White spot lesions after orthodontic fixed appliance treatment
Beerens, M.W.

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White Spot Lesions after Orthodontic Fixed Appliance Treatment

The effectiveness of MI Paste Plus® as a remineralising agent
a randomised controlled trial

Moniek Willemien Beerens

1. MI Paste Plus®, a remineralisation agent, does not show its intended effect in patients who have white spot lesions (WSL) after orthodontic treatment with full fixed appliances.
2. WSL tend to regress over time but do not disappear after removal of the appliances.
3. After bracket removal the microbial composition gradually changes towards a more healthy composition. This is a gradual change that does not occur immediately after bracket removal.
4. The ICDA5 scoring system on clinical oral photographs does not have enough discriminatory power to address changes in WSL severity.
5. Comparing respective clinical oral photographs taken over time provides discriminatory power in assessing changes in WSL. This method of monitoring WSL over time is useful for clinical decision making in the management of WSL.
6. Quantitative light induced fluorescence (QLF) is confirmed as a useful outcome measure in detecting and monitoring WSL over time.
7. The DGGE-banding pattern software, GelCompar II is susceptible to bias when used with the provided settings.
8. Denaturing Gradient Gel Electrophoresis (DGGE) analysis of microbial composition does not have a predictive value for caries risk assessment of the formation of WSL in orthodontic patients treated with full fixed appliances.
10. This thesis describes the first randomised control trial assessing the effectiveness of MI Paste Plus® in vivo for the treatment of WSL after orthodontic fixed appliance on the long term (12 months) and stipulates the importance of combining independent outcome measures in research.
White Spot Lesions after Orthodontic Fixed Appliance Treatment

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The research described in this thesis was performed as a research collaboration between the Department of Orthodontics and the Department of Preventive Dentistry of the Academic Centre for Dentistry Amsterdam (ACTA), the combined faculty of dentistry of the University of Amsterdam and the VU University Amsterdam, the Netherlands.

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**PROMOTIECOMMISSIE**

Promotor:
Prof. dr. J.M. ten Cate  Universiteit van Amsterdam

Co-promotor:
Dr. ir. M.H. van der Veen  Vrije Universiteit Amsterdam

Overige leden:
Dr. T.J. Algera    Universiteit van Amsterdam
Prof. dr. C. van Loveren  Universiteit van Amsterdam
Prof. dr. B. Prahl-Andersen Vrije Universiteit Amsterdam
Prof. dr. Y. Ren  Rijksuniversiteit Groningen
Prof. dr. F.R. Rozema  Universiteit van Amsterdam

Faculteit der Tandheelkunde

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Faculteit der Tandheelkunde
Therapeutic reports with controls tend to have no enthusiasm, reports with enthusiasm tend to have no controls.

David Lawrence Sackett (1934 – 2015) was an American-Canadian medical doctor and a pioneer in evidence-based medicine. He is known as one of the fathers of Evidence-Based Medicine.
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General introduction
and outline of the thesis
Orthodontics and dentofacial orthopaedics is a specialty field of dentistry that deals primarily with the diagnosis, prevention and correction of malpositioned teeth and the jaws (Mosby’s Medical Dictionary, 2012). Malocclusions may cause problems with the oral function, for example temporo-mandibular joint dysfunction, mastication, swallowing and speech problems. Greater susceptibility to trauma, periodontal disease and tooth decay are also related to malocclusions (Proffit et al., 2013). However, the principal objective for most patients seeking orthodontic treatment is to achieve a detectable improvement in their dentofacial appearance while their secondary goal of treatment is an oral health benefit (Ackerman, 2010). Therefore, most orthodontic patients are treated for esthetic reasons, with only a small number of patients receiving treatment for primarily a medical or dental indication (van der Kaaij et al., 2015).

