be carried out.

Previous *in vivo* studies carried out have shown regression of white spot lesions but not complete resolution and no difference in the regression of these white spot lesions when using a fluoride toothpaste (Brochner et al 2011).

Tooth Mousse™ Plus is a more recent formulation that incorporates 900ppm of fluoride to help form a CPP-ACPF complex. The theory is that the CPP-ACPF complex delivers fluoride to the subsurface part of the lesion, as well as the surface lesion thereby promoting remineralisation and resolution of white spot lesions. Beerens et al (2010) have shown in a randomized clinical trial comparing Tooth Mousse™ Plus to a fluoride-free calcium based paste in post-orthodontic patients no difference to the regression of white spot lesions over 12 weeks.

1.3.3 Tooth Mousse™ (GC Corporation, Tokyo, Japan)

The manufacturers of Tooth Mousse™ and Tooth Mousse™ Plus recommend usage of the product in a number of situations (http://www.toothmousse.info/index.html); 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, susceptible to dehydration from sporting activities or outdoor work, suffering from morning sickness during pregnancy, increased risk of decay as a result of medical conditions (e.g. diabetes) and a low saliva flow rate as a result of prescription medications.

The instructions on how to apply Tooth Mousse™ (Plus) are described below:

<u>Direct Application</u>

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.

Then use your tongue to spread the applied crème throughout the mouth.

3. Hold in the mouth for a further 1-2 minutes. The longer GC Tooth Mousse™ (Plus) and saliva are maintained in the mouth, the more effective the result.

4. Expectorate thoroughly and if possible avoid rinsing. Any GC Tooth Mousse™ (Plus) remaining on the tooth surface can be left to gradually dissipate.

5. 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 crème 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, 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™ (Plus) and saliva are maintained in the mouth, the more effective the result.

6. Expectorate thoroughly and if possible avoid rinsing. Any GC Tooth Mousse™ (Plus) remaining on the tooth surface can be left to gradually dissipate.

7. Do not eat or drink for 30 minutes following application.

1.3.4 Tooth Mousse™ Plus (GC Corporation, Tokyo, Japan)

The recommendation is that Tooth Mousse™ Plus is used in patients older than 6 years of age. This is due to the risk of fluorosis to the developing dentition especially if the

child is using it in addition to a fluoride toothpaste. The majority of crown development of anterior teeth is completed by age 7.5 years (Welbury et al 2005) although there will be individual variability and so there could still be a risk of unaesthetic fluorosis occurring if Tooth Mousse™ Plus is used in children between 6 and 8 years of age. The recommendation however, is to expectorate excess Tooth Mousse™ Plus to prevent ingestion.

CPP- ACP has been incorporated into other products to exert a topical effect. These include products such as chewing gum, mints, gels and sports drinks. Incorporating CPP-ACP into glass ionomer cement has been investigated (Mazzaoui et al 2003, Al Zraikat et al 2011) and maybe an alternative way of delivering remineralisation agents to the tooth surface to areas at greatest risk of demineralisation, which does not rely on compliance. Previous investigations have shown *in vivo* the fluoride releasing impact of glass ionomer cements in orthodontic patients (Benson et al 2004) and this may have added benefit for the patient.

1.4 Methods of trial techniques to evaluate enamel demineralisation

There a number of different experimental models used to study the process of enamel demineralisation and these include:

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

1.4.1 *In vitro*

In vitro is a study, in which the enamel samples, human or animal are tested in the laboratory and thus the techniques of quantification can be destructive to the sample being studied.

White (1992, 1995) discusses the advantages and disadvantages of *in vitro* models:
The advantages of an *in vitro* system is that it is relatively inexpensive, is not time consuming and under tight control.

The disadvantages of *in vitro* systems are; inadequate simulation of biological aspects of caries. Neither microbiology nor the salivary effect can be simulated. It is difficult to simulate the volume and composition of saliva as well as use the coverage/clearance factors that will affect the dynamic variations under plaque. It will also affect the uptake and reactivity of fluoride, which is much lower *in vivo* than *in vitro*. The actual rates of demineralisation and remineralisation are faster *in vitro* than *in vivo*. There is difficulty in matching solid/solution ratios occurring *in vivo* and there may be artifacts associated with the substrate choice/reaction conditions.

There have been a number of demineralisation systems developed in order to mimic the demineralisation of teeth *in vivo*, in the *in vitro* and *in situ* setting. This way remineralisation or further demineralisation can be studied whereas sound enamel will only allow the study of demineralisation.

Bovine enamel has been used instead of human enamel due to availability in the production of artificial lesions. However bovine enamel is softer and more porous than

human enamel and demineralises more readily. The same demineralisation systems are used for both human and bovine enamel. Human enamel is more representative of the true situation and so the results of experimentation should be more applicable and relevant (Mellberg 1992).

1.4.2 *In Vivo*

In vivo is when the experiment is carried out within the living organism, and so in situations where human beings are the organism, generally non-destructive techniques are used, unless the teeth being investigated are destined for extraction.

In vivo caries models have involved the banding or bracketing of teeth for a period followed by extraction and examination of the teeth.

Mellberg (1992) discusses the disadvantages of *in vivo* techniques, which include: That there is a lack of availability of these teeth, as only patients requiring extractions can take part. There is less control over lesion reproducibility and restrictions regarding lesion location.

The patient cannot commence orthodontic treatment until the tooth is extracted otherwise the patient's treatment will be unduly prolonged and so the experiment is confined to the initial stages of treatment, usually the first month whereas orthodontic treatment can take up to 2 years. This technique is therefore unable to monitor changes in the enamel throughout the duration of treatment (Benson 1999). The study will only be able to manage a relatively small sample size, and there will be inadequate controls.

The advantages are however, that the teeth are in their most natural state in the natural oral environment.

1.4.3 *In situ*

Zero (1995) defines the *in situ* model as involving the use of appliances or other devices which create defined conditions in the human mouth that simulate the process of dental caries. They represent a bridge between the *in vitro* study and the clinical trial. This is represented in Figure 1.4.1.

Figure 1.4.1 Illustration of how *in situ* models can be placed on the continuum between clinical trials and *in vitro* models (Zero 1995)

Clinical Trials	*In situ* models	*In Vitro* Models
Natural		Artificial
High clinical relevance		Possibly decreased clinical relevance
Limited scientific control		High level of scientific control
High variation		Low variation
Insensitive detection methods		Highly sensitive detection methods
Large "n"		Small "n"

The *in situ* technique entails using a specimen of enamel, placed in a customised holder for investigating the caries process. One *ex vivo* specimen of the same tooth may be kept

as a control or an additional specimen measured *in vivo* throughout the duration of the experiment.

The experimental production of artificial carious lesions on the specimen of enamel is used in both extra and intraoral cariogenicity tests in order to gain further insight into factors affecting caries formation and prevention. This allows us to study changes but with some the more destructive *in vitro* methods of quantification and so overcomes ethical issues.

In situ caries models are different from epidemiological studies and clinical trials in that they use small numbers of subjects. The intent of these model systems is to mimic what occurs in the natural caries process, yet provide clinically relevant information such as the degree of remineralisation or demineralisation occurring with a particular intervention in a relatively short period of time without causing irreversible tissue changes in the natural dentition.

Zero (1995) describes the advantages of the *in situ* appliance; Studies are performed in the human mouth, in contrast to *in vitro* or animal studies and therefore the model includes all the natural elements of the oral environment that may contribute to caries process. It is possible to control the experimental variables and provide flexibility of the experimental design to allow crossover studies. The studies allow the integration of various basic scientific analytical techniques. This will increase the sensitivity and scientific validity of the experiment compared with clinical trials that use cruder and more insensitive means of measurement and recording eg probing or visual scoring. The ability to accurately quantify the mineral change gives the study validity. They are

generally short-term, thereby overcoming the ethical implications of long-term clinical trials and reducing cost. Most importantly they mimic the natural caries process of a specimen without causing irreversible damage to the host. There is a generally a favourable cost factor compared with long-term clinical trials. In addition, this model can be used at any stage of treatment in the orthodontic patient and will not affect the treatment itself.

The disadvantages of the *in situ* model are (Zero 1995): The technique is very demanding on both clinical and analytical expertise. Due to the large amount of laboratory and analytical work, the number of subjects is limited to between 5 and 40. In terms of sample size this may not be representative of the general population. These studies are generally heavily dependent on compliance by the test subjects. A lack of compliance will have a major effect on the experimental outcome. The conduct of high-quality *in situ* studies is demanding, requiring both clinical and analytical expertise.

1.5 The subjects

The participants should be representative of the population for which the study is intended. However if the subject panel reflects the breadth of variation in the population then the study may not have sufficient power to detect a significant difference. Zero (1995) advocates standardisation according to a number of parameters that include age, gender, race, all of which may influence the model. The medical health status is important in that the subjects need to be in good health and not have received antibiotics two months before or during the study, as this may alter the composition of the oral flora. Their dental health status should be representative of the population we are looking at. Therefore it is desirable to select subjects with a range of past caries

experiences to maximise the relevance of the *in situ* study. The subjects need to have a minimum salivary flow level and similar exposure to fluoride as this can also affect the demineralisation/ remineralisation of *in situ* models (Stookey 1992).

Stookey (1992) recommended that adults rather than children are more appropriate as panelists for *in situ* studies when one considers that caries rate and response to fluoride treatment are similar for adults and children as they are more likely to comply with protocols.

1.6 Randomised Controlled Trials

The definition of a randomised controlled trial (RCT) is a study in which participants are allocated randomly to receive one or more clinical interventions. They are used to measure an effect of an intervention in a design, which reduces influences of the following; Bias and Confounding factors

1.6.1 Bias

Bias is defined as any factor or process that tends to deviate the results or conclusions of a trial systematically away from the truth (Jadad 1998). The main types of bias that can occur in an RCT can originate at any point, from the selection of the participants, to the delivery of the interventions, followed by measurement of the outcomes and statistical analysis.

1.6.2 Confounding factors

A confounding factor is a factor that has a relationship with both the potential cause and the outcome but is not the causal pathway (Bruce et al 2008). These can under or over

exaggerate results of the study and are often unknown. To reduce the effect of confounding factors, randomisation is carried out.

1.6.3 Randomisation

Random allocation means that all the participants have the same chance of being assigned to each of the study groups. By randomising the interventions, confounding factors that may influence the study, but cannot be known or quantified are kept to a minimum. Therefore the effect of the interventions can be quantified (Jadad 1998). In order to achieve randomisation, one can generate random sequences of allocation using software programmes specifically for the purpose.

1.6.4 Blinding

Blinding is used to reduce the risk of bias in a study. This can be done to the participant in relation to the intervention, known as single blind trial or to both the participant and the investigator, called a double blind trial. A double blind trial will prevent the participant from changing his behaviour due to the intervention and the investigator from being biased during analysis. Coding of samples collected so the investigator remains blind to origin of sample will also reduce bias when the investigator is analysing the samples and the data (Jadad 1998). This type of bias is called ascertainment bias.

1.6.5 Cross-over and Washout effect

A cross-over design is when each participant is given all the study interventions in successive periods. The order of the interventions is determined by randomisation. Cross-over trials allow within patient comparisons to be carried out. As each participant

acts his/her own control, the study can be statistically and clinically significant with fewer participants (Jadad 1998). Washout periods are used to eliminate any carry-over effects of the interventions. The quantity of time needed for the washout period will depend on the half-life of the drug (Altman 1999). If the interventions were given to the participants in the same order, this would introduce bias. Instead they can be randomised into all possible combinations of order and this can be done in blocks so the all possible combinations are equally distributed.

1.7 Methods of mineral evaluation techniques

Tooth minerals are lost and regained constantly in a normal, human environment. Assessing the amount of mineral content is a good method of diagnosing the presence of, and monitoring the progression of, dental caries. Quantitative measurements allow accurate measurement of small changes in individual lesions and thus their progression (Ten Bosch and Angmar-Mansson, 1991).

1.7.1 The International Caries Detection and Assessment System II (ICDAS II)

ICDAS II is a visual scoring method developed from the best elements of previously published systems of the previously published systems and is based upon the most robust evidence available (Pitts 2009). It has been used in research and was used by Bailey et al (2009), in assessment of remineralising cream containing CP-ACP on white-spot lesions. ICDAS II has been shown to be a reproducible method following training of examiners however there still remains some subjectivity about this method (Pitts 2009).

Several other novel non-destructive methods have been used in *in vivo* experiments described by Pitts (2009). These include laser fluorescence, quantitative light fluorescence, subtraction radiography and electrical caries measurements.

1.7.2 Laser fluorescence

Laser fluorescence is used in the Diagnodent device, 655 nm monochromatic light is emitted from an optical tip/sensor, which can also detect the amount of back-scattered fluorescence. The method relies upon the light entering the enamel, which will either go unhindered into the dentine or be partially scattered. A more regular crystalline structure such as mature enamel is more transparent as light can pass through the enamel layer with only little deflection. If the enamel layer in less homogeneous, the light is diffracted and scattered. The scattered portion of light then excites either the dental hard substance (called autofluorescence) or fluorophores within the lesion. The fluorophores have been identified as bacterial protoporphyrins and are excited by a specific wavelength of electromagnetic energy. Therefore theoretically the amount of back-scattered fluorescence is proportional to the amount of bacterial infection, pore volume and lesion depth and is measured on the Diagnodent between values of 0 to 99. The drawback of this method is that it has to be used in non-frozen samples due to the degradation of the bacterial porphyrins that occurs and thus introduces bias into *in vitro* studies. Systematic reviews have shown the method to be more sensitive (Type 1 error - can detect a positive result) on the occlusal aspect of posterior teeth but is less specific (Type 2 error - good at detecting a negative result) than clinical visual examination. The ability to detect caries between teeth is affected by the presence of stain, polishing pastes or adjacent fillings and has ranged from good to moderate. (Pitts 2009).

1.7.3 Quantitative Light-Induced Fluorescence (QLF)

QLF is a diagnostic device that has been employed in the detection of early demineralisation in enamel and, with the addition of a fluorescent dye, dentine. This technique has been used in the detection of early caries in permanent teeth, adjacent to restorations and orthodontic brackets, as well as the detection of failing fissure sealants (de Josselin de Jong et al 1995, Pretty et al 2004).

QLF is based on the dark appearance of a white spot in otherwise highly fluorescent enamel. White light is directed at the tooth surface and is passed through a blue filter. The excitation of enamel with blue light (370 nm) causes it to fluoresce in the yellow-green region. By using a yellow high-pass (540 nm) filter to cut out the blue portion, this fluorescence can be observed using a small intraoral camera. If a lesion is present on the surface, an increase in light scattering is observed relative to the surrounding enamel. The net result of this is that the contrast between sound enamel and a lesion is seen as being dark on a light green background. Bacterial porphyrins will fluoresce red (Pitts 2009).

It is the change in fluorescence between sound enamel and the lesion that is used to measure relative mineral loss. This is done using a computer program as it simulates the fluorescence radiance of sound enamel at the lesion site by reconstructing fluorescence radiance values of a sound tooth based on intact enamel values based on a rectangle around the lesion. This is calculated as the percentage difference between the actual and reconstructed fluorescence surface (de Josselin de Jong et al 1995).

This method was adapted for clinical use with the development of a small portable machine. It has been compared to microradiographic and chemical analysis and shown to be sensitive and reproducible method, which is able to quantify lesions up to 400µm. Thus it is suitable for an *in vivo* method of quantifying lesions (Pitts 2009), particularly for the quantification of smooth-surface initial caries. It has been applied in *in vivo* clinical studies to test the natural behaviour of white spot lesions after removal of orthodontic appliances (Van der Veen et al 2007, Mattousch et al 2007) as well as to assess lesion activity (Meller et al 2006). QLF has been shown to be comparable to TMR, (Al Khateeb et al 1997, Lovel 2008) as a technique to measure demineralisation. Other methods that have been used to measure demineralisation are Subtraction Radiography and electrical caries measurement.

1.7.4 Destructive methods of assessment

These techniques are destructive of the tooth sample and require a single cut or two cuts of the specimen. Chemical analysis requires microsamples to be removed from the specimen in order to be assessed. Another method, Microhardness determination, is an indirect way of measuring mineral loss through deformation of the sample. Polarised Light Microscopy (PLM) is a method using 2 plane-polarised rays at right angles to each other through a small slice giving a quantitative mineral measurement. All these methods have various advantages and disadvantages however none are as accurate as tranverse microradiography (TMR).

1.7.5 Microradiography

Microradiography was first described by Thewlis (1940) and made quantitative by Angmar et al (1963). There are 3 types of microradiography techniques;

1. Transverse microradiography (TMR)
2. Longitudinal microradiography (LMR)
3. Wavelength independent microradiography (WIM)

LMR and WIM are both non-destructive methods unlike TMR. The TMR technique is the most practical technique, and is now considered as the gold standard due its reasonable accuracy (Damen et al 1997).

1.7.6 Theory of TMR

The basis of TMR is the measurement of x-ray absorption by a tooth section compared with absorption by a simultaneously exposed standard (Ten Bosch and Angmar-Mansson, 1991). In this technique planoparallel sections are cut from the sample to be investigated (80μm in section). The sections are cut perpendicular to the anatomical tooth surface, placed on high-resolution photographic film along with an aluminium calibration stepwedge and irradiated with monochromatic x-rays (Arends and Ten Bosch 1992). Absorption of X-rays by the tooth sample and stepwedge is directly reflected in the optical density of the developed film (microradiograph).

Analysis of mineral content and distribution is calculated by means of Angmar's formula (Angmar et al 1963) from the optical density of the tooth sample and stepwedge images. Densitometry-based systems and more recently, image analysis systems comprising a

video (CCD) camera and dedicated software are used for evaluation of microradiograph optical densities and for the generation of mineral content profiles (Lagerweij et al, 1994).

From the analysis of mineral content profiles, three main parameters are obtained: mineral loss ΔZ (vol%μm), lesion depth L_d (μm) and lesion width L_w (μm). ΔZ is the integrated difference between the microradiograph of the sample with mineral loss and that of the sound sample, whilst Ld and Lw values are determined from the mineral distribution. Arends and ten Bosch (1992) report that the accuracy of TMR for enamel in lesion depth (L_d) is approximately 5 μm and in ΔZ about 220 vol%μm. The advantages of TMR is that mineral loss or gain is measured quantitatively with reasonable accuracy as well as determining the change in mineral distribution. The disadvantages of TMR are that it is a technique, which destroys the sample due to the cutting procedure. In addition, phenomena less than 10 μm from the anatomical surface are not measured due to finite densitometer slit width and specimen curvature. The presence of ions with a very high absorption coefficient for x-rays can lead to a misinterpretation of TMR data (for example, the presence of Sn ions in outer enamel surface after SnF_2 application causes a very strong x-ray absorption by the Sn ions which can easily be misinterpreted as remineralisation. White (1992) also discusses the problems with accurately cutting plano-parallel sections and the effect on radiographic precision. Consistent sections depth has been improved with the use of ball bearings for polishing. TMR is accepted as the gold standard in direct quantification of mineral change and lesion characteristic. Other techniques such as QLF are measured against TMR to validate them.

1.8 Summary

Studies of CPP-ACP containing toothpaste reported to show remineralisation promotion has been varied. Studies *in vitro* by Yamaguchi et al (2007); Rahiotis et al 2007; Sudjalim et al (2007); Kumar et al 2008; Jayarajan et al (2011) have shown a positive effect and *in vivo* by Bailey et al (2009) and Brochner et al (2011) and *in situ* by Srinivasan et al (2010). However in our local cariology group at the University of Liverpool, Lovel 2008 and Benson 2009 showed no such effect *in vitro*. This study sets out to determine the remineralising properties of calcium based toothpastes *in vivo* using an *in situ* design. *In situ* studies have been shown the model to be useful in studying the effectiveness of remineralisation regimes through controlled experimentation in the laboratory and in the mouth (Benson 1999).

2.0 Study: Aims and Objectives

2.1 Aims

To measure demineralisation/remineralisation of sub-surface caries-like lesions placed *in situ* on an orthodontic appliance and treated with Tooth Mousse™.

2.2 Objectives

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

3.0 Null Hypothesis

This study will test the null hypothesis that there is no difference between the remineralising abilities of Tooth Mousse™ with normal fluoride toothpastes in orthodontic patients.

4.0 Materials and Method

4.1 Ethics

Ethical approval was sought and obtained from the Liverpool Children's Local Research and Ethics Committee and the NHS/HSC Research and Development Offices.

REC Reference number: 10/H1005/85

Some minor alterations were made to the Information Sheets and the Consent and Assent forms following ethical approval.

4.2 Study Design

This was a randomised cross-over *in situ* study. This involved the use of prepared enamel lesions on previously extracted premolar teeth being placed *in situ*. The interventions were given to the orthodontic participants in a randomised order with a wash out phase constituting a cross-over study.

4.3 Study Setting

The Oral Health Research laboratories, and The Orthodontic Clinic. Liverpool University Dental Hospital (LUDH).

4.4 Sample Size Calculation

The primary outcome was change in mineral loss (vol%µm). Using data from a previous *in vitro* study (Benson 2009), the residual standard deviation is estimated at 189 vol%µm. Four Latin square blocks, with a total of 12 participants, will allow detection of pairwise differences between groups of 300 vol%µm, with 90% power, at the 5%

confidence level. Therefore the planned recruited number will be 15 to allow for any dropouts from the study.

4.5 Subject Selection

Patients attending the Orthodontic Department of the Liverpool University Dental Hospital for treatment with myself that met the inclusion criteria were identified.

4.5.1 Inclusion Criteria

The subject was required to be between the ages of 12 to 16 years undergoing orthodontic treatment. The subject had to have adequate space in between the lower 2nd premolar region and lower 1st molar region in order to provide space to place the carrier on the archwire. The subject had to be in good health.

4.5.2 Exclusion Criteria

If the subject was allergic to milk products and/or had taken antibiotics in the last 2 months the patient was excluded.

4.6 Consent

At the routine orthodontic appointment, eligible patients were invited to participate in the study. Patient information leaflets were provided to the parents and the children in 'plain English' explaining the purpose of the study (appendix 10.2). Consent forms were completed for inclusion in the study at the next orthodontic appointment (appendix 10.2). An inclusion/exclusion criteria checklist was also completed at the same appointment (appendix 10.3)

4.7 Randomisation

Each eligible subject was coded and randomly allocated to a subject number using a random number generator programme in blocks of 3 from random number tables by the statistician who was not involved in the recruitment or allocation of the interventions. The method of randomisation was by random number generation which was then subject to envelope concealment. A Latin square design was used, where each paste was given to every patient in the same order but at different start points in order to remove any wash out effect. The order of interventions in the Latin square design as designated by the random number generator programme is shown in table 4.7.1. Once the patient had consented, the next envelope was retrieved and opened by the patient. The paper revealed the order of interventions and the appropriate pack and instructions were given to the patient at the time.

Table 4.7.1 Subject and randomised order of interventions generated by the statistician

Subject	Intervention	Intervention	Intervention
1	B	C	A
2	A	B	C
3	C	A	B
4	C	A	B
5	B	C	A
6	A	B	C
7	C	A	B
8	B	C	A
9	A	B	C
10	B	C	A
11	C	A	B
12	A	B	C
13	C	A	B
14	A	B	C
15	B	C	A

4.8 Intervention

The interventions were as follows;

A. A standard fluoride toothpaste (1450ppm).

B. A standard fluoride toothpaste (1450ppm) and a separate, topically applied Tooth Mousse™ Plus toothpaste (GC Corporation, Tokyo, Japan), which incorporated CPP-ACP and fluoride (900ppm) to the carrier only.

C. A standard fluoride toothpaste (1450ppm) and separate, topically applied Tooth Mousse™ (GC Corporation, Tokyo, Japan) to be applied to the carrier only.

The carrier contains the previously demineralised enamel sample.

'Tesco Value Toothpaste 75Ml' was chosen as the standard fluoride toothpaste due to the minimal ingredients, and the least number of additives present compared to other toothpastes available.

The ingredients of Tesco Value Toothpaste Ingredients were 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. Batch number; F1110414.

The ingredients of Tooth Mousse™ (GC Corporation, Tokyo, Japan) were 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.

Tooth Mousse™ Plus has the same constituents of Tooth Mousse™ plus in addition to 900ppm of Sodium Fluoride.

4.9 Blinding in the clinical phase

The Tooth Mousse™ tubes were covered in insulation tape in order to blind the patients and the principal investigator as the contents of each tube. This was in order to reduce performance in the clinical phase and the measurement bias in the laboratory phase.

Once the enamel samples were removed from the carrier following the *in situ* phase, the samples were recoded by a Research Technician not involved in the study. The samples were then sectioned and analysed so the principal investigator was blinded to the subject and intervention during analysis. Following completion of TMR analysis the coding was revealed.

4.10 Creation of artificial subsurface lesions

Human premolar teeth, which were extracted for orthodontic purposes, were collected and stored in distilled water containing a few grams of thymol. The teeth were carefully examined and those, which were devoid of stains, caries or cracks, were selected. The 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. The teeth were then varnished with acid resistant nail varnish (Max Factor, Nailfinity, Weybridge, UK) except for 2 rectangular windows approximately 4mm by 1mm on the buccal surface.

The teeth were then mounted on glass rods using greenstick compound and immersed into an acid buffer demineralising gel as described by Edgar (1983) and shown in Figure 4.10.1. 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. The volume of gel made up was based on the fact that 20ml of gel was required per tooth. 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". The approximate value for the gel's viscosity was greater than 10 Pa.S (Ns/M). The teeth

were submerged for a period of 5-7 days. The time period was judged according to a visual examination of the lesion, when removed.

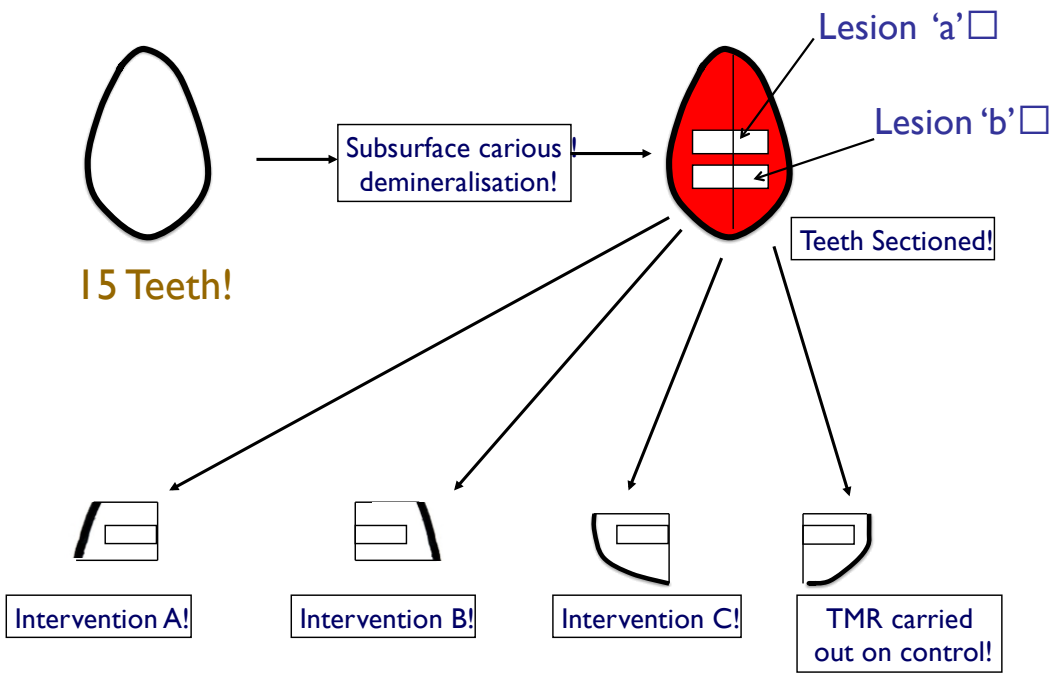
Figure 4.10.1 Example of premolar tooth submerged in demineralising gel

After withdrawal from the gel the sections were then rinsed thoroughly in distilled water, gently blotted dry with a paper towel and left to air dry. The block of enamel containing the lesion was then cut from the crown of the tooth, together with a margin of sound enamel, which had been under the varnish above and below the lesion. This was done using a diamond disc (Skilldental, High Wycombe, UK). The lesions were then divided to give 4 sections; 3 experimental and 1 control as shown by Figure 4.10.2. The control samples were cut perpendicular to the surface using a diamond wire saw (Well, Walter Ebner, Le Locle, Germany) and mounted on a brass anvil as shown in Figure 4.14.3 using nail varnish (Max Factor, Nailfinity, Weybridge, UK).

The samples were then polished using the diamond impregnated disc as shown in Figure 4.14.4 on both sides to give planoparallel specimens of approximately 100μm thickness after which they were soaked off with acetone (VWR International Ltd, Poole, England) to remove them from the brass anvil. The sections were then examined Leica Leitz DMRB optical microscope (Leica, Wetzlar, Germany) for a subsurface lesion of even quality. Samples with evidence of surface lesions or lesions of poor quality were rejected. The control sections from each acceptable lesion were placed together with a 12-step aluminium stepwedge (25μm thickness), on a high-definition photoplates (HTA Enterprises, California, USA). They were then radiographed in a Phillips X-ray set with a copper target and nickel filter with an exposure time of 35 minutes at 20kV and 10mA. The anode to film distance was set at 40cm. The microradiograph images were developed to make a radiographic plate. The microradiographs were then examined using a Leica Leitz DMRB optical microscope (Leica, Wetzlar, Germany). The images of the enamel sections were then captured using a CCD video camera (Sony, Tokyo, Japan), which was connected to a computer (Hewlett Packard Pavilion t3000, USA).

The images of the lesions were captured using the software, TMR 2000 v 2.0.27.16, Inspektor Research System BV, Amsterdam, The Netherlands. The images of the lesions were then analysed using a bespoke software package developed by de Josselin de Jong (TMR 2006 v 3.0.0.10, Inspektor Research System BV, Amsterdam, The Netherlands) The mineral content of the sections was expressed as mineral loss (Vol%μm), lesions depth (l_d), lesion width (l_w).

Figure 4.10.2 Diagrammatic representation of enamel lesion sectioning.



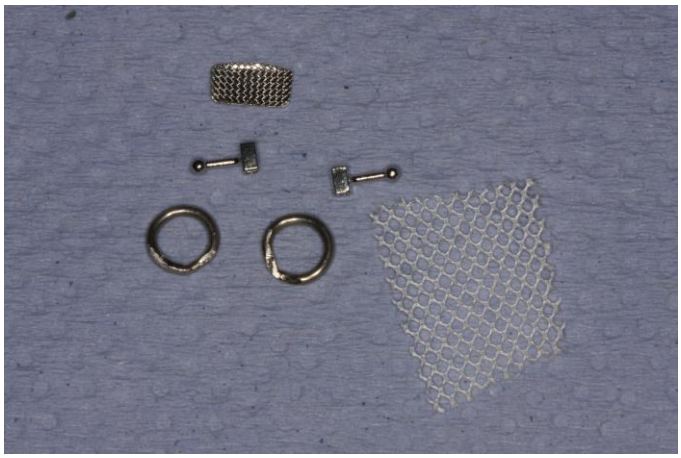
In total, 40 teeth were prepared in this way. Following TMR analysis of the teeth, only teeth with lesions in the range of a mineral loss of 900-1700 vol%µm were selected. Of these, only 14 teeth, in which all sections could be used were found to be adequate. Lesions from 2 other teeth were combined for the last participant. The teeth were numbered from the outset and the letters 'a' and 'b' denoted from which enamel lesion the section originated as shown in Figure 4.10.2.

4.11 Creation of Customised Enamel holder

This was a modified version of that used by and described by Benson (2000).

4.11.1 Manufacture

Figure 4.11.1 Basic components of carrier construction



The basic components of carrier construction are shown in Figure 4.11.1 prior to assembly. A small ring of 0.8mm stainless steel wire was formed to enable it to just cover a stainless steel molar bracket base (American Orthodontics, 1714 Cambridge Ave, Sheboygan, WI 53081 USA, Ref 996-016). The ring was welded at the cross over point and then the ends smoothed using a green polishing stone in a straight hand piece. The bracket base edges that lay beyond the metal ring were smoothed using a green stone in a straight hand piece to prevent irritation to the participants' soft tissues. The result should look as shown in Figure 4.11.2.

Figure 4.11.2 Stainless steel ring welded to bracket base and edges polished

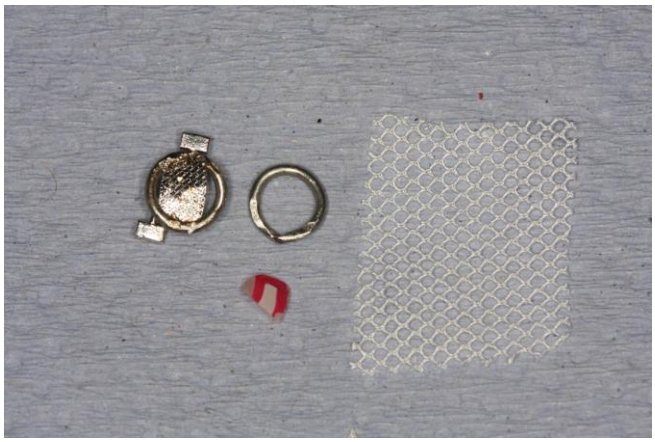
Two 2mm cast slide-on surgical ball hooks (Orthodontic Technology, 17401 Commerce Park Blvd, Tampa, FL 33647 USA) with internal dimensions 0.021”x 0.025” were then spot-welded to the back of a stainless steel molar bracket base, trying to avoid damaging the mesh. The ball was placed approximately in the middle, with the shaft of the hook running parallel to the longest part of the rectangular bracket base as shown in Figure 4.11.3.

Figure 4.11.3 Bracket base and steel ring with ball hooks welded to base

The second surgical ball hook was then spot-welded to the bracket base, in the exact same way but in the opposite direction. The additional hook was placed in case of weld failure or need for ligation in the clinical phase. The enamel specimens were then ground to fit inside the ring using a diamond disc (Skilldenta, High Wycombe, UK) in a high-speed hand piece. The specimen was then attached to the bracket base using clear nail varnish (Max Factor, Nailfinity, Weybridge, UK).

A piece of Dacron gauze (polyester mesh P/N: PETKM3002 Textile Development Associates Inc, Connecticut, USA) was placed over the enamel specimen and with another ring of 0.8mm stainless steel positioned over the Dacron gauze and the base ring. The individual components of the carrier prior to welding are shown in Figure 4.11.4.

Figure 4.11.4 Components of carrier prior to being welded



This arrangement was then spot welded together using mosquito forceps. The resulting carrier is shown in Figure 4.11.5.

Figure 4.11.5 Carrier completed

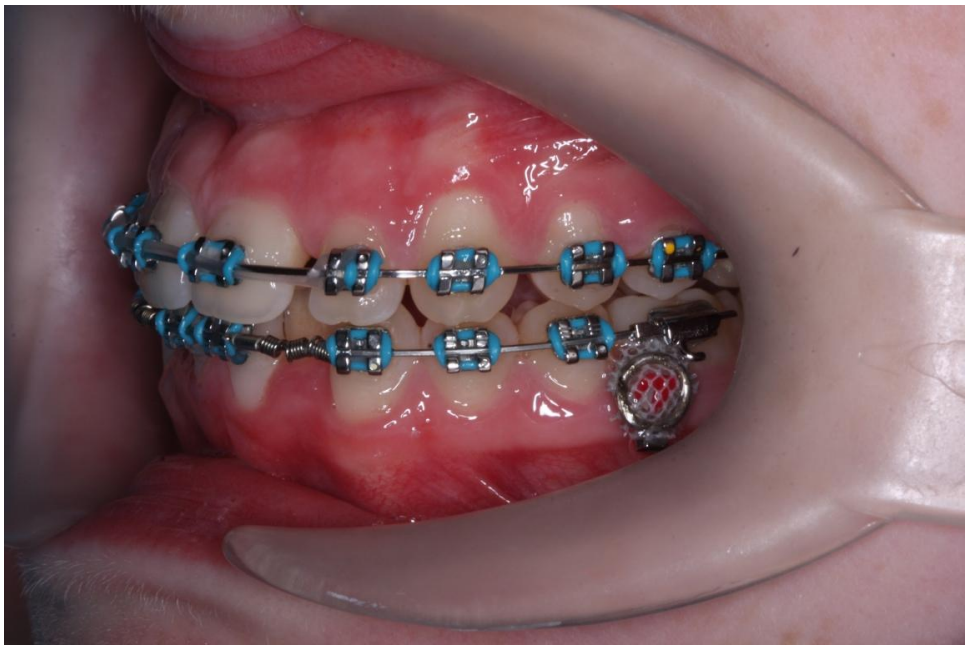
The carrier was then placed in a 1.5ml-capped tube with a small drop of water to maintain moisture content until it was possible for the carriers to undergo sterilisation. Sterilisation was carried out with the carrier inside the 1.5 ml capped tube. The carrier was then sterilised by gamma irradiation with a dose of 4080 Grays over three days under a Cobalt source. This dose sterilises the enamel sample of bacteria, without causing discoloration or change in mineral loss values (Amaechi et al 1999). The carrier was then ready to be placed in the patient. During storage the 1.5ml capped tubes containing the carriers were kept inside the fridge at 3°-5°.

4.12 Carrier Placement

The carrier was threaded onto the archwire into a position between the lower 2nd premolar and the 1st premolar. It was then stabilised using a wire ligature (0.010 stainless steel wire) threaded through the most gingival surgical ball hook and around

the molar tube where necessary. Impingement onto the gingivae was checked and the carrier was then adjusted to eliminate any discomfort experienced by the patient.

Figure 4.11.1: Carrier *in situ*



4.13 Study Procedure

The study consisted of 3 distinct 4-week phases. Each participant was provided with the relevant toothpaste and toothbrush and asked to brush their teeth for two minutes twice a day with a pea sized amount for the 4-week phase. The participants were asked not to use any other toothpaste or mouth rinses during the 4-week phase. The instructions were provided in written form (Appendix 10.5). The following is an example only of the process.

Treatment A paste/s was given to the subject 1 and instructed to use it for 4 weeks. At the 4-week appointment a routine orthodontic appointment was carried out and the

carrier with the enamel specimen was removed from the mouth and taken to the laboratory for analysis. Subject 1 was then instructed to continue with their normal oral hygiene procedures during the following 4 weeks using the Tesco Value Toothpaste that had been provided. This was the washout period. Subject 1 then returned for another routine orthodontic appointment and insertion of a new enamel specimen was carried out. Subject 1 was then given treatment B paste/s to use for 4 weeks. This continued until the subject had used all the interventions in the order set out in the envelope allocated to the subject.