Orthodontics and white spot lesions

Three main types of appliances are used in orthodontic therapy: active, passive and functional. These can be fixed or removable. Particularly fixed active appliance treatment has become an integral part of modern orthodontics (Graber et al., 2011). Unfortunately, this type of treatment can cause adverse effects. Among these, white spot lesions (WSL) are prominent. These lesions have a negative effect on the esthetic outcome of orthodontic treatment (Gorelick et al., 1982). These WSL manifest themselves as subsurface enamel porosities that might progress into carious lesions and are therefore a problem of clinical relevance. The overall prevalence of WSL in orthodontic patients was reported between 2% and 97% (Boersma et al., 2005; Chapman et al., 2010; Julien et al., 2013). The highest incidence occurs on the maxillary lateral incisors followed by the maxillary canine, premolar, and central incisors, respectively (Chapman et al., 2010).

WSL are the result of prolonged accumulation of bacterial plaque on the enamel surface adjacent to the fixed appliances (O’Reilly and Featherstone, 1987), commonly due to inadequate oral hygiene (Chapman et al., 2010), and a frequent intake of carbohydrates (Feyerskov and Kidd, 2008; Maltz et al., 2017), resulting in a disbalance between remineralization and demineralization with

Orthodontics

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various stages being either reversible or irreversible. If the demineralization process is not being stopped, an intact enamel surface eventually collapses and cavitates. WSL are considered the precursor of enamel caries but can, in principle, be reversed (Sudjalim et al., 2006; Chambers et al., 2013). WSL developed during orthodontic treatment, however, have limited ability to regress after appliance removal and in many cases these lesions will therefore remain visible as permanent scars of the enamel (Mattousch et al., 2007).

Management of white spot lesions (WSL)

Modern management of dental caries associated with orthodontic fixed appliance treatment has three major components: prevention, control and atraumatic care of existing WSL after orthodontic treatment with fixed appliances (Willmot, 2004; Ekstrand et al., 2009).

The majority of research addresses the primary prevention of WSL, showing that there is scientific evidence that fluoride has a positive effect on the primary prevention of WSL adjacent to fixed orthodontic appliances. A Cochrane review concluded that there is evidence that daily mouth rinses with 0.05 % NaF with can reduce the occurrence and severity of WSL during orthodontic treatment (Benson et al., 2004). Despite the clinical effect of daily home use of mouth rinses, compliance can be a problem, as only up to 50% of the studied participants was compliant (Geiger et al., 1988; Geiger et al., 1992). A treatment not requiring patient compliance is the application of high concentration (36 000 ppm) fluoride varnishes. It reduces the formation and decreases enamel lesion depth adjacent to bonded brackets during treatment with fixed appliances (Stecksen-Blicks et al., 2007; Farhadian et al., 2008; Shafi, 2008)

Secondary prevention of existing WSL after orthodontic treatment can be divided into three different approaches. The strategy using fluorides (Zero, 2006; Reynolds et al., 2008; Huang et al., 2013) and phosphate-based remineralizing agents (Reynolds et al., 2008; Yengopal and Mickenautsch, 2009; Robertson et al., 2011; Chen et al., 2013; Li et al., 2014) addresses the biologic repair process, aiming to reverse the carious process. A strategy focusing on cosmetic improvement of the lesions is infiltration by resins (Kielbassa et al., 2009; Kugel et al., 2009; Senestraro et al., 2013). Other options can be categorized as invasive strategies such as micro abrasion (Murphy et al., 2007),
bleaching (Knösel et al., 2007) and preparation and restoration (Shungin et al., 2010) applied only on inactive lesions. From a minimally invasive perspective, fluoride has been shown to arrest the development and progression of caries lesions during orthodontic treatment (Marinho, 2009). Concentrated fluoride is not recommended for treatment of WSL on the labial surfaces of the teeth in the esthetic zone. Concentrated fluoride results in a hyper-mineralized subsurface of the lesion (Øgaard et al., 1988; Willmot, 2004), which inhibits the ion movement through the subsurface (ten Cate and Arends, 1980). This prevents further demineralisation but also remineralisation, of the deeper layer of the lesion is inhibited. The lesion remains as a white scar. Therefore, minimally invasive approaches with other applications than fluoride are preferred, to effectively remineralize WSL associated with orthodontic treatment, (Cochrane et al., 2010).