To check compliance with using the pastes the patient was asked to bring the tube of paste with them on their review appointment. The tube was weighed to assess the amount used in the four-week period.

4.14 Preparation of Sections for TMR

On returning the enamel sample to the lab following the 4-week intervention *in situ* the carrier was placed in a 1.5ml capped tube and covered with distilled water. Once a number of samples were available for preparation they were then carefully removed by squeezing gently at the nail varnish/bracket interface with a narrow pair of tweezers (UKGE Ltd). The carrier was then discarded in a sharps bin. The sample was then mounted on the diamond saw (Precision Diamond Wire Saw 3242, Well, Walter Ebner, Le Locle, Germany) as shown in Figure 4.14.1 using greenstick compound. The samples were then cut perpendicular to the anatomical surface the lesion. If possible, 2 sections per sample were obtained, however this was dictated by the size of the lesion. The widths of the sections were 280µm and these were then attached to brass anvils (Figure 4.14.3) 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) (Figure 4.14.4) using ball bearings of a known diameter. Sections were ground on both sides, which ensured that they were plano-parallel after which they were soaked off with acetone (VWR International Ltd, Poole, England) to remove them from the brass anvil. Following cleaning of all nail varnish residue with acetone (VWR International Ltd, Poole, England) the sections were then stored in mircrotubes submerged in distilled water prior to manufacture of the TMR plate.

Figure 4.14.1: Water cooled diamond saw

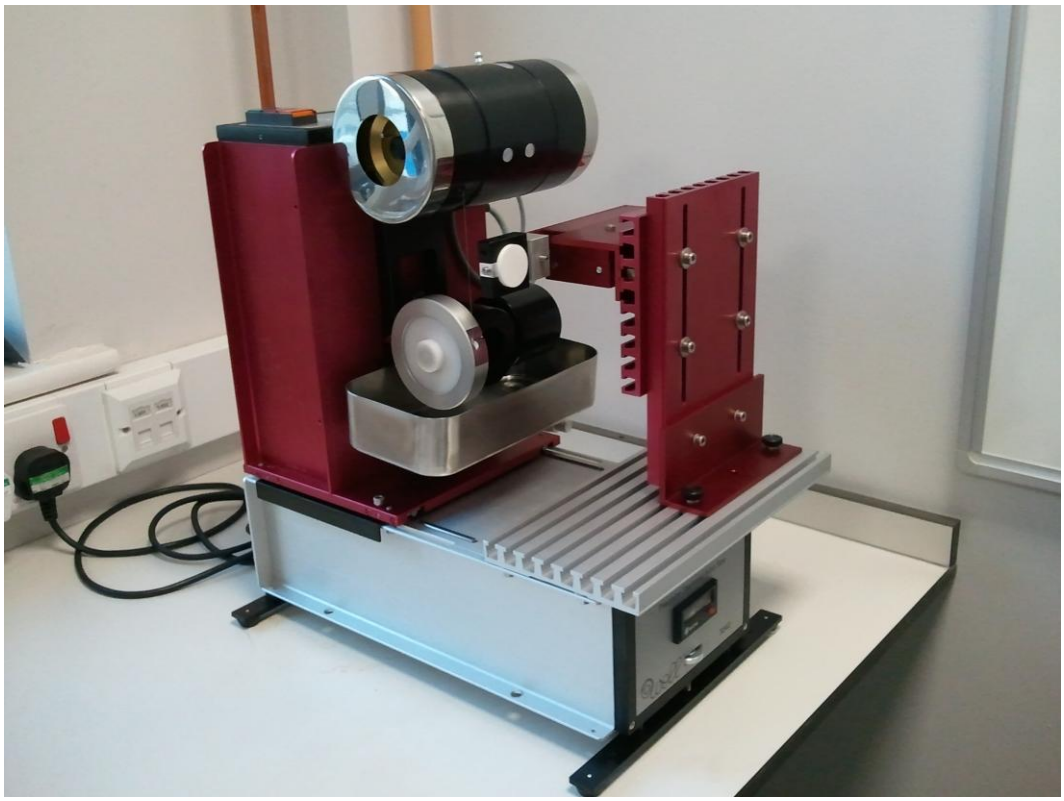


Figure 4.14.2 Sample mounted in greenstick compound showing the perpendicular direction of sectioning

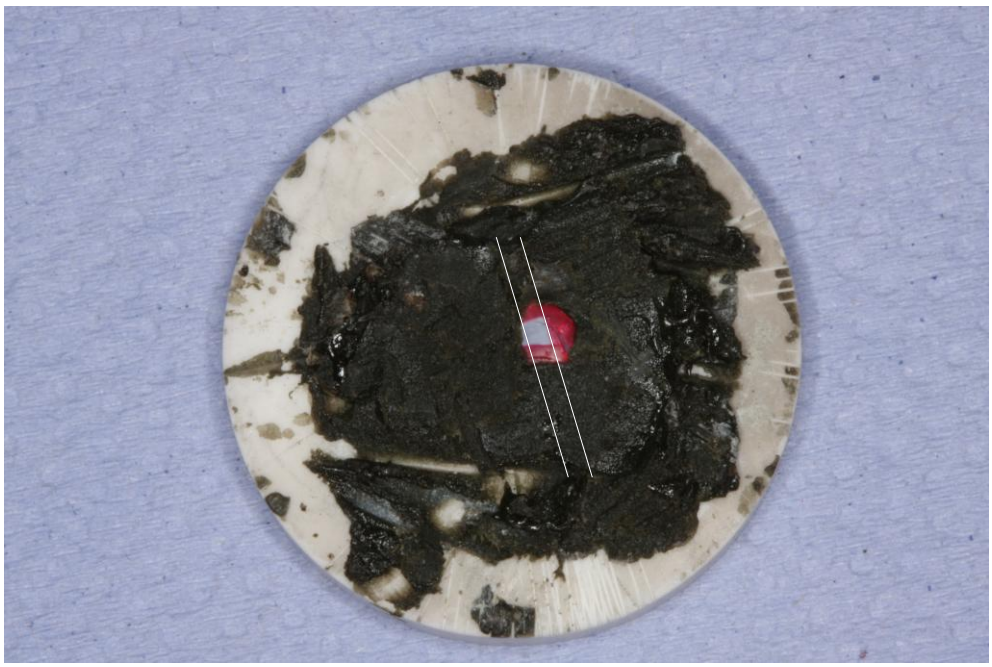


Figure 4.14.3: Brass anvil with mounted enamel sections using nail varnish

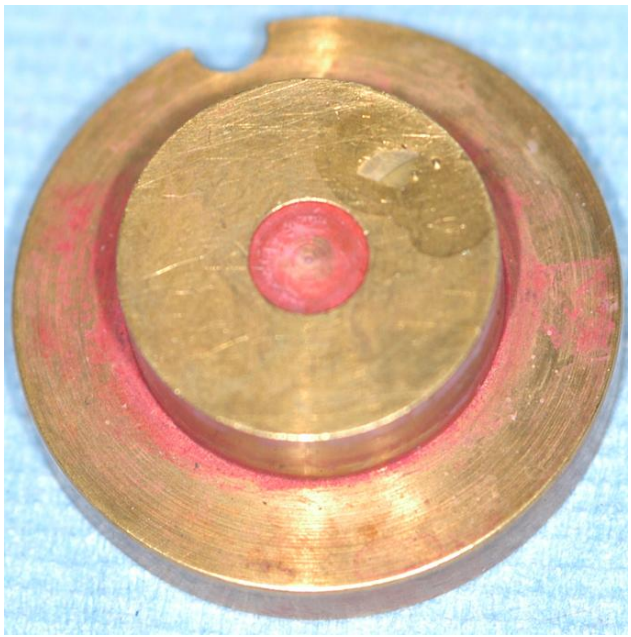
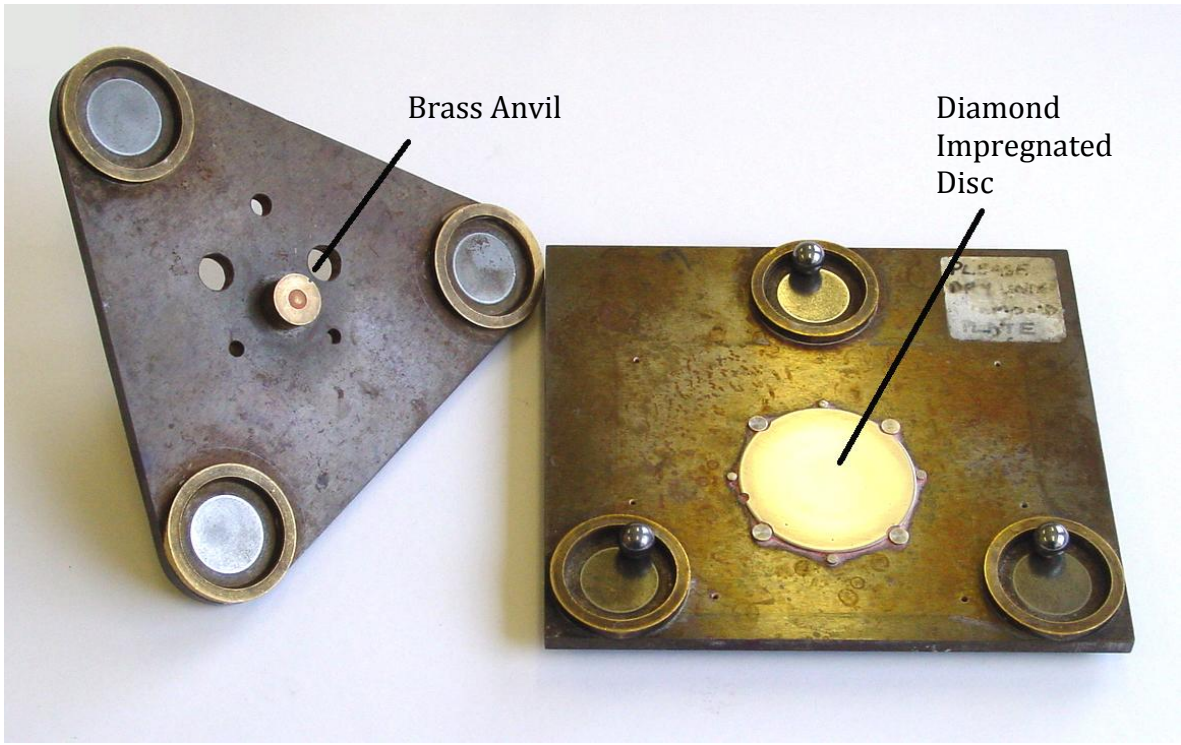


Figure 4.14.4: Brass anvil and diamond disc



The ground enamel sections are then mounted on a Perspex template covered in double sided sticky tape (Staples) as shown in Figure 4.14.5. This was then mounted onto a holder bearing an aluminium stepwedge with twelve 25µm steps. X-rays were generated using a Philips X-ray set (Philips B. V, Eindhoven, The Netherlands) from a copper anode with a nickel filter operating at 20Kv and 10mA with a focus distance of 40cm. A microradiograph was then taken with a 35 minute exposure onto a high resolution photographic plate (HTA Enterprises, California, USA). This time taken to expose was based on previous studies (Lovel 2008) to obtain a good radiographic contrast suitable for analysis later.

Figure 4.14.5: Mounted enamel sections on Perspex template

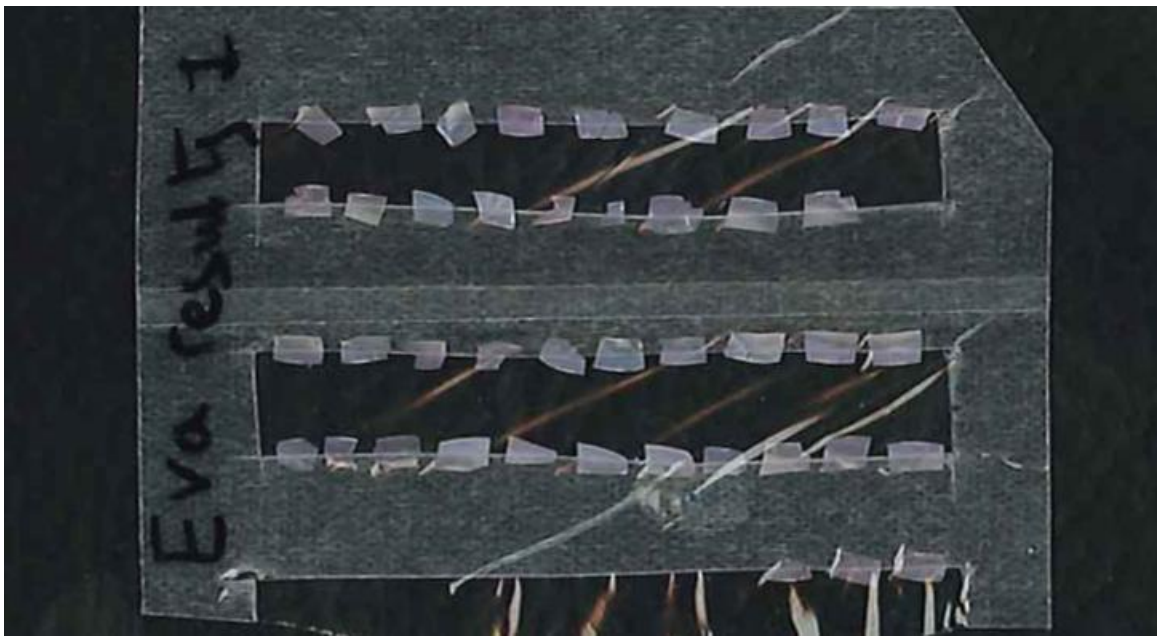
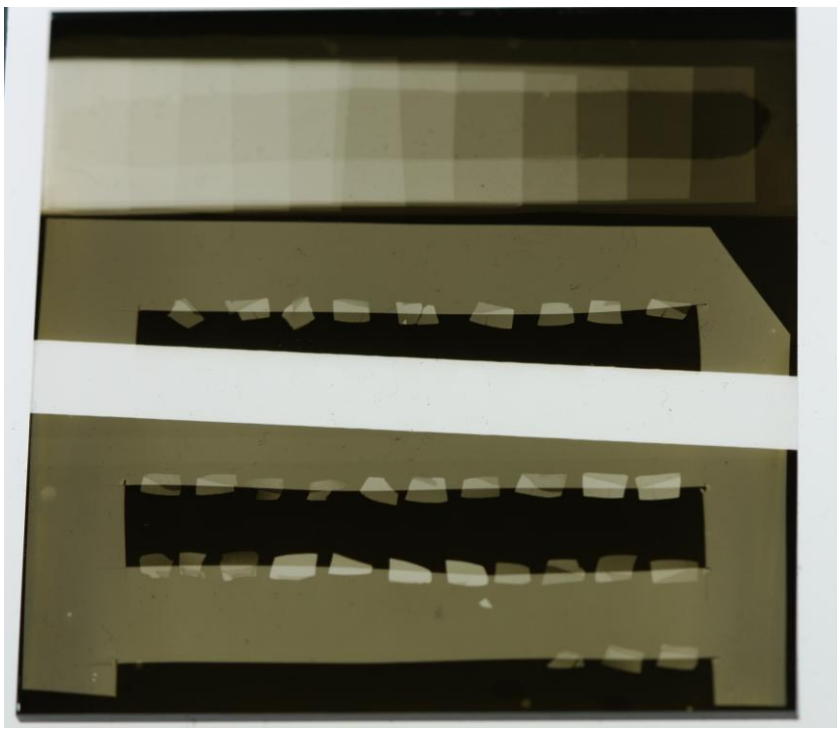


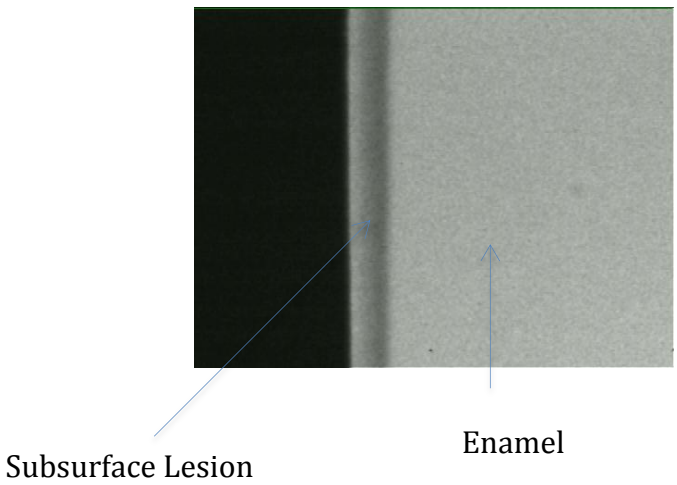
Figure 4.14.6 Micro radiograph of sections with stepwedge



4.15 TMR Imaging and Analysis

The microradiographs were then examined using a Leica Leitz DMRB optical microscope (Leica, Wetzlar, Germany). The images of the enamel sections were then captured using a CCD video camera (Sony, Tokyo, Japan), which was connected to a computer (Hewlett Packard Pavilion t3000, USA). Their typical appearance is shown in Figure 4.15.1.

Figure 4.15.1 Transverse microradiographs of a subsurface lesion



Step wedge calibration was then carried out. Ideally a correlation of 1.00 should be achieved with the line passing through 0, however 0.999 was deemed acceptable as shown in Figure 4.15.2. A typical radiographic image of a subsurface lesion is demonstrated in Figure 4.15.2. These were captured using the software TMR 2000 v 2.0.27.16, Inspektor Research System BV, Amsterdam, The Netherlands.

The lesions were then analysed using a bespoke software package developed by de Josselin de Jong (TMR 2006 v 3.0.0.10, Inspektor Research System BV, Amsterdam, The Netherlands) in order to assess the change in mineralisation.

Figure 4.15.2 Example of correlation following calibration of step wedge

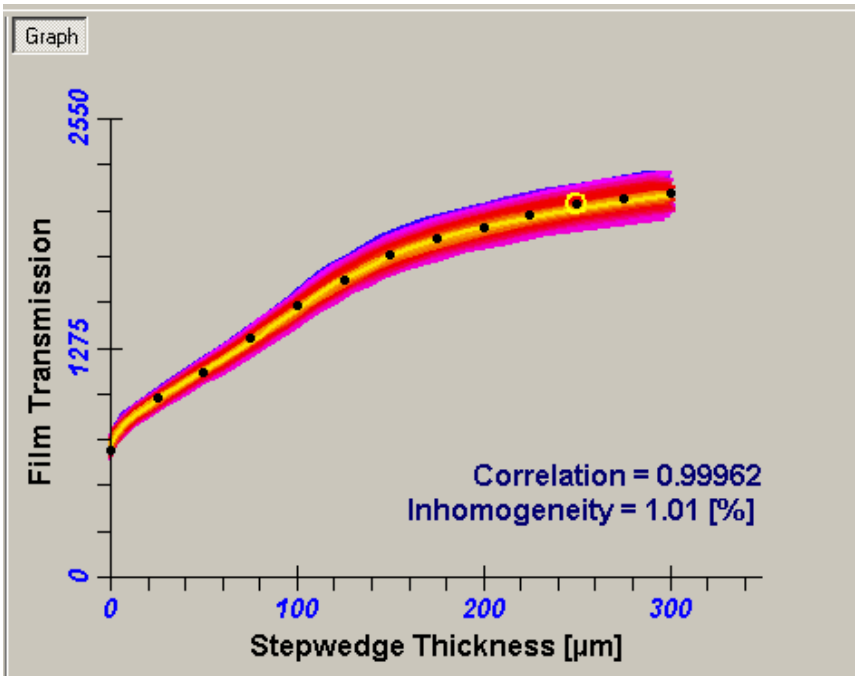
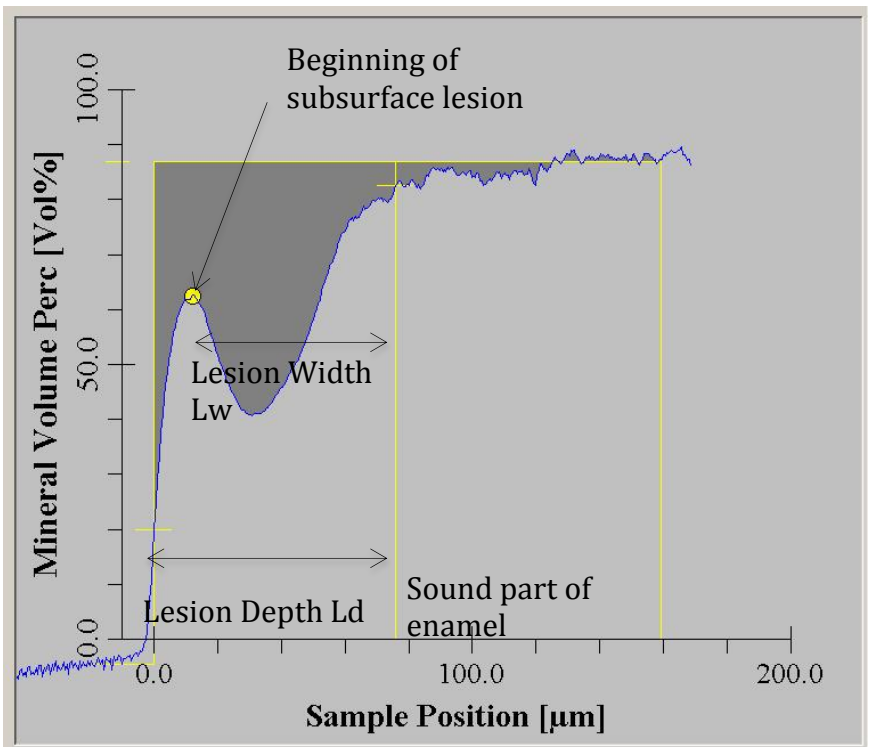


Figure 4.15.3 Analysis of TMR image shown from study with TMRW v1.22 software



4.16 Outcome Measurements

The difference in mineral loss/gain between the control specimens and those used in the subjects. Analysis of mineral content profiles, three parameters were obtained; mineral loss (vol%µm) lesion depth Ld (µm) and lesion width Lw (µm).

From this, a percentage change in mineral loss, lesion depth and lesion width was calculated. This was carried out by dividing the sample value by the control value and multiplying by 100 as described by Strang et al (1987). 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 enamel in lesion depth (L_d) is approximately 5 µm and in ΔZ about 220 vol%µm (Arends and ten Bosch 1992).

4.17 Statistical Analysis

Data was entered into the Statistical Package for Social Sciences software (SPSS version 20), which calculated the descriptive statistics including mean, standard deviation, minimum and maximum values of percentage mineral loss for each intervention group and frequency histogram of percentage mineral loss for each intervention group. The data was analysed with a 3-way analysis of variance (ANOVA) to test for differences in the percentage change in mineral loss, lesion depth and lesion width between the treatment groups, controlling for order and patient effects using a random model. Significance level for the statistical test was predetermined as $p < 0.05$.

5.0 Results

5.1 Baseline Lesions

Of 40 teeth prepared, 15 were needed, one for each subject. Only teeth with lesions in the range of a mineral loss of 900-1700 vol%µm were selected. Of these, only 14 teeth, in which all sections were found to be adequate (35%) and their mineral loss lay in the range of 990 -1450 vol%µm. Lesions from 2 other teeth (11a and 49a) were combined for the last subject as shown. The letters 'a' and 'b' denote the lesion from which the sample originated as shown in Figure 4.10.2. Sections were cut from each sample. The number of sections were either 1 or 2 depending on the size and quality of enamel. These values are the result of an average of 2 of readings taken where possible from the lesion. Depending on the size of the lesion 1 to 4 readings were taken from each tooth.

Table 15.1.1 Mineral Loss Parameters of Baseline Lesions selected

Tooth number	Mineral Loss (Vol%µm)	Lesion depth (µm)	Lesion width (µm)
3a	1110	45.9	38.5
3b	1080	44.8	35.45
10a	1170	45.6	36
10b	1010	41.7	32.1
11a	1080	46.1	38.87
13a	1287.5	46.85	37.43
13b	1283.3	47.23	38.47
16a	1380	51.2	42.9
16b	1400	52.7	38.8
18a	1010	46.25	38.95
18b	1180	45.5	40.3
22a	990	49.4	41.25
22b	1140	40.3	28.7
23a	1445	48.15	37.18
23b	1372	47.92	37
28a	1192.5	44.7	35.68
28b	1217.5	45.25	35.55
32a	995	43.625	33.13
32b	1383.33	46.5	38.57
34a	1073.33	48.2	38.73
34b	1130	46.67	38.07
36a	1375	54.68	40.18
36b	1380	50.7	43.45
38a	1325	50.65	42.98
38b	1070	45.9	37.7
40a	1422.5	59.48	47.93
40b	1330	56.1	44.45
41a	1210	47.2	38.7
41b	1160	45.1	36.1
49a	1103.33	44.83	36.43

5.2 Order of Interventions and Specimens

Table 5.2.1 Order of Interventions and specimens used in each subject

	Phase 1		Phase 2		Phase 3		Phase 4		Phase 5	
Subject	Intervention	Specimen	Intervention	Specimen	Intervention	Specimen	Intervention	Specimen	Intervention	Specimen
1	B	41A1	C	41B1	A	41A2				
2	A	38A1	B	38A2	C	38B1	A	38B2		
3	C	10A1	A	10B1	B	10B2				
4	C	13B1	A	13A1	B	13A2	C	11A2		
5	B	28A1	C	28B1	A	28B2				
6	A	22A1	B	22B1	C	22A2				
7	C	16A1	A	16B1	B	16B2	C	16A2		
8	B	3B1	C	3A1	A	3B2	B	3A2		
9	A	40A1	B	40B1	C	40B2	A	40A2	A	49A1
10	B	32A1	C	32B1	A	32B2				
11										
12	A	23A1	B	23B1	C	23B2				
13	C	34B1	A	34A1	B	34A2				
14	A	36A1	B	36B1	C	36B2	C	13B2		
15	B	18A1	C	18B1	A	18B2	C	18A2		

Key

Lost specimen
Dropout

Table 5.2.1 shows the order in which each subject received the intervention and the tooth number. There was one dropout, subject 11, as shown by the yellow line and one subject, subject 13, who completed 2 of the 3 interventions. Table 5.2.1 also shows the number of lost specimens occurred in the greatest number in the first phase, five altogether. One occurred in the second phase, 2 in the third and 1 in the fourth phase. The fourth and fifth phases were due to lost specimens *in situ* and the need to repeat those legs of the trial.

Figure 5.2.1 Flow diagram of patients through trial

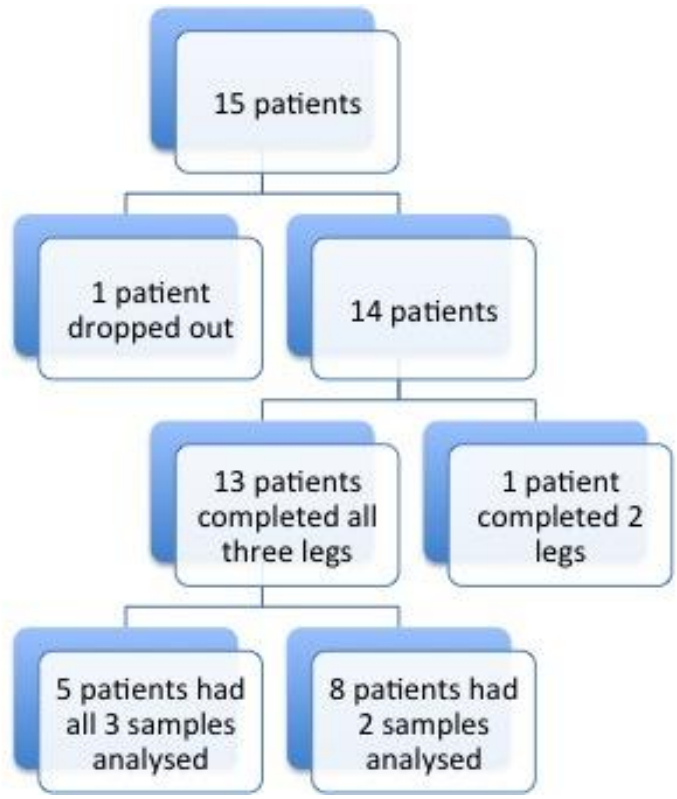
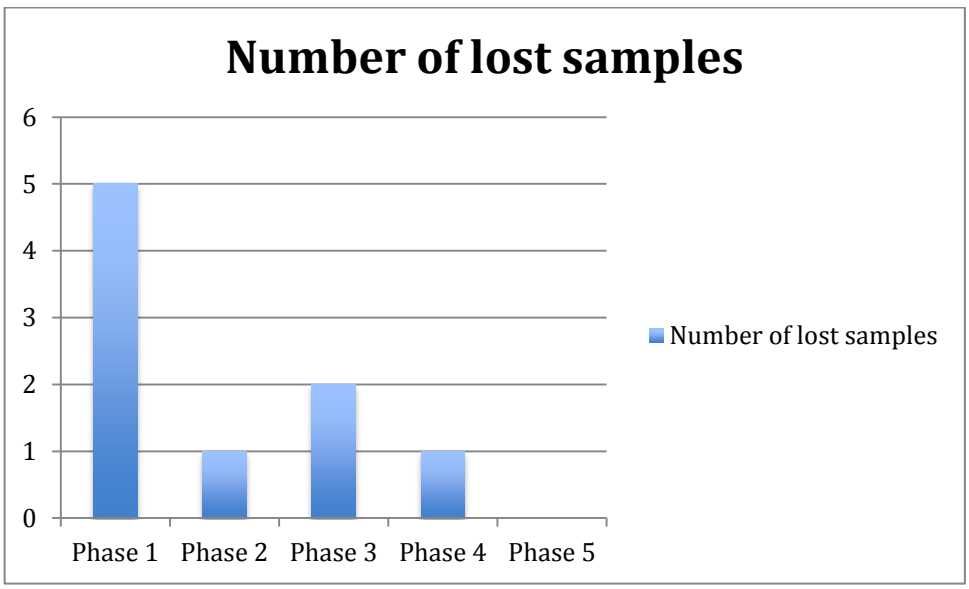


Figure 5.2.2 Table showing lost samples during *in situ* phases



The interventions were then revealed and are shown in the Table 5.2.2

Table 5.2.2 Coding and Interventions

Intervention code	Intervention
A	Toothpaste
B	Toothpaste and Tooth Mousse™ Plus
C	Toothpaste and Tooth Mousse™

5.3 Main Results

To test the change in parameters of mineral loss, lesion depth and lesion width, the percentage change in these respective variables was calculated in order to make the results comparable between each sample and its control. The data were normalised by dividing the sample value by the control value and multiplying by 100 as per Strang et al (1987). 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. For lesion depth and lesion width a value below 100 signifies a decrease in lesion size and a value above 100 signifies increase in lesion size. These results were calculated using the raw data presented in Appendices 10.6. The results are shown in Tables 5.3.1, 5.3.2, and 5.3.3 with an indication of which samples were lost and for what reason. Overall a further 6 samples were lost during the sectioning process and 3 samples in the polishing process. The flowchart in figure 5.3.4 collates what occurred to all the samples. The number of samples in each group is not equal due to dropouts and loss during the polishing and sectioning process. There were 11 samples in Group A, 9 samples in Group B and 12 samples in Group C. Overall only 5 patients had all three samples available for analysis at the end of treatment. 8 patients had two samples available and one patient had 1

sample available. This equated to a 22% loss rate due to the lab process excluding subject dropouts.

There was large intra-subject variation with both demineralisation and remineralisation occurring within subjects. Lesion width changes were in the order of 5-10μm. The accuracy of TMR for enamel in lesion depth (L_d) is approximately 5 μm, thus these changes could be nil if error is accounted for.

Table 5.3.1 Change in mineral content of each sample following in situ phase (%)

	Phase 1			Phase 2			Phase 3			Phase 4			Phase 5		
Subject	Intervention	Specimen	% mineral change	Intervention	Specimen	% mineral change	Intervention	Specimen	% mineral change	Intervention	Specimen	% mineral change	Intervention	Specimen	% mineral change
1	B	41A1	49	C	41B1	117	A	41A2							
2	A	38A1		B	38A2	94	C	38B1	143	A	38B2	105			
3	C	10A1		A	10B1		B	10B2	32						
4	C	13B1		A	13A1	62	B	13A2		C	11A2	98			
5	B	28A1	114	C	28B1	116	A	28B2	71						
6	A	22A1	195	B	22B1	109	C	22A2	81						
7	C	16A1		A	16B1		B	16B2	80	C	16A2	86			
8	B	3B1		C	3A1	95	A	3B2	52	B	3A2	74			
9	A	40A1		B	40B1		C	40B2	98	A	40A2		A	49A1	66
10	B	32A1	110	C	32B1		A	32B2	77						
11															
12	A	23A1	59	B	23B1		C	23B2	63						
13	C	34B1	110	A	34A1	150	B	34A2							
14	A	36A1	85	B	36B1		C	36B2		C	13B2	112			
15	B	18A1	100	C	18B1		A	18B2	70	C	18A2	108			

Key

Lost	During sectioning
Lost	During polishing
Lost	During in situ
	Drop out

Table 5.3.2 Change in Lesion Depth (%)

	Phase 1			Phase 2			Phase 3			Phase 4			Phase 5		
Subject	Intervention	Specimen	Change Lesion Depth (%)	Intervention	Specimen	Change Lesion Depth (%)	Intervention	Specimen	Change Lesion Depth (%)	Intervention	Specimen	Change Lesion Depth (%)	Intervention	Specimen	Change Lesion Depth (%)
1	B	41A1	96	C	41B1	156	A	41A2							
2	A	38A1		B	38A2	103	C	38B1	115	A	38B2	109			
3	C	10A1		A	10B1		B	10B2	108						
4	C	13B1		A	13A1	118	B	13A2		C	11A2	133			
5	B	28A1	128	C	28B1	125	A	28B2	110						
6	A	22A1	163	B	22B1	127	C	22A2	87						
7	C	16A1		A	16B1		B	16B2	100	C	16A2	112			
8	B	3B1		C	3A1	125	A	3B2	111	B	3A2	93			
9	A	40A1		B	40B1		C	40B2	112	A	40A2		A	49A1	115
10	B	32A1	102	C	32B1		A	32B2	117						
11															
12	A	23A1	99	B	23B1		C	23B2	98						
13	C	34B1	130	A	34A1	122	B	34A2							
14	A	36A1	99	B	36B1		C	36B2		C	13B2	123			
15	B	18A1	105	C	18B1		A	18B2	117	C	18A2	106			

Key

Lost	During sectioning
Lost	During polishing
Lost	During in situ
	Drop out

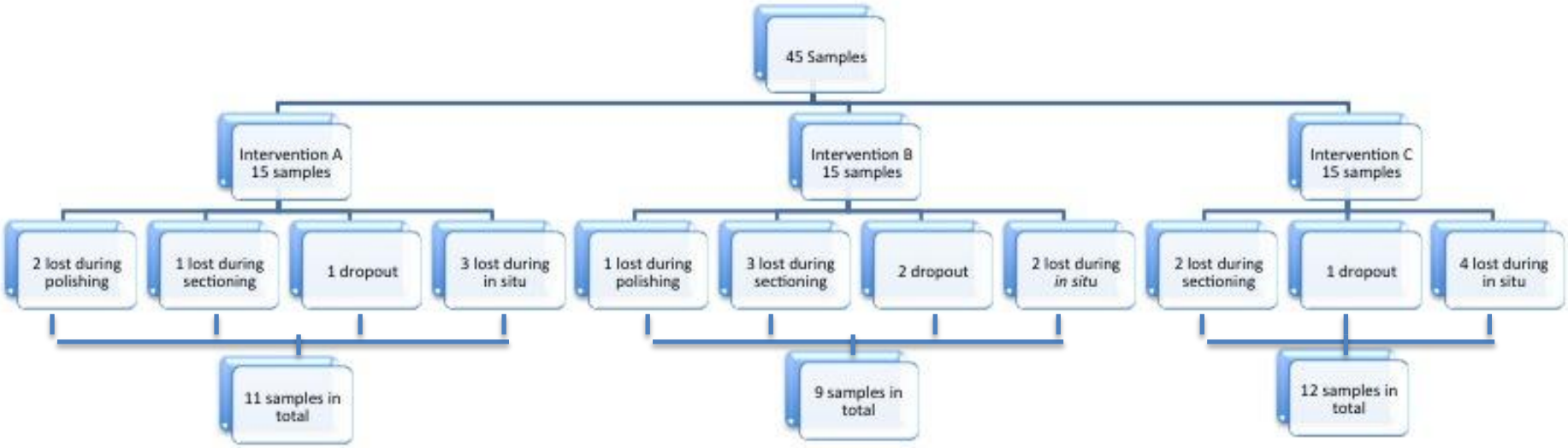
Table 5.3.3 Change in Lesions Width (%)

	Phase 1			Phase 2			Phase 3			Phase 4			Phase 5		
Subject	Intervention	Specimen	Change Lesion Width (%)	Intervention	Specimen	Change Lesion Width (%)	Intervention	Specimen	Change Lesion Width (%)	Intervention	Specimen	Change Lesion Width (%)	Intervention	Specimen	Change Lesion Width (%)
1	B	41A1	95	C	41B1	181	A	41A2							
2	A	38A1		B	38A2	106	C	38B1	121	A	38B2	109			
3	C	10A1		A	10B1		B	10B2	107						
4	C	13B1		A	13A1	85	B	13A2		C	11A2	130			
5	B	28A1	138	C	28B1	136	A	28B2	119						
6	A	22A1	159	B	22B1	156	C	22A2	77						
7	C	16A1		A	16B1		B	16B2	121	C	16A2	112			
8	B	3B1		C	3A1	131	A	3B2	126	B	3A2	91			
9	A	40A1		B	40B1		C	40B2	117	A	40A2		A	49A1	126
10	B	32A1	117	C	32B1		A	32B2	110						
11															
12	A	23A1	107	B	23B1		C	23B2	93						
13	C	34B1	144	A	34A1	124	B	34A2							
14	A	36A1	121	B	36B1		C	36B2		C	13B2	135			
15	B	18A1	106	C	18B1		A	18B2	111	C	18A2	108			

Key

Lost	During sectioning
Lost	During polishing
Lost	During in situ
	Drop out

Figure 5.3.4 Flow Chart of clinical phase Note: those samples lost *in situ* were replaced.