**Minimal intervention Paste Plus®**
Within the minimal invasive approach the CPP-ACP technology has been developed by Professor E. Reynolds and his team at the University of Melbourne Dental School. Casein phosphopeptide (CPP) is a milk derived protein able to bind calcium and phosphate ions and stabilise these ions as amorphous calcium phosphate (ACP). CPP-ACP adheres intra-orally to plaque pellicle, mineral as well as soft tissues. When dissolving it supplies bioavailable calcium and phosphate to saliva and plaque pellicle enabling it to stimulate remineralisation. Also this CPP-ACP complex binds with about twice the affinity of the bacterial cells for calcium (Rose, 2000). The newer form of CPP-ACPF products contains fluoride. The fluoride ions in amorphous calcium phosphate fluoride are stabilised in this ion complex and able to diffuse through the subsurface layer (Ahmadi Zenouz et al., 2015). This formulation is the main active ingredient of a commercially available remineralisation agents called MI Paste Plus®. This product contains 900 parts per million fluoride in a mole ratio with the calcium and phosphate of 5 calcium, 3 phosphate and 1 fluoride, considered the ideal ratio for building fluorapatite into the enamel structure (Cross et al., 2004; Reynolds, 2008).

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CHAPTER 1
Screening and monitoring methods
The management of WSL relies on methods screening the caries risk, incidence of new caries lesions and monitoring of the severity of existing caries lesions. Given the rapid development of WSL during orthodontic treatment, such methods need to have excellent discriminatory power, such that even small changes in caries risk, caries prevalence and caries severity can be detected.

Caries risk assessment is currently based on past caries experience or DMFS scores complemented by current oral hygiene level (Sundell et al., 2013). Low hygiene levels result in high amount of plaque and especially a high amount of mature plaque (Marsh, 1994). Orthodontic fixed appliance treatment is associated with a rapid increase of dental plaque, lower pH and shift in the composition of the bacterial flora (Chatterjee and Kleinberg, 1979; Marsh, 2010) towards higher levels of acidogenic bacteria, such as *Streptococcus mutans* and *lactobacilli* (Lundstrom and Krasse, 1987). Therefore, monitoring changes in the microbial composition may help guide prevention and treatment of caries (Lucchese and Gherlone, 2013).

Traditional detection methods, such as visual inspection and intra-oral photography are commonly available in clinical practice. Visual inspection is the first choice to screen for the presence or absence of caries. Visual inspection according to the international caries assessment and detection system (ICDAS) includes the assessment of early WSL and scores the severity of lesions (Ismail et al., 2007). An advantage that the use of intra-oral photographs has over visual examination methods, is the ability to archive intra-oral photographs, remote scoring, allowing multiple scorers to score images and enabling longitudinal analysis (Wenzel et al., 1991). Also it is beneficial in studies when examiner blinding is required and in practice based RCTs (Boye et al., 2013).

Quantitative light-induced fluorescence imaging (QLF) has been developed for the longitudinal assessment of early WSL and changes in WSL severity (Hafstrom-Bjorkman et al., 1992). The technique is based on tooth illumination by a broad beam of blue-violet light (405 nm). The resulting fluorescence of the enamel in the yellow-green region (520 nm) is observed through a yellow high-pass filter, which filters out all reflected and back-scattered light. The difference between the measured values and the reconstructed values gives
the resulting fluorescence loss in the lesion. Three quantities are obtained providing information on the lesion area, depth (fluorescence loss) and volume (integrated fluorescence loss) (Tranaeus et al., 2005).

The use of a combination of several independent outcome measures can be useful to assess caries risk, caries prevalence and caries severity.
AIM OF THE STUDY

The main ingredient of MI Paste Plus®, CPP-ACPF was shown to be effective in remineralizing WSL when tested in *in vitro*, animal, and human *in situ* caries models. Its effectiveness has been described for both primary prevention as well as for the regression of WSL in a controlled environment (Chen et al., 2013). There is a lack of reliable evidence to support the effectiveness of CPP-ACPF present in the commercially available remineralisation MI Paste Plus® product especially in post-orthodontic white spot lesions (Chen et al., 2013; Li et al., 2014). This thesis assesses the effectiveness of MI Paste Plus® as a remineralisation agent in patients with existing WSL after fixed orthodontic appliance treatment. Therefore, a prospective, double-blind, randomized, placebo-controlled trial has been conducted and analysed.

In Chapter 2, changes in white spot lesions in former orthodontic patients were assessed over a 12 months period by comparing two outcome measures: 1. scoring visual changes and ICDAS on clinical oral photographs, and 2. quantitative light-induced fluorescence imaging (QLF), aiming to assess discrimination accuracy in monitoring changes in lesion severity.

In Chapter 3, dental plaque, in orthodontic patients who were scheduled for appliance removal, was assessed by two outcome measures: 1. Denaturing Gradient Gel Electrophoresis (DGGE), and 2. conventional microbiology, aiming to test the predictability of DGGE as a risk indicator for the development of WSL during orthodontic treatment.

In Chapter 4, the effectiveness of MI Paste Plus® as a remineralizing agent was analysed over time, for a period of 3 months, by assessing two outcome measures: 1. QLF imaging, and 2. conventional microbiology.
In Chapter 5, the long term effectiveness of MI Paste Plus® as a remineralizing agent was analysed over time, for a period of 12 months, by assessing several outcome measures:
1. QLF Imaging,
2. conventional microbiology,
3. measuring acidogenicity of plaque by capillary ion analysis (CIA), and
4. assessing ICDAS-scores on clinical photographs.
REFERENCES


White spot lesions after orthodontic treatment with fixed appliance assessed on clinical photography and by quantitative light-induced fluorescence imaging; a retrospective study

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White spot lesions after orthodontic treatment assessed by clinical photographs and by quantitative light-induced fluorescence imaging; a retrospective study.
ABSTRACT

Objective
White spot lesions (WSL) are an important side-effect of orthodontic full fixed appliance treatment. Standardized monitoring of such WSL may help in caries management.

Materials and methods
In this retrospective study the discriminatory power of caries assessment on routine digital oral photographs was compared to quantitative light-induced fluorescence (QLF) imaging in monitoring WSL development after debonding of orthodontic appliances. Oral and QLF photographs captured directly after debonding (T1) and 1 year thereafter (T2) of 51 subjects, treated with full fixed orthodontic appliances were used. Oral photographs were assessed using The International Caries Detection and Assessment System (ICDAS) at both time points independently and by side-by-side comparison to assess visual transition (VT). QLF photographs were categorized based on integrated fluorescence loss at T1 and T2.

Results
At T1 433 and 384 lesions on 918 buccal surfaces were detected using ICDAS and QLF, respectively. For both methods these numbers were reduced at T2. Changes within ICDAS scores were recorded by VT and showed mainly lesion improvement within ICDAS score 2.

Conclusion
The oral and QLF photographs both showed regression of WSL after debonding of fixed orthodontic appliances. The VT evaluation was found to have higher discriminatory power in comparison to ICDAS.
Enamel demineralisation and white spot lesions (WSL) occur often during orthodontic full fixed appliance treatment and can remain after treatment (Artun and Brobakken, 1986; O’Reilly and Featherstone, 1987; Willmot, 2004; Murphy et al., 2007). Prolonged exposure to bacterial plaque caused by deficient/inadequate oral hygiene is an important causal factor (Adams, 1967; Sakamaki and Bahn, 1968). There seems to be a difference in progression rate between non-orthodontic caused caries formation and white spot lesions induced by deficient oral hygiene combined with fixed appliance treatment (O’Reilly and Featherstone, 1987; Øgaard et al., 1988a; Øgaard et al., 1988b; Øgaard and Ten Bosch, 1994). It has been shown that visible white spot lesions can develop within 4 weeks of the start of fixed appliance treatment. It is known that not all lesions progress to cavities (Ferreira Zandona et al., 2012). Although orthodontic lesions may remineralise to some extent after appliances are removed, they remain as scars, influencing the aesthetical appearance of the teeth (Mattousch et al., 2007). It is important for clinical decision-making not to focus just on detecting caries at one point in time, as this is of limited use without monitoring caries activity and optical behaviour of WSL over time (Topping et al., 2009).