Frequency histograms were produced for % change in mineral loss for each intervention. This shows the distribution of the data.

Figure 5.3.1 Frequency histogram of change in mineral loss (%) for Intervention A

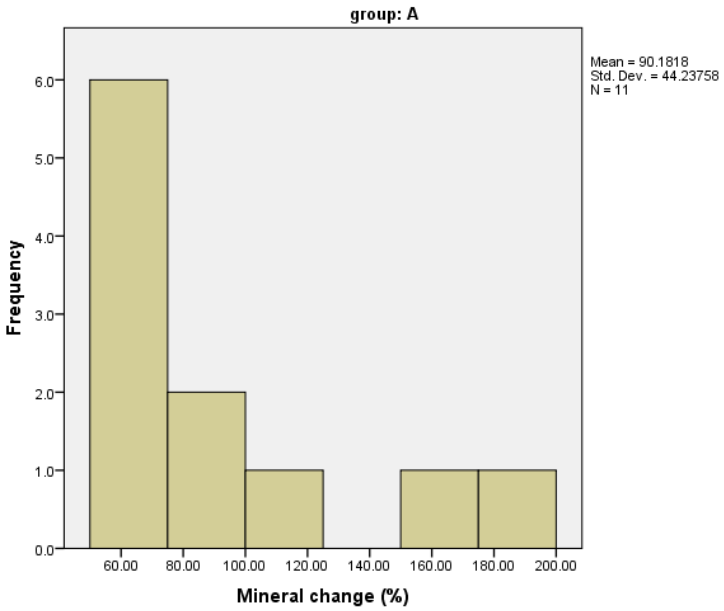


Figure 5.3.2 Frequency histogram of change in mineral loss (%) for Intervention B

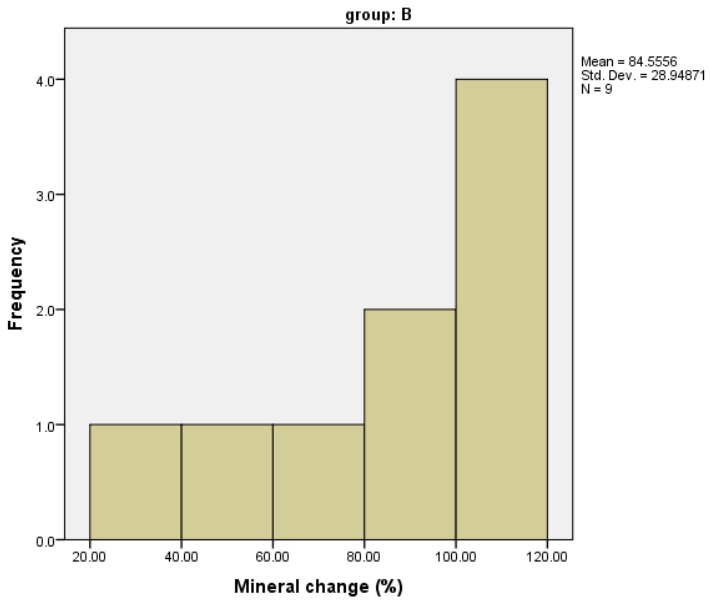


Figure 5.3.3 Frequency histogram of change in mineral loss (%) for Intervention C

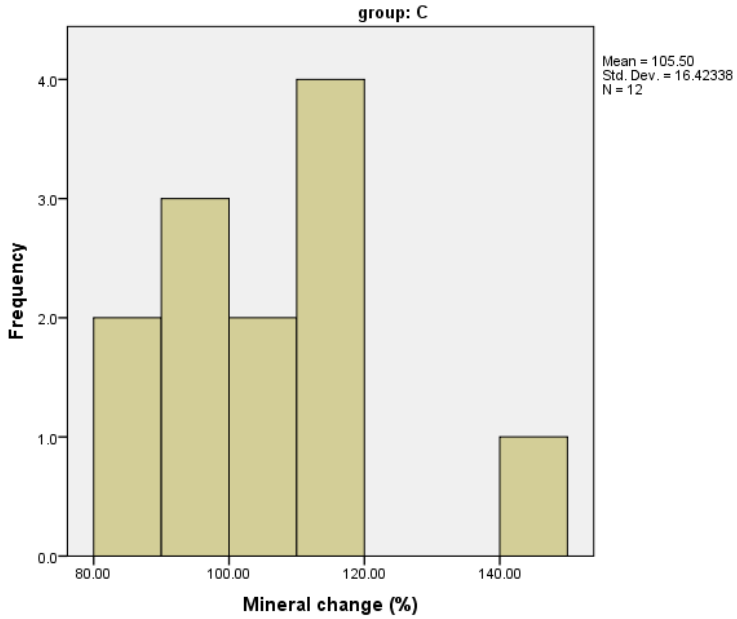


Figure 5.3.4 Frequency histogram of change in lesion depth (%) for Intervention A

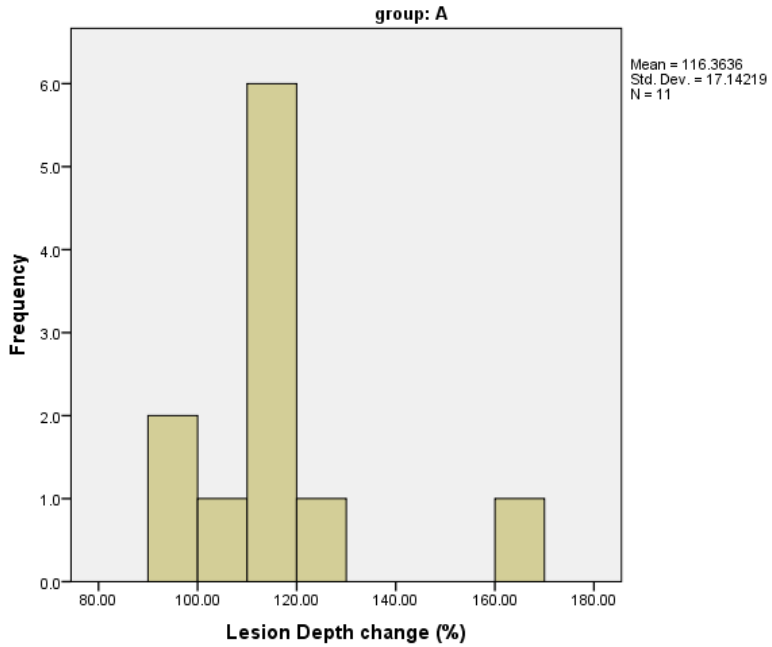


Figure 5.3.5 Frequency histogram of change in lesion depth (%) for Intervention B

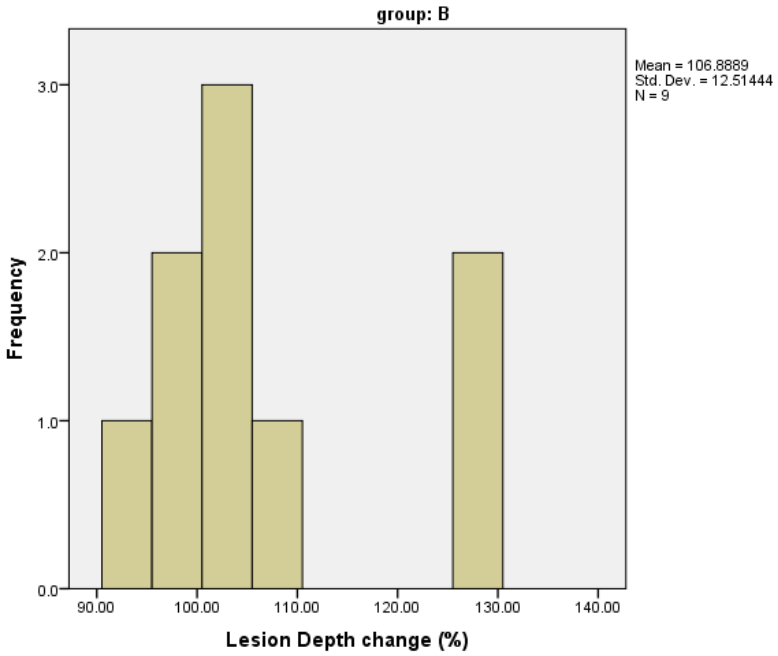


Figure 5.3.6 Frequency histogram of change in lesion depth (%) for Intervention C

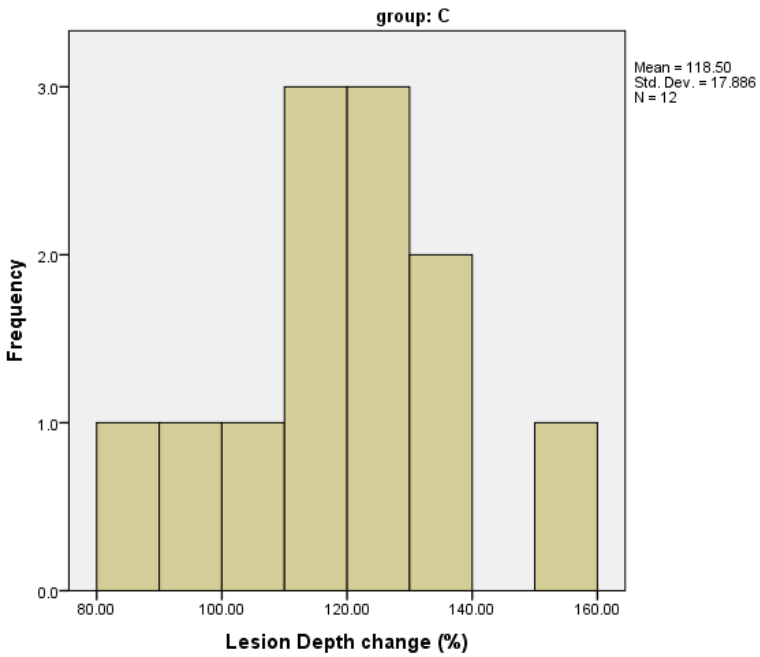


Figure 5.3.7 Frequency histogram of change in lesion width (%) for Intervention A

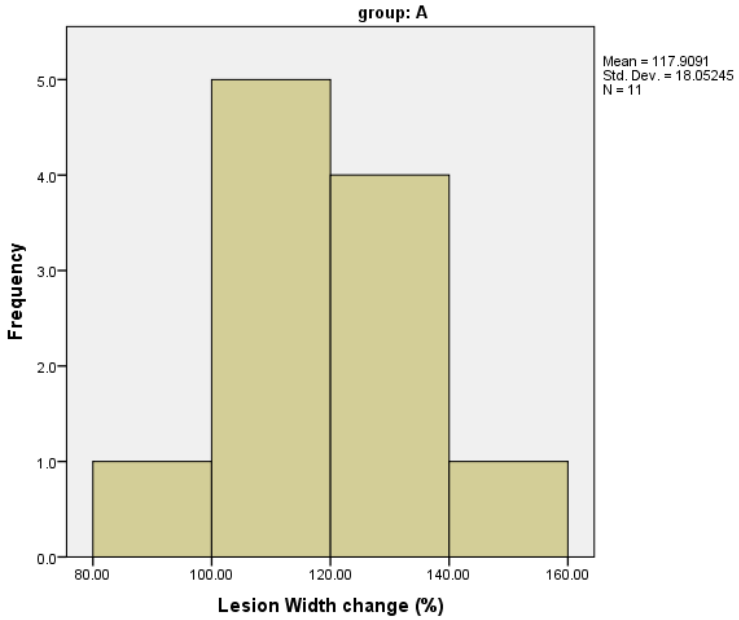


Figure 5.3.8 Frequency histogram of change in lesion width (%) for Intervention B

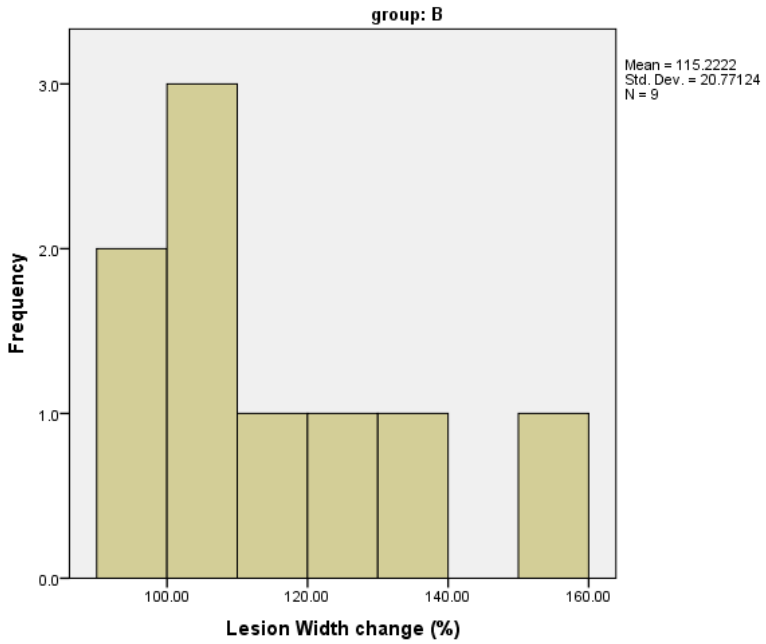
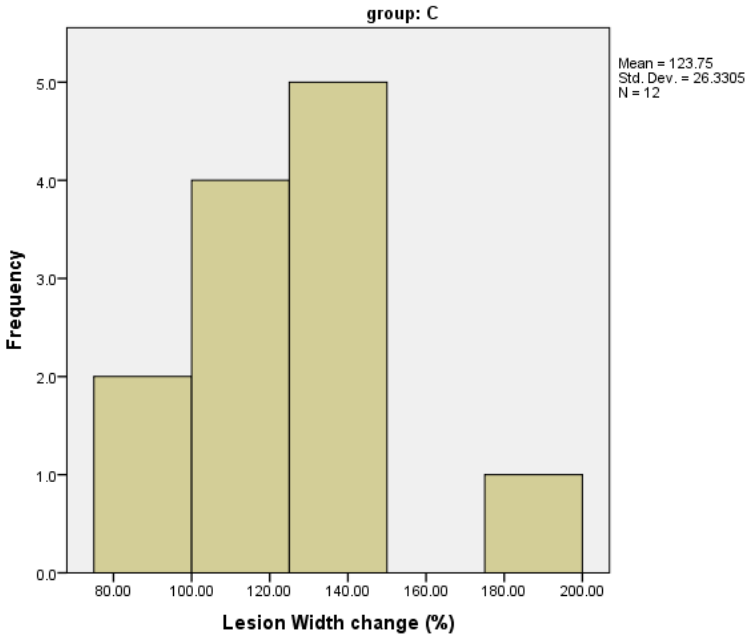


Figure 5.3.9 Frequency histogram of change in lesion width (%) for Intervention C



These frequency histograms for Interventions A, B and C were to identify if the samples were normally distributed. Following statistical advice this is not possible to identify with certainty, as the sample sizes are too small. However the graphs do show some elements of being normally distributed for the purposes of this statistical analysis were assumed to be normally distributed.

Table 5.3.4 Mean and standard deviation of Change in mineral loss, Lesion depth and Lesion width

Intervention		Change in mineral loss (%)	Lesion Depth (%)	Lesion Width (%)
A	Mean	90	116	118
	N	11	11	11
	Std. Deviation	44	17	18
B	Mean	85	107	115
	N	9	9	9
	Std. Deviation	29	13	21
C	Mean	106	119	124
	N	12	12	12
	Std. Deviation	16	18	26
Total	Mean	94	115	119
	N	32	32	32
	Std. Deviation	32	17	22

Table 5.3.4 shows the mean change in mineral loss, lesion depth and lesion width for each intervention. The mean change in mineral loss in Group A, (standard toothpaste) was 90% with a standard deviation of 44%, in Group B (standard toothpaste and Tooth Mousse™ Plus) the mean change was 85% with a standard deviation of 29% and Group C (standard toothpaste and Tooth Mousse™) the mean mineral loss was 106% with a standard deviation of 16%.

Figure 5.3.10 Mean change in mineral loss (%) per intervention

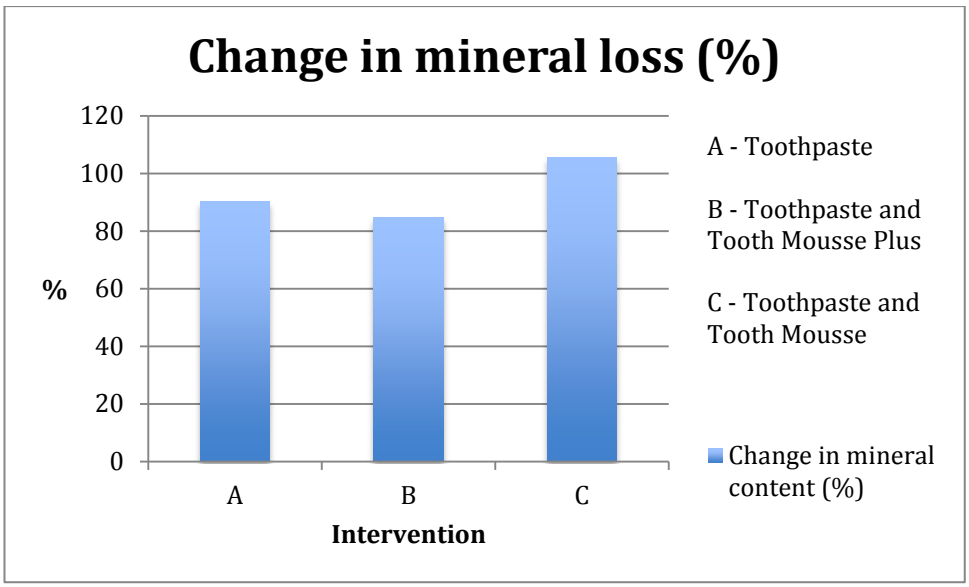


Figure 5.3.11 Mean change in lesion depth and lesion width (%) per intervention

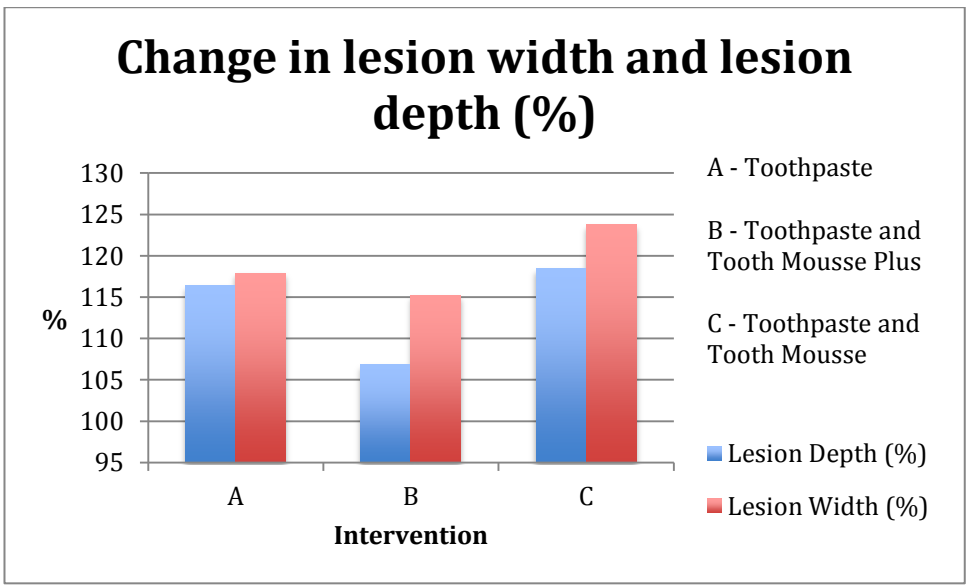


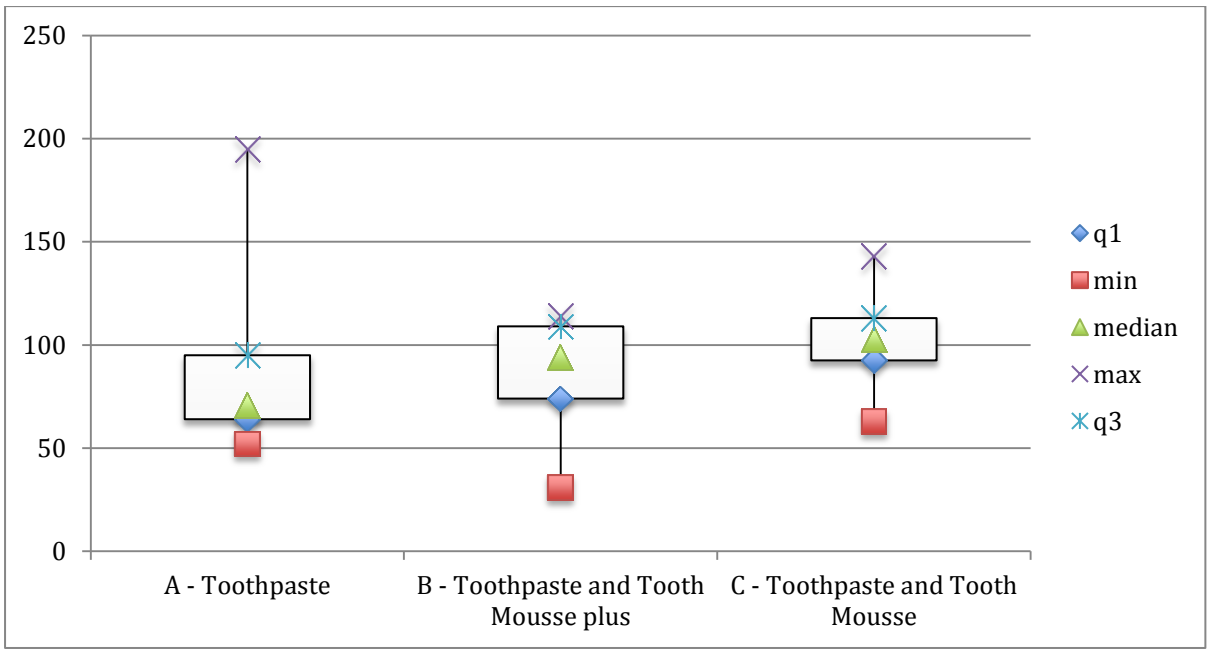
Figure 5.3.10 and figure 5.3.11 demonstrates the mean changes in mineral loss, lesion depth and lesion width. Remineralisation occurred with A, the standard toothpaste and

B, the standard toothpaste and Tooth Mousse™ Plus. Conversely there was mean demineralisation that occurred with intervention C, the standard toothpaste and Tooth Mousse™, but this was just greater than 100% indicating little change in mineral content. The mean lesion depth and width increased with all three interventions. Due to the large range of percentage change in mineralisation for all interventions, the mean is not a good way of interpreting the effect of the interventions due to the outliers. However the outliers could be just normal variability. The data in Table 5.3.5 were transformed into the box and whisker plot shown in Figure 5.3.12. This allows graphical presentation of the distribution of the data with the median being more resistant to outliers. Figure 5.3.12 demonstrates the range for the percentage change in mineral loss and for intervention A was 52% to 195%, intervention B was 31% to 114% and intervention C was 63% to 143%.

Table 5.3.5 Statistical data for Mineral Change (%)

statistic	Intervention A	Intervention B	Intervention C
q1	64	74	92.5
min	52	31	63
median	71	94	103
max	195	114	143
q3	95	109	113

Figure 5.3.12 Box and whisker plot of mineral change (%)



5.4 Statistical Analysis: ANOVA testing

Analysis of variance (ANOVA) testing is a parametric model for comparing continuous data between more than two groups. The null hypothesis being that the populations have identical means. One-way ANOVA and two-way ANOVA differ in that the groups in two-way ANOVA have two categories of defining characteristics instead of one. Because we are comparing three groups with three interventions and we want to compare the means of these groups, the three–way ANOVA test is the most appropriate in this setting (Altman 1999, Bruce et al, 2008).

It has the following assumptions (Bruce et al, 2008):

- Continuous data
- Three or more independent groups with normal distributions

- Groups should have similar standard deviations
- Probability distribution (F-distribution)

The data were tested for normal distribution as shown by the histograms in Figure 5.3.1-9, however following statistical advice there is not an adequate amount of data to be able be certain of normal distribution, however there are some elements of normal distribution and so this has been assumed. The standard deviation for percentage change in mineral loss for Group A, B and C was 44, 29 and 20 respectively. This was judged to be similar enough for the sake of the statistical test but it could be argued that these values are too far apart. The standard deviations of both the percentage change in lesion width and lesion depth were more similar. The F-distribution is calculated by the taking the degrees of freedom for between groups minus 1 and the degrees of freedom for within groups is calculated the total number of subjects minus the number of groups being compared. The F-ratio is then compared to tables of the F-distribution and degrees of freedom to check for significance at a certain p-value (Bruce et al 2008).

This model contained three categorical independent variables (participant number, tooth number of the sample and intervention) and one continuous dependent variable (% change in mineral loss). This model was used with percentage change in lesion depth and lesion width as the continuous dependent variable separately.

<u>Null Hypotheses (Ho) Testing for change in mineral loss</u>

1. Ho 1: Mineral loss was independent of order

   P=0.625 ➔ Ho is not rejected

2. Ho 2: Mineral loss ΔZ was independent of intervention

P=0.278 ➔Ho is not rejected

3.Ho 3: Mineral loss ΔZ was independent of subject

P=0.66➔Ho is not rejected

These tests are summarized in Table 5.4.1

Table 5.4.1 Tests of between subject effects: Mineral loss (%)

Dependent Variable: Mineral loss (%)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Order	1884.845	3	628.282	0.603	0.625
Intervention	2945.209	2	1472.604	1.413	0.278
Subject	10720.664	13	824.666	0.791	0.66

Table 5.4.1 shows that there was no significant effect on the change in mineral loss (%) for the order in which they received the intervention (p=0.625), the intervention (p=0.278) or for the subject (p=0.66).

Null Hypotheses (Ho) Testing Lesion Depth

4.Ho 4: Lesion depth was independent of order

P=0.474➔Ho is not rejected

5.Ho 5: Lesion depth was independent of intervention

P=0.184➔Ho is not rejected

6.Ho 6: Lesion depth was independent of subject

P=0.957➔Ho is not rejected

These tests are summarized in Table 5.4.2

Table 5.4.2: Tests of between subject effects: Lesion depth (%)

Dependent Variable: Lesion depth (%)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Order	911.86	3	303.953	0.887	0.474
Intervention	1324.839	2	662.42	1.933	0.184
Subject	1655.956	13	127.381	0.372	0.957

Table 5.4.2 shows that there was no significant effect on the change in lesion depth for the order in which they received the phase (p=0.474), intervention (p=0.184), or for the subject (p=0.957).

Null Hypotheses (Ho) Testing Lesion Width

7.Ho 7: Lesion width was independent of order

P=0.582➔Ho is not rejected

8.Ho 8: Lesion width was independent of intervention

P=0.553➔Ho is not rejected

9.Ho 9: Lesion width was independent of subject

P=0.977➔Ho is not rejected

Table 5.4.3 Tests of between subject effects: Lesion width (%)

Dependent Variable: Lesion width (%)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Order	1414.944	3	471.648	0.676	0.582
Intervention	866.537	2	433.269	0.621	0.553
Subject	2838.746	13	218.365	0.313	0.977

Table 5.4.3 shows that there was no significant effect on the change in lesion width for the order in which they received the intervention (p=0.582), the intervention (p=0.553), or for the subject (p=0.977).

The groups were unbalanced due to drop outs and lost samples, group A having 11 subjects, group B having 9 subjects and group C having 12 subjects. This situation is not ideal for an ANOVA test and may have had an effect on the results. If the data are assumed to be non-parametric regression testing is suggested by Altman (1999). Statistical advice was that the global ANOVA should be performed first, and only if this is significant should the treatment means be compared pairwise, using for example Tukey's test.

5.5 Quantities of Toothpaste and Tooth Mousse™ (Plus) applied and correlation testing

Table 5.5.1 shows the quantity of Toothpaste and Tooth Mousse™ (Plus) used by each subject. Figure 5.5.1 is a box and whisker plot and shows from these data that the average quantity of toothpaste used was 37g per 4 week phase and ranged from 12g to 68g. The average quantity of Tooth Mousse™ (Plus) used was 6g and ranged was 2g to 18g. However only 64% of the Toothpastes were returned for weighing and 79% of the Tooth Mousse™ (Plus) tubes were returned.

Figure 5.5.1 Box and Whisker Plot of amounts of Toothpaste and Tooth Mousse™ used.

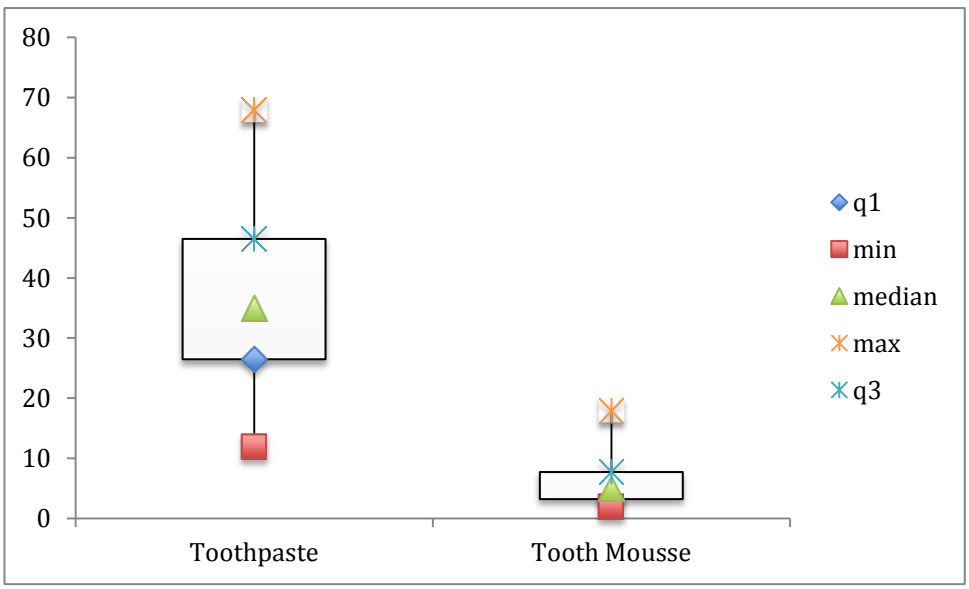


Table 5.5.1 Amount of Toothpaste and Tooth Mousse™ used (g)

	Phase 1				Phase 2				Phase 3				Phase 4				Phase 5			
Subject	Intervention	Specimen	TP (g)	TM (g)	Intervention	Specimen	TP (g)	TM (g)	Intervention	Specimen	TP (g)	TM (g)	Intervention	Specimen	TP (g)	TM (g)	Intervention	Specimen	TP (g)	TM (g)
1	B	41A1	UK	3	C	41B1	57	2	A	41A2	UK	NA								
2	A	38A1	26	NA	B	38A2	18	5	C	38B1	UK	3	A	38B2	27	NA				
3	C	10A1	41	4	A	10B1	UK	NA	B	10B2	45	3								
4	C	13B1	UK	12	A	13A1	UK	NA	B	13A2	UK	UK	C	11A2	UK	18				
5	B	28A1	38	5	C	28B1	35	5	A	28B2	21	NA								
6	A	22A1	UK	NA	B	22B1	48	12	C	22A2	UK	UK								
7	C	16A1	31	5	A	16B1	42	NA	B	16B2	22	4	C	16A2	54	7				
8	B	3B1	UK	4	C	3A1	68	3	A	3B2	UK	NA	B	3A2	UK	UK				
9	A	40A1	40	NA	B	40B1	60	7	C	40B2	UK	UK	A	40A2	UK	NA	A	49A1	UK	NA
10	B	32A1	21	5	C	32B1	52	10	A	32B2	UK	NA								
11																				
12	A	23A1	UK	NA	B	23B1	33	5	C	23B2	43	8								
13	C	34B1	UK	UK	A	34A1	UK	NA	B	34A2	12	UK								
14	A	36A1	27	NA	B	36B1	48	8	C	36B2	UK	UK	C	13B2	33	10.5				
15	B	18A1	UK	3	C	18B1	26	3	A	18B2	31	NA	C	18A2	UK	5				

Key

Lost	During sectioning	TM	Tooth Mousse™
Lost	During polishing	TP	Toothpaste
Lost	During in situ	Weight of TP	100g
	Drop out	Weight of TM	53g
UK	Unknown		
NA	Not Applicable		

5.6 Correlation Testing: Mineral loss vs quantity of Tooth Mousse™

Correlation analysis is used to describe the strength and direction of the linear relationship between the two data sets. A Pearson correlation coefficient is for parametric data of two continuous variables. The test gives a range of values from -1 to +1, -1 being a perfect negative relationship and +1 being a perfect positive and 0 being no linear relationship (Bruce et al, 2008).

The two variables in this case being the Tooth Mousse™ used and percentage demineralisation change. Table 5.6.1 was entered in to SPSS and generated the data in table 5.6.2. A correlation of +0.144 shows no correlation between the variables, being close to zero.

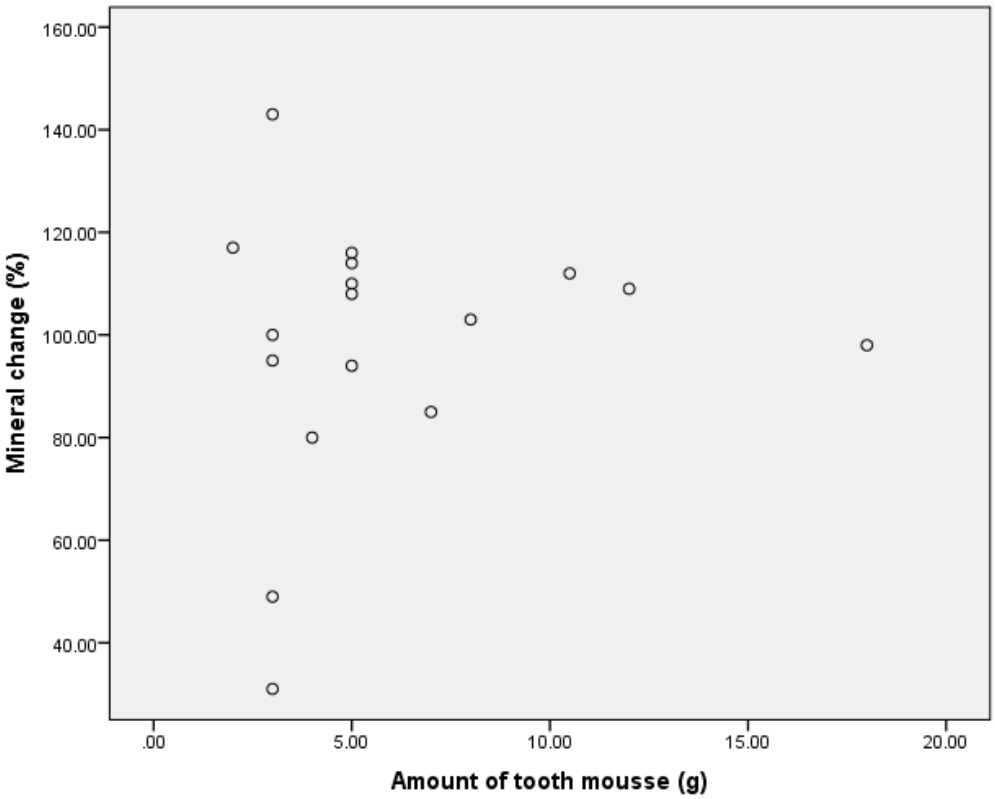
Table 5.6.1 Amount of Tooth Mousse™ (Plus) used in comparison to % mineral loss

Tooth Mousse™ (g)	mineral loss (%)
3	49
5	114
5	110
3	100
2	117
5	94
5	116
12	109
3	95
3	143
3	31
4	80
8	103
18	98
7	85
10.5	112
5	108

Table 5.6.2 Pearson's correlation of Tooth Mousse™ vs Mineral loss data

		Amount of Tooth Mousse™ (g)	Mineral loss (%)
Amount of Tooth Mousse™ (g)	Pearson Correlation	1	0.144
	Sig. (2-tailed)		0.582
	N	17	17
Mineral loss (%)	Pearson Correlation	0.144	1
	Sig. (2-tailed)	0.582	
	N	17	17

Figure 5.6.1 Scatter plot of Mineral loss (%) Vs Tooth Mousse™ (g)



5.7 Intervention and Washout periods

Table 5.7.1 shows the interventions with the number of days in each intervention and washout period. There was a variety in the number of days for both the intervention period and the washout period. 30 samples were *in situ* for 28 days. Only 2 samples had intervention period of 27 days. 11 samples had a washout of 28 days. 5 samples had washout periods of greater than 28 days, which was between 32 and 42 days. 3 samples had washout periods of 27 days. The differences in washout and intervention period were due to ability of patients to attend the appointments, due to prior commitments.

Table 5.7.1 Intervention and washout periods (days)

	Phase 1				Phase 2				Phase 3				Phase 4				Phase 5			
Subject	Intervention	Specimen	Time with carrier (days)	Wash out period (days)	Intervention	Specimen	Time with Carrier (days)	Wash out period (days)	Intervention	Specimen	Time with carrier (days)	Wash out period (days)	Intervention	Specimen	Time with carrier (days)	Washout period (days)	Intervention	Specimen	Time with carrier (days)
1	B	41A1	28	28	C	41B1	28	28	A	41A2	28								
2	A	38A1	28	29	B	38A2	27	28	C	38B1	28	42	A	38B2	28				
3	C	10A1	29	28	A	10B1	28	42	B	10B2	28								
4	C	13B1	28	32	A	13A1	28	28	B	13A2	28	35	C	11A2	28				
5	B	28A1	28	31	C	28B1	28	38	A	28B2	28								
6	A	22A1	28	42	B	22B1	27	28	C	22A2	28								
7	C	16A1	28	28	A	16B1	28	28	B	16B2	28	27	C	16A2	28				
8	B	3B1	28	28	C	3A1	28	28	A	3B2	28	28	B	3A2	28				
9	A	40A1	29	27	B	40B1	32	28	C	40B2	28	24	A	40A2	32	24	A	49A1	28
10	B	32A1	28	28	C	32B1	28	28	A	32B2	28								
11																			
12	A	23A1	28	27	B	23B1	28	28	C	23B2	28								
13	C	34B1	28	27	A	34A1	28	57	B	34A2	28								
14	A	36A1	28	28	B	36B1	28	28	C	36B2	28	28	C	13B2	28				
15	B	18A1	28	28	C	18B1	28	27	A	18B2	28	28	C	18A2	28				

Lost	During sectioning
Lost	During polishing
Lost	During in situ
	Drop out

6.0 Discussion

6.1 Summary of study findings

This study demonstrated:

1. Transverse microradiography analysis revealed that there was no significant effect on the percentage change in mineralisation for the intervention (p=0.278), the order in which they received the intervention (p=0.625) or for each subject (p=0.66).