Since 2005, The International Caries Detection and Assessment System (ICDAS) is the instrument generally used to score caries clinically (National Institute of Health, 2001). This scoring system was developed to define visual caries detection criteria at an early non-cavitated stage that could inform on diagnosis, prognosis and clinical management. The ICDAS system enables detection of early non-cavitated (white spot) lesions and provides an opportunity to explore lesion changes after orthodontic fixed appliance treatment in order to determine the progression or regression over time in a standardized way. However with ICDAS, progression or regression can only be observed when lesions transfer to a different score. Given that most orthodontic related caries lesions remain visible as scars despite remineralisation (van der Veen et al., 2007), such ICDAS transitions are expected to be rare.

In orthodontic practice it is custom to capture oral photographs at the start, during and after orthodontic treatment and retention and these photographs offer the opportunity to monitor caries lesion development by comparison of photographs longitudinally in time.
At the department of orthodontics at the Academic Centre for Dentistry (ACTA) all patients are photographed using quantitative light induced fluorescence (QLF) as part of a caries prevention programme. These QLF photos have proven useful in monitoring of the development of caries lesion after conclusion of orthodontic treatment (Boersma et al., 2005; Mattousch et al., 2007; Beerens et al., 2010). However, QLF is a time-consuming and expensive method and, hence, not generally available in orthodontic practice.

In this retrospective study we aimed to assess the use of ICDAS and visual transition on photographs and QLF for clinical caries detection. The discriminatory accuracy of assessing routine digital oral photographs and QLF images in monitoring incidence and severity of WSL are compared from immediately post-orthodontic fixed appliance treatment to 12 months thereafter.

The tested hypothesis is that changes in white spot lesions from immediately post-orthodontic treatment to 1 year follow-up, using ICDAS or visual transition (VT) scoring on clinical oral photography and QLF imaging, have comparable accuracy in discriminating changes in lesion severity.

MATERIALS AND METHODS

This retrospective study was performed to assess the discriminatory power of three methods to detect caries lesions and assess caries lesion progression and regression seen on clinical and QLF photographs obtained from subjects immediately after removal of the full fixed appliances (T1) and 1 year thereafter (T2).

QLF photographs used were obtained as part of a caries clinical trial aimed at regression of white spot lesions after orthodontic treatment (Beerens et al., 2010). The medical ethical committee of the University Medical Centre of the Free University Amsterdam, the Netherlands, approved this study protocol (MEC 07/213).

Clinical photographs used are routinely made before, during and after orthodontic treatment at the department of orthodontics.

Clinical and QLF photographs were scored on all buccal surfaces in the upper and lower jaw from second pre-molar to second pre-molar. Clinical photographs were assessed using ICDAS scores adapted for photographs as described below on post-debond and 1-year photographs independently as well as by visual transition scores (VT) comparing the post-debond and 1 year post-orthodontic fixed appliance treatment to 12 months thereafter.

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photographs side-by-side. The QLF photographs were assessed for changes in integrated fluorescence loss (IFL) (de Josselin de Jong et al., 1995).

**Data collection**

Subjects whose QLF photographs were used fulfilled the following criteria:
1. treated with orthodontic fixed appliances in both arches with debond between January 2008 and August 2009,
2. healthy and between the age of 12–19 at debond, and
3. have developed two or more WSL without open cavitation on previously bracketed buccal surfaces.

All subjects who completed the caries clinical trial with QLF photographs available from T1 and T2 were eligible for enrolment in the current study. Subjects were enrolled after having signed informed consent, which, in case of minors was co-signed by parents/guardians. The clinical photographs of these subjects at the same time points were retrieved from the patient database. Only complete datasets were considered. Furthermore, clinical and QLF photographs should be available from T1 and T2. Subjects were enrolled after signed informed consent, which was in the case of minors co-signed by parents/guardians.