2. Transverse microradiography analysis also revealed there was no significant effect on the lesion depth for the intervention (p=0.184), the order (p=0.474) or the subject (p=0.957).

3. Transverse microradiography analysis also revealed there was no significant effect on the lesion depth for the intervention (p=0.553), the order (p=0.582) or the subject (p=0.977)

4. Remineralisation and demineralisation occurred with all three interventions and within each subject with an overall pattern tending towards remineralisation for all interventions. There were large intra-subject and inter-subject ranges with no association between intervention and degree of mineral loss or gain.

5. There was no correlation between the amount of mineral loss and the amount of Tooth Mousse™ or Tooth Mousse™ Plus used (r=0.144).

6. The lesions were difficult to produce with only 14 whole teeth adequate out of a total of 40 teeth (35%).

6.2 Discussion

The aim of this study was to investigate if Tooth Mousse™ or Tooth Mousse™ Plus had a remineralisation effect in orthodontic patients using an *in situ* study design. The subjects were a representative group with fixed appliances. The results showed great variability both between interventions and within each subject with no overall trend. This was similar to that seen by other studies (Benson et al 1999).

The average mineral loss for each intervention shows remineralisation for intervention A, standard toothpaste and intervention B, standard toothpaste with Tooth Mousse™ Plus. Conversely the average of intervention C, standard toothpaste and Tooth Mousse™ shows demineralisation. However means are not resistant to outliers, so this is not a reliable method of interpretation of what has occurred in this study. Box and whisker plots show the range and interquartile range and can be used to indicate an overall trend. This shows huge range in percentage mineral loss with a range of 52% to 195% for standard toothpaste and 31% to 114% for standard toothpaste with Tooth Mousse™ Plus and 63% to 143% for standard toothpaste and Tooth Mousse™. Thus with all the interventions both remineralisation and demineralisation occurred. The trend appears to favour more demineralisation with Intervention C, standard toothpaste with Tooth Mousse™ than with Intervention A, which was a standard toothpaste only. However, the interquartile ranges for both Intervention B and C cross the 100% line, which indicates no or little change in the mineral loss.

Testing of the null hypothesis (1-9) using the ANOVA test found that the hypothesis could not be rejected for mineralisation change, lesion depth or lesion width for the

intervention, the order in which they received the intervention or for the subject. Due to the lack of balanced groups the ANOVA test could have been affected.

The major flaw in this study is the loss of data. This could have affected the outcome of the study. In all, only five subjects had a complete set of data for each intervention. Eight patients had 2 sets of data and one patient had 1 set of data. This left the groups unbalanced, which is not ideal for an ANOVA test. Increasing the sample size, as well as improving the in situ technique would help to reduce the loss of data in the future. Allowing for a learning curve in the TMR technique by carrying out a pilot study would also help to reduce the loss of data.

The sample size calculation was based on a previous *in vitro* study. This has implications as an *in vitro* study has controlled conditions and thus the standard deviation would be less than in an *in vivo* study. This was shown by Benson (2009) finding a standard deviation of 189 vol%μm in comparison with this study which had a much larger standard deviation of 328 vol%μm. It would have been preferable to use a previous *in situ* study for the sample size calculation. If the study had used a standard deviation of 328 vol%μm, we would be looking for a clinically significant difference in the order of at least twice this figure.

The ANOVA statistics used were advised to be the most appropriate. ANOVA assumes independent groups, however the enamel samples were not independent due to the cross-over design of the trial. The sample size calculation was based on mineral change (vol%μm), however the statistics used percentage change. This was felt to be more appropriate for comparison between different teeth.

A Pearson correlation coefficient of 0.144 was found between the between the amount of Tooth Mousse™ (Plus) used and percentage mineral loss on an individual basis. This indicates there was no relationship between the two variables. The average amount of Tooth Mousse™ (Plus) used was very small. The fact that such a small amount of paste was used on the carrier can be argued that this could have had an effect on the results of the study such that individual factors, which are discussed in further detail below, would have had a greater influence on the outcome.

Remineralisation has been shown to even occur in distilled water (Lovel 2008). In this study demineralisation and remineralisation occurred. This is likely to be due to the individual environmental factors such as diet, buffering ability of saliva and composition and thickness of the biofilm and fluoride ion concentration in the oral fluids. Orthodontic patients are instructed in a non-cariogenic diet, however this depends on the compliance of the patient. The subjects have individual buffering ability, which was not measured, and will be different at different times according to frequency of food intake, amount of saliva flow and composition of saliva. Individuals will have varying compositions of biofilm, which will have different cariogenic properties. Instructions regarding oral hygiene were given to the subjects (appendix 10.5).

It was noted that when the carriers were removed from the subjects, that they were nearly always coated in plaque. The gauze was very effective at trapping and retaining the plaque. However as discussed previously the amount and composition of the biofilm will have an effect on its cariogenic potential. If this plaque was high in streptoccus species, this may have accounted for the demineralisation seen in some samples.

Further research could have been done to test the bacterial composition of the plaque seen.

It was also observed that a number of the lesions once removed from the carrier had a black colouration. This has been noticed in other studies in the cariology group. The aetiology of this colouring may be due to chromogenic bacteria. A study carried out by Saba et al (2006) used the Polymerase Chain Reaction to identify bacteria causing the black discolouration. *Porphyromonas gingivalis* and *Prevotella melaninogenica* have previously been implicated, however in this study (Saba et al 2006) *Actinomyces* could have been involved because its presence was demonstrated in half the patients with black staining. *Actinomyces* is one of the later colonisers and is associated with caries thus could have been responsible for further demineralisation seen. There was also some concern that by placing the carriers straight into the capped tubes, submerged in distilled water there may have been further action by the biofilm coating and thus exposing the sample to demineralisation. This could have changed the mineral content of the samples when left over a period of time. However the viability and vitality of *streptococcus salivarius* (Roger et al, 2011) and *streptococcus mutans* (Tong et al 2011, Zhongchun et al 2011) has been shown not be maintained in starvation conditions and thus placement in sterile water should not have affected the results.

Behavioural factors that affect the anticariogenic properties of the interventions used include frequency of toothpaste use, length of brushing, rinsing practices after brushing, the time of day the toothpaste is applied and the amount of toothpaste applied used (Zero 2006). However, the cross-over design of the study should over-come these confounding factors because each crossover patient serves as his or her own control.

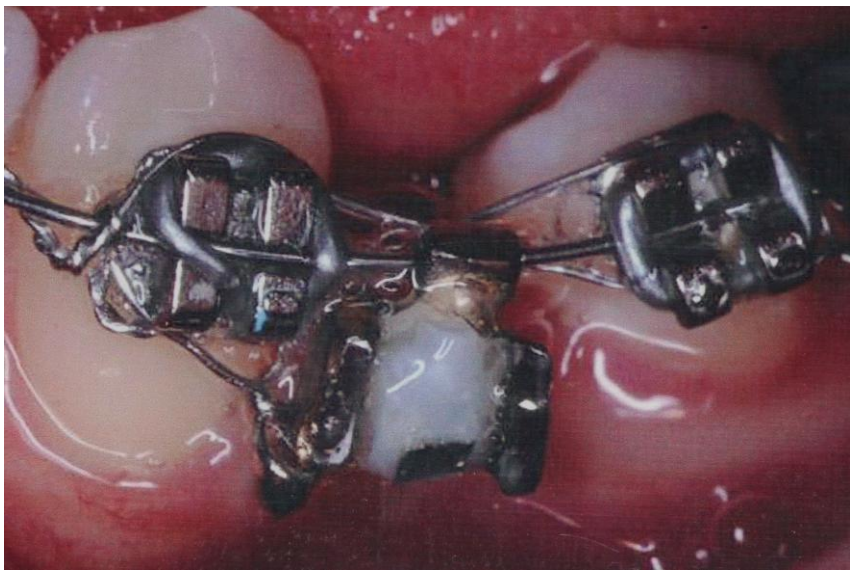
The subjects were given instructions to maintain consistency with oral hygiene procedures (Appendix 10.5). The subjects were asked not to use any additional products and asked not to rinse following brushing. Rinsing has an effect on the amount of fluoride found in the saliva, reducing the amount in the oral cavity. Nordström (2009) showed no significant difference of fluoride concentration in the mouth between a 5000ppm and a 1450ppm toothpaste when rinsing was carried out with the 5000ppm toothpaste and no rinsing with the 1450ppm toothpaste. Patients' individual habits and compliance with the instructions would thus have an affect on the amount of fluoride present in the saliva and the remineralisation/demineralisation found in the sample. However previous studies (Adeyemi et al 2010) found no difference in the remineralisation of post-orthodontic white lesions between the difference types of toothpastes, which contained 250, 1100 or 2800 ppm amounts of fluoride. Willmot (2004) found that use of a low fluoride mouth rinse made no significant difference in the size reduction of post-orthodontic white lesions.

Ethical approval for this study was granted with the topical use of Tooth Mousse™ (Plus) to the carrier only with no further application to the rest of the mouth. There was some concern that there would be too much fluoride applied when Tooth Mousse™ Plus was used however this would not pose a risk of fluoride toxicity (Welbury 2005). The total maximum amount of fluoride that would have been used if the whole mouth application were instigated would have been 2300ppm. This is still well below any toxic level as demonstrated and ethical approval could have been granted for a whole mouth application of Tooth Mousse™ and Tooth Mousse™ Plus.

6.3 Development of carrier design

Benson (2000) developed the design of a holder for the enamel following experimentation of attaching the enamel sample to an orthodontic band (Figure 6.3.1). This was found to be unsuccessful at retaining the enamel sample. Following this, the bracket holding the enamel sample was designed to be retained on the archwire. It was also found that the acid-etch technique could be used on the human enamel sample and this could reasonably be compared to the control that has not undergone this procedure. Benson (2000) found this design reflected more accurately the position of the bracket in the arch. The specimen was protected from trauma and toothbrush abrasion. The holder design was a more comfortable design for the patient and was easier to place and remove.

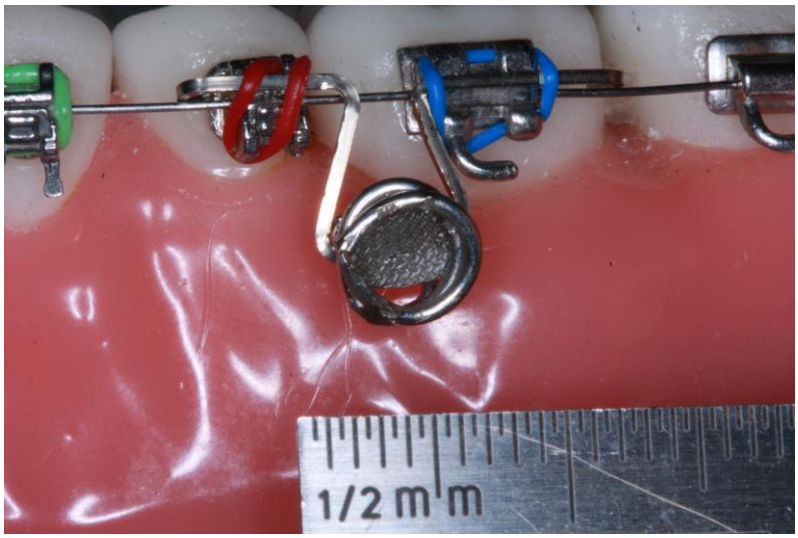
Figure 6.3.1: Design used by Benson (2000)



Since this design was developed, more importance has been placed on the need for a biofilm to be present for remineralisation to take place. The biofilm acts as a reservoir

for fluoride and calcium ions. Thus in this design the incorporation of a gauze over the enamel sample to create a biofilm was deemed important by the Cariology group. Other options were considered that would incorporate Dacron gauze between 2 metal rings. The Dacron gauze is then able to hold a biofilm on the surface of the lesion. With the Dacron gauze *in situ* this would also mean the acid-etch technique would not be necessary to retain the enamel sample in the carrier.

Figure 6.3.2: Design developed in order to incorporate Dacron gauze demonstrated on a typodont.



The design shown in Figure 6.3.2 shows 2 metal rings welded to a molar bracket base. The molar base incorporates a tube, which allows a stainless steel wire to be inserted and then formed, using orthodontic wire bending pliers, in order that it can be tied over the archwire present. This idea was developed to solve the problem of the subject being in flexible archwires. However there was concern in being able to tie the carrier to the brackets securely with this design, especially if the patient was in full-size archwires.

Wire ligatures would have to be used in preference to elastic modules to prevent loss of the carrier. The bulkiness of the design may have made it uncomfortable for the subject.

The design was further developed and the method described below was a modified version of that used by Benson (2000). The most ideal situation for carrier placement in orthodontic terms, was a rectangular archwire, either stainless steel or nickel titanium. This prevented movement of the carrier and thus possible irritation to the participant's buccal mucosa. If this was not possible, and the carrier was placed on a round archwire, a stainless steel ligature (0.010 " stainless steel) could also be placed through the second ball hook on the carrier and around the hook on the first molar tube or band. This would also stabilise the carrier and prevent it from moving. It was important not to affect the patient's treatment by using a wire that would not help with treatment progress and so this method of stabilisation was also used on round nickel titanium archwire. If the position of the carrier was such that it was pressing on the gingiva, the ball hook was bent using a tucker to move the carrier into a more comfortable position.

6.4 Limitations of the study

This study had a number of limitations. These were divided into clinical and laboratory based limitations.

6.4.1 Clinical Phase

Delicacy and bulkiness of the carrier necessitated its position in the buccal sulcus of the mandibular arch, as this is anatomically the only place on a buccal appliance that would be comfortable for the participant. A number of failures of the carrier occurred;

loosening of the Dacron gauze possibly due to over-welding of the rings and burning of the gauze, failure of the welded rings, failure of the weld of the ball hook to the bracket base. These failures could have been exacerbated by positioning of the carrier on the left-hand side of the mouth in a right-handed brusher. Following a number of initial failures, the carriers' position was changed to the right-hand side of the mouth and this reduced the number of failures. This change, however, could have affected remineralisation of the lesions as a right-handed brusher would spend less time brushing the right-hand side of the mouth and therefore there would be less toothpaste delivery to the lesion. However a previous study (Benson et al 1999) found no difference between placing *in situ* enamel on the dominant and non-dominant side of the mouth.

Other possible positions of the lesion could have been in a palatal position on an upper removable appliance. This, however, would not be representative of an orthodontic patient with a buccally placed fixed appliance as it would be subject to other environmental factors such as the tongue and may not be brushed.

The use of Dacron gauze could be eliminated altogether and the use of an acid etch technique with resin bonding could be used to secure the composite to the bracket base. The sample surface should attract biofilm without the Dacron gauze in place and prevent the large build up of plaque over the sample.

Compliance with the intervention could have biased the results although randomisation of the order of compliance should reduce the possibility of this. Means of assessing compliance should be included with most protocols (Wefel 1995). In this study

compliance was measured indirectly by requesting the patients to bring in their used tubes of toothpaste and Tooth Mousse™. In all, 64% of the toothpaste tubes and 79% of the Tooth Mousse™ tubes were returned and on weighing of the tubes, showed that there was compliance with both the toothpaste and the Tooth Mousse™. The patient could however, have squeezed out the tube to appear as though they had complied with the intervention. A modification could have been made by asking the patients to complete a calendar; self-reporting has been shown to help compliance with headgear as reported by Cureton et al (1993a, 1993b).

The consistency of Tooth Mousse™ is such that it has a very slippery consistency and in my own subjective view is a little tricky to place. It tends to slide off the tooth. So in this case where only a small amount is being applied to the carrier using one's finger, it is possible that the Tooth Mousse™ may slide away and be swallowed. The gauze component of the carrier however would have helped maintain some of the Tooth Mousse™ *in situ*. The recommendations by GC Tooth Mousse™ are not to eat for 30 min following application of the Tooth Mousse™. In retrospect this should have been applied to this study to prevent loss of the Tooth Mousse™ from the oral environment. If the patients ate and drank following brushing, this may have affected the results.

This study was blinded using insulation tape on the Tooth Mousse™ and Tooth Mousse™ Plus. The lids of these Tooth Mousse™ tubes, however were a different colour, one being red and one being white. Ideally both tubes should have been identical to be a truly blinded study. This could have affected the subjects' behaviour and caused performance bias (Jadad 1998).

Intervention and washout phases should have been 28 days in length. However due to patient commitments this was not possible. A pragmatic approach had to be adopted in order to maintain commitment to the study. The majority of samples were *in situ* for the correct amount of time. In total, 30 of the samples were *in situ* for 28 days. Only 2 samples had an intervention period of 27 days. A 1 day difference would have had very little impact as shown by the results as one of these samples showed demineralisation and 1 sample demonstrated remineralisation. The washout phases were less consistent with only 11 samples having washout phases of 28 days. The remaining 8 samples had washout phases of greater or 1 day less than 28 days. Having a reduced washout period would only affect the results if the intervention used had good retention in the mouth and a long half-life. As these interventions are topical this is unlikely to occur and the variation in the washout periods was unlikely to have affected the study.

In the subjects that lost the sample, it was decided to continue with the study and repeat the lost intervention at the end. This would thus impact upon order, such that not all the subjects received the interventions in the order allocated to them by the randomisation process. The statistical analyses, however demonstrated that there is not enough evidence to reject the null hypothesis that the order of intervention had an effect on the mineralisation of the sample and thus this would not have had a significant impact on the results.

6.4.2 Laboratory Phase

Creating the lesion is a wasteful process. 40 human teeth were demineralised at the start of the study. Only 14 whole teeth were within the demineralisation parameters

chosen. It was attempted to have one whole tooth for each patient but this was not possible. Due to failures during the clinical part of the study, some lesions were taken from different teeth, however a range of demineralisation was selected of between 900-1700%volμm. As the data are looking at the percentage change in demineralisation the variation in baseline should not have had an effect.

As brackets failed new carriers were made. These new carriers were not modified as it was felt this could affect the results. There was a learning process associated with the manufacture of the brackets. Figure 5.2.1 demonstrates the reduced loss rate as the study progressed, from losing 5 carriers/samples at the beginning of the study to 1 carrier at the end of the study. More attention was paid to the welding of the rings and the hook due to previous failures in these areas. However in some the gauze was found to pull away with the rest of the carrier intact. This could have been due to over welding and thus burning of the Dacron gauze, which would make it easier to pull away from the rings. The retention of the enamel sample could have been improved using an acid-etch technique to hold the sample within the carrier and this would be a recommendation if the study were to be repeated. Benson et al (1999) describes how etching of the enamel makes very little difference to the overall mineral loss. However, this would be another way in which the control would have been treated differently to the experimental section. Removing such a small enamel sample, in this case 1 x 2mm may have increased the risk of damage.

Enamel samples may become damaged during the whole experimental process. It is not possible to assess if the sample is damaged during the *in situ* phase or due to sectioning and the TMR process. It is possible that some of the lesions could have actually been

affected by erosive changes during the *in situ* phase instead of being due to the processing. The final TMR pictures showed surface damage the type of which is both seen in erosion or due to enamel breaking off during sectioning which is discussed below.

Benson (2009) reported there was a 13% loss due to TMR processing. There was a 22% loss of data due to TMR processing in this study. Benson (2009) used bovine enamel, which has a different structure to human enamel and the size of each lesion was 5mm by 5mm in comparison to the lesion size in my study being 1mm by 2mm. The larger the sample the more robust it will be to the sectioning and polishing process.

In order to sterilise the carrier it was irradiated. The control sample however was not irradiated. This is unlikely to affect the enamel hardness or its resistance to demineralisation (Rodrigues et al 2004); however ideally the control should also be irradiated so all samples were treated in the same way. As we were looking at percentage change in mineral loss from the control this shouldn't have had any effect overall.

Systematic and random errors in taking the TMR readings are possible. This could make a significant result subject to a type II error, finding no difference when there is one (Bruce et al 2008). Benson (1999) carried out a reproducibility assessment of 2 readings taken and found there was systematic error in the recording of mineral loss and lesion width. To identify if this occurred in this study, repeat measurements would have to be taken on 2 separate occasions.

Problems with sectioning

Six samples were lost during the sectioning process. This occurred because the samples disintegrated during cutting. The enamel layer would separate from the underlying dentine and due to the brittle nature of the enamel it would then disintegrate. Partway through the laboratory process the diamond wire saw was upgraded to a new model, which uses direct vision to section the enamel. The old model used indirect vision and this required a learning curve to achieve consistent results. The new diamond wire saw made the sectioning process much easier and no samples were lost with the new model. TMR requires plano-parallel sections for analysis (White 1992). This however is done using subjective judgment and is very difficult to assess to an accurate degree once the samples are mounted in the wax. This could be something to address in the future by the manufacturers of the diamond saw.

The lesions used in this study were 1mm x 4mm. Once sectioned into two, the lesions were 1mm x 2mm in size approximately. Only a maximum of two sections could be gained from the sample taking account of the width of the diamond wire, however in the majority only one section could be cut from the sample. Obtaining a greater number of samples would increase the number of readings and thus the mineral change would be more representative of what had occurred. If this study was to be repeated the windows painted on the premolar teeth should be enlarged to allow a slightly larger lesion to be created. This could be one large rectangle that could then be sectioned into four and thus more sections could be gained.

7.0 Conclusions

There were large intra-subject and inter-subject ranges with no association between intervention and degree of mineral loss or gain.

Remineralisation and demineralisation occurred with all three interventions and within each subject with an overall pattern tending towards remineralisation for all interventions.

There was no significant difference between the three interventions on mineral gain or loss.

Due to the large number of samples lost, this could have had a large effect on the results
In this study the null hypothesis that there was no difference between the remineralising abilities of Tooth Mousse™ with normal fluoride toothpastes in orthodontic patients could not be rejected.

Based on the results of this study, orthodontic patients are at risk of enamel demineralisation during treatment and attentiveness by the clinician should be maintained at all times. Consistently effective preventive regimes to prevent demineralisation in patients with fixed orthodontic appliance need to be developed. Targeting of remineralisation and prevention regimes based on sex, pretreatment age, oral hygiene, and clinical status of the first molars can be used as predictors for the development and severity of white spot lesions during orthodontic treatment (Al Maaitah et al 2011).

The aims of this study have been met. However, further improvements of the *in situ* method are required and a movement towards in vivo method is needed in order to advance our knowledge of the remineralising potential of this group of agents.

8.0 Future Research

Following this study, future research should concentrate on developing the *in situ* model such that it is representative as closely as possible to the *in vivo* situation. The work completed in this study should form the basis for subsequent investigation.

In order to assess the full remineralising efficacy of casein phosphopeptide, development of the *in situ* model is recommended. The other possibility is to move to an *in vivo* model, which would require a non-destructive method of measuring demineralisation such as QLF.

Given that there is a research that both shows Tooth Mousse™ (Plus) to significantly affect remineralisation of white spot lesions (Brochner et al 2011) and conversely that it makes no difference to post orthodontic white spot lesions (Beerens et al 2010), a randomised controlled model is an ideal method to answer this question. The use of QLF which is non-destructive and comparable to TMR in measuring demineralisation (Benson 2008 and Lovel 2007) could be used *in vivo* on subjects undergoing orthodontic treatment or who have previously sustained white spot lesions following orthodontic treatment and treated with the interventions used in this study, however with the whole mouth application of the Tooth Mousse™ (Plus). Further work needs to done to ascertain the remineralisation potential of Tooth Mousse™.

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Instructions for Tooth Mousse™ available from;

http://www.toothmousse.info/index.html

10.0 Appendices

10.1 Patient Information sheets

10.1.1.Participant information sheet

version 1.3

Department of Clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place, Liverpool L3 5PS
Research Ethics Committee; NW 2 REC Liverpool Central

February 2011

PARTICIPANT INFORMATION SHEET

REMINERALISATION PROPERTIES OF CALCIUM BASED TOOTHPASTE: IN SITU CROSS -OVER STUDY

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 which makes toothbrushing more difficult. Fluoride mouth rinses have been recommended to prevent the occurrence of these white spots, however this may not lead to full repair of the white spot. Calcium based toothpastes are now available and have been shown in some studies to promote better repair of these white spots. The purpose of this study is to find out if calcium based toothpastes which contain fluoride are better at repairing these white spots than fluoride toothpaste alone.

Has the study been approved?

Yes. The Liverpool Central Research Ethics Committee has given the 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 cosponsoring the study.

Who will be conducting the study?

The study is being run by Prof. Susan Higham (Professor of Oral Biology), Prof. Neil Pender (Professor of Orthodontics and Consultant in Orthodontics), and Miss Eva Bryniarska(Specialist Registrar in Orthodontics).

Why have I been asked to take part? You have been asked 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 enamel will be attached to the wire part of your brace, positioned towards the back part of your lower brace. This will not affect your brace.

You will be given a toothbrush and a standard fluoride toothpaste to use during the study period. You will also be given two smaller tubes at different times that contain pastes for application to the piece of enamel in the holder once each day. As normal, you will be instructed to brush your teeth for 2 minutes twice a day with a pea sized amount of the toothpaste until your next routine orthodontic appointment which will be 4 weeks later. You will be asked not to use any

other toothpaste or mouth rinses during the 4 week period. At that following 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 carry out your usual oral hygiene procedures until your next routine brace appointment with no carrier on your brace. At this appointment a new carrier will be attached to the brace and you will use the normal toothpaste as before but in addition will be asked to apply the paste from one small tube to the piece of enamel in the holder.

You will have in total 3 carriers attached. Two of the carriers will need paste from a small toothpaste tube whilst the third will not need additional paste except for the standard fluoride toothpaste provided for you. The pastes will be labelled with a letter so that the researcher and the participants won't know the identity of the pastes, this is called blinding. However we do know that the two small tubes of paste contain calcium with either a small amount of fluoride or calcium alone.

How long will the study last?

The study will last for approximately 6 months of your treatment.

What if I don't want to take part?

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

What if I have a question or there is a problem on the trial?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions 0151 7065252. If you remain unhappy and wish to complain formally, you can do this through the Patient Advice Liaison service 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 kept private. 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 Professor Neil Pender, the Chief investigator of the Study. Data will be stored for ten years.

What do I do if I want to take part?

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

THANK YOU FOR TAKING THE TIME TO READ THIS

10.1.2 Guardian Information sheet

version 1.3

Department of Clinical Dental Sciences
Liverpool University Dental Hospital and School of Dentistry
Pembroke Place, Liverpool L3 5PS
Research Ethics Committee; NW 2 REC Liverpool Central

February 2011

PARTICIPANT INFORMATION SHEET FOR PARENT/ LEGAL GUARDIAN

REMINERALISATION PROPERTIES OF CALCIUM BASED TOOTHPASTE: IN SITU CROSS -OVER STUDY

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 which makes toothbrushing more difficult. Fluoride mouth rinses have been recommended to prevent the occurrence of these white spots, however this may not lead to full repair of the white spot. Calcium based toothpastes are now available and have been shown in some studies to promote better repair of these white spots. The purpose of this study is to find out if calcium based toothpastes which contain fluoride are better at repairing these white spots than fluoride toothpaste alone.

Has the study been approved?

Yes. The Liverpool Central Research Ethics Committee has given the approval for this study.

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Who will be conducting the study?

The study is being run by Prof. Susan Higham (Professor of Oral Biology), Prof. Neil Pender (Professor of Orthodontics and Consultant in Orthodontics), and Miss Eva Bryniarska (Specialist Registrar in Orthodontics).

Why have I been asked to take part? You have been asked because your child is undergoing orthodontic treatment with fixed braces on their teeth.

What will I have to do?

During your child's routine brace appointment a small carrier containing a piece of sterilised human enamel will be attached to the wire part of their brace, positioned towards the back part of your child's lower brace. This will not affect your child's brace.

Your child will be given a toothbrush and a standard fluoride toothpaste to use during the study period. Your child will also be given two smaller tubes at different times that contain pastes for application to the piece of enamel in the holder once each day. As normal, your child will be instructed to brush your teeth for 2 minutes twice a day with a pea sized amount of the toothpaste until your next routine orthodontic appointment which will be 4 weeks later. Your child will be

asked not to use any other toothpaste or mouth rinses during the 4 week period. At that following appointment the carrier will be removed from your brace and sent to the laboratory for analysis. Your child's brace will be adjusted as usual. Your child will then be instructed to carry out their usual oral hygiene procedures until their next routine brace appointment with no carrier on their brace. At this appointment a new carrier will be attached to the brace and your child will use the normal toothpaste as before but in addition will be asked to apply the paste from one small tube to the piece of enamel in the holder.

Your child will have in total 3 carriers attached. Two of the carriers will need paste from a small toothpaste tube whilst the third will not need additional paste except for the standard fluoride toothpaste provided for them. The pastes will be labelled with a letter so that the researcher and the participants won't know the identity of the pastes, this is called blinding. However we do know that the two small tubes of paste contain calcium with either a small amount of fluoride or calcium alone.

How long will the study last?

The study will last for approximately 6 months of your treatment.

What if I don't want to take part?

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

What if I have a question or there is a problem on the trial?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions 0151 7065252. If you remain unhappy and wish to complain formally, you can do this through the Patient Advice Liaison service 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 kept private. 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 Professor Neil Pender, the Chief investigator of the Study. Data will be stored for ten years.

What do I do if I want to take part?

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

THANK YOU FOR TAKING THE TIME TO READ THIS

10.2 Consent forms

10.2.1 Patient Assent form

version 1.2

UNIVERSITY OF LIVERPOOL

October 2010

Centre Number:

Study Number:

Patient Identification Number for this trial:

ASSENT FORM

Title of Project: **REMINERALISATION PROPERTIES OF CALCIUM BASED TOOTHPASTE:IN SITU CROSS-OVER STUDY**

Name of Researcher: Eva Bryniarska

	Please initial box
1. I confirm that I have read and understand the information sheet dated February 2011 (version 1.3) 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 medical care or legal rights being affected.	☐
3. I understand that the data collected during the study will be tested by the study investigators. I give permission for these individuals to have access to my records.	☐
4. I agree to take part in the above study.	☐

_____	_____	_____
Participant	Date	Signature

_____	_____	_____
Name of Person taking assent	Date	Signature

10.2.2 Patient Consent form

version 1.2

October 2010

Centre Number:

Study Number:

Patient Identification Number for this trial:

CONSENT FORM FOR PARENT/LEGAL GUARDIAN

Title of Project: **REMINERALISATION PROPERTIES OF CALCIUM BASED TOOTHPASTE:IN SITU CROSS-OVER STUDY**

Name of Researcher: Eva Bryniarska

Please initial box

1. I confirm that I have read and understand the information sheet dated February 2011 (version 1.3) 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 child's medical care or legal rights being affected. ☐

3. I understand that the data collected during the study will be analysed by the study investigators. I give permission for these individuals to have access to my child's records. ☐

4. I agree for my child to take part in the above study. ☐

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

10.3 Inclusion criteria sheet

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

UNIVERSITY OF LIVERPOOL

REMINERALISATION PROPERTIES OF CALCIUM BASED TOOTHPASTE: IN SITU CROSS-OVER STUDY

INCLUSION & EXCLUSION CRITERIA CHECKLIST

Subject's name .....

D.O.B. .....

Name of Researcher: *Eva Bryniarska*

Criterion	YES	NO
I am aged between 12 and 16 years		
I consider myself to be in good health		
I have no allergies to any milk-based products		
I have had no antibiotics in the last 2 months		

Signed by investigator..... Date.....

10.4 Example of Timetable for study

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

UNIVERSITY OF LIVERPOOL

REMINERALISATION PROPERTIES OF CALCIUM BASED TOOTHPASTE: IN SITU CROSS-OVER STUDY

DATASHEET FOR CLINICAL NOTES

Subject Name ..... Subject code ☐

Visit 1 Date ..... 1st carrier inserted: YES / NO Toothpaste/Mousse provided: A / B / C Specimen No. ☐ ☐ ☐	**Visit 2** Date ..... 1st carrier removed: YES / NO TMR carried out: YES / NO
Visit 3 Date ..... 2nd carrier inserted: YES / NO Toothpaste/Mousse provided: A / B / C Specimen No. ☐ ☐ ☐	**Visit 4** Date ..... 2nd carrier removed: YES / NO TMR carried out: YES / NO
Visit 5 Date ..... 3rd carrier inserted: YES / NO Toothpaste/Mousse provided: A / B / C Specimen No. ☐ ☐ ☐	**Visit 6** Date ..... 3rd carrier removed: YES / NO TMR carried out: YES / NO

10.5 Patient Instruction Sheets

10.5.1 Instruction sheet for Toothpaste

Instruction sheet

Brush your teeth with the toothbrush and toothpaste 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 as this may affect the results of the study.

10.5.2 Instruction sheet for Tooth Mousse™

Instruction sheet

Brush your teeth with the toothbrush and toothpaste 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 from the smaller second tube which is covered in grey coloured tape. Wipe this across your teeth, including the carrier. Do not rinse.

Brush your teeth in this way twice a day.

Do not use any other mouth rinses or toothpastes as this may affect the results of the study.

10.6 Raw data

10.6.1 End Mineral Loss

	Phase 1			Phase 2			Phase 3			Phase 4			Phase 5		
Subject	Intervention	specimen	End mineral loss (%Volµm)	Intervention	Specimen	End mineral loss (%Volµm)	Intervention	Specimen	End mineral loss (%Volµm)	Intervention	Specimen	End mineral loss (%Volµm)	Intervention	Specimen	End mineral loss (%Volµm)
1	B	41A1	590	C	41B1	1355	A	41A2	Lost						
2	A	38A1	Lost	B	38A2	1240	C	38B1	1525	A	38B2	1122.5			
3	C	10A1	lost	A	10B1	Lost	B	10B2	320						
4	C	13B1	Lost	A	13A1	795	B	13A2	Lost	C	11A2	1055			
5	B	28A1	1355	C	28B1	1415	A	28B2	867.5						
6	A	22A1	1930	B	22B1	1245	C	22A2	800						
7	C	16A1	Lost	A	16B1	Lost	B	16B2	1120	C	16A2	1185			
8	B	3B1	Lost	C	3A1	1050	A	3B2	673.3	B	3A2	817.5			
9	A	40A1	Lost	B	40B1	Lost	C	40B2	1305	A	40A2	Lost	A	49A1	725
10	B	32A1	1090	C	32B1	Lost	A	32B2	1070						
11															
12	A	23A1	850	B	23B1	Lost	C	23B2	860						
13	C	34B1	1240	A	34A1	1610	B	34A2	Lost						
14	A	36A1	1170	B	36B1	Lost	C	36B2	Lost	C	13B2	1485			
15	B	18A1	1015	C	18B1	Lost	A	18B2	824	C	18A2	1092.5			

Lost	During sectioning
Lost	During polishing
Lost	During in situ
	Drop out

10.6.2 End Lesion Depth

	Phase 1			Phase 2			Phase 3			Phase 4			Phase 5		
Subject	Intervention	Specimen	End Lesion Depth (µm)	Intervention	Specimen	End Lesion Depth (µm)	Intervention	Specimen	End Lesion Depth (µm)	Intervention	Specimen	End Lesion Depth (µm)	Intervention	Specimen	End Lesion Depth (µm)
1	B	41A1	45.4	C	41B1	70.45	A	41A2							
2	A	38A1		B	38A2	52.05	C	38B1	52.8	A	38B2	50.15			
3	C	10A1		A	10B1		B	10B2	45.1						
4	C	13B1		A	13A1	55.3	B	13A2		C	11A2	61.3			
5	B	28A1	57.355	C	28B1	56.4	A	28B2	46.9						
6	A	22A1	80.65	B	22B1	51	C	22A2	43						
7	C	16A1		A	16B1		B	16B2	52.9	C	16A2	57.3			
8	B	3B1		C	3A1	57.5	A	3B2	49.73	B	3A2	42.55			
9	A	40A1		B	40B1		C	40B2	63.05	A	40A2		A	49A1	51.65
10	B	32A1	44.35	C	32B1		A	32B2	54.47						
11															
12	A	23A1	47.6	B	23B1		C	23B2	46.9						
13	C	34B1	60.5	A	34A1	59	B	34A2							
14	A	36A1	54.3	B	36B1		C	36B2		C	13B2	57.9			
15	B	18A1	48.5	C	18B1		A	18B2	53.2	C	18A2	48.85			

Lost	During sectioning
Lost	During polishing
Lost	During in situ
	Drop out

10.6.3 End Lesion Width

	Phase 1			Phase 2			Phase 3			Phase 4			Phase 5		
Subject	Intervention	Specimen	End Lesion Width (µm)	Intervention	Specimen	End Lesion Width (µm)	Intervention	Specimen	End Lesion Width (µm)	Intervention	Specimen	End Lesion Width (µm)	Intervention	Specimen	End Lesion Width (µm)
1	B	41A1	36.8	C	41B1	65.3	A	41A2							
2	A	38A1		B	38A2	45.35	C	38B1	45.65	A	38B2	40.95			
3	C	10A1		A	10B1		B	10B2	34.35						
4	C	13B1		A	13A1	31.9	B	13A2		C	11A2	50.55			
5	B	28A1	49.25	C	28B1	48.15	A	28B2	42.1						
6	A	22A1	65.4	B	22B1	44.85	C	22A2	37.8						
7	C	16A1		A	16B1		B	16B2	46.8	C	16A2	48			
8	B	3B1		C	3A1	50.35	A	3B2	44.73	B	3A2	35.1			
9	A	40A1		B	40B1		C	40B2	51.8	A	40A2		A	49A1	45.9
10	B	32A1	38.75	C	32B1		A	32B2	42.53						
11															
12	A	23A1	39.8	B	23B1		C	23B2	34.5						
13	C	34B1	54.7	A	34A1	48.1	B	34A2							
14	A	36A1	48.7	B	36B1		C	36B2		C	13B2	51.75			
15	B	18A1	41.2	C	18B1		A	18B2	44.66	C	18A2	42.125			

Lost	During sectioning
Lost	During polishing
Lost	During in situ
	Drop out