**Clinical Oral Photograph galleries**

Clinical oral photographs were captured using a Nikon digital camera D3000 camera body with CCD (charge coupled device), DCS (digital still camera) from Kodak (Odijk, The Netherlands) with an image size of 1012 x 1524 pixels or 4.5 Mb. The camera had a 2.8/105 mm macro lens (that combined with the CCD surface size, resulted in images comparable to those made with a 160-mm lens). The camera was equipped with a Nikon SB 29 s macro speed light flasher. Photographs were stored on a computer. At each time point a set of front teeth in maximal occlusion, front teeth in open position; left and right lateral views in occlusion were captured. Frontal pictures were captured under an angle of 0° in maximal occlusion and in opened position approximately 3 mm from occlusion. Left and right lateral pictures were captured under an angle of 45° (Figure 1).

Frontal pictures were captured using a rounded cheek retractor (DB orthodontics, Silsden, UK, Set large. Double end large DB04-0175). Oval shape

Cheek retractors were used for lateral pictures (Pelz & Partner, Lindenberg im Allgäu, Germany. Set: medium & large double end, for lateral area 720-0007). Pictures were taken by trained orthodontic residents. Clinical plaque removal was performed prior to photography.

For each subject and time point pictures were collected and combined in a photography gallery comprising four pictures per patient (Figure 1) and printed on high quality photographic paper. Each photograph gallery was assigned a random number by M.H.V., according to a computer-randomization scheme, to ensure examiners (F.B. and M.W.B) were blinded for subject and time point of recording.

Photography galleries were analysed by two calibrated examiners (F.B.) and (M.W.B). Photographs were analysed in random order for subject and time using the ICDAS-II criteria (Health., 2001), where code 1, First Visual Change in Enamel, seen only after prolonged air drying, was not used, given that no air drying was applied before photographs were made. Also, at T1 ICDAS codes 5 and 6 were not given due to exclusion criteria (Table 1).

### Table 1. Code description for ICDAS, Visual transition (VT) and QLF.

<table>
<thead>
<tr>
<th>CODE</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICDAS</td>
<td>Description</td>
</tr>
<tr>
<td>0</td>
<td>Sound</td>
</tr>
<tr>
<td>1</td>
<td>First Visual Change in Enamel (excluded in this research)</td>
</tr>
<tr>
<td>2</td>
<td>Distinct Visual Change in Enamel</td>
</tr>
<tr>
<td>3</td>
<td>Localized Enamel Breakdown (without clinical visual signs of dentinal involvement)</td>
</tr>
<tr>
<td>4</td>
<td>Underlying Dark Shadow from Dentin</td>
</tr>
<tr>
<td>5</td>
<td>Distinct Cavity with Visible Dentine (excluded in this research)</td>
</tr>
<tr>
<td>6</td>
<td>Extensive Cavity with Visible Dentine (excluded in this research)</td>
</tr>
<tr>
<td>RESTORATION</td>
<td>Restoration Applied</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>VISUAL TRANSITION</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Sound</td>
</tr>
<tr>
<td>2</td>
<td>Distinct Visual Change in Enamel</td>
</tr>
<tr>
<td>3</td>
<td>Localized Enamel Breakdown (without clinical visual signs of dentinal involvement)</td>
</tr>
<tr>
<td>IMPROVED</td>
<td>Lesion Improved at T2</td>
</tr>
<tr>
<td>RESTORATION</td>
<td>Restoration Applied at T2</td>
</tr>
<tr>
<td>SAME</td>
<td>Lesion Same at T1 and T2</td>
</tr>
<tr>
<td>WORSE</td>
<td>Lesion Worsened at T2</td>
</tr>
<tr>
<td>QLF IFL (%mm²)</td>
<td>Description</td>
</tr>
<tr>
<td>0</td>
<td>Integrated fluorescence loss =0, i.e. sound</td>
</tr>
<tr>
<td>1</td>
<td>Integrated fluorescence loss &gt; 0 to ≤ 5</td>
</tr>
<tr>
<td>2</td>
<td>Integrated fluorescence loss &gt;5 to ≤25</td>
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<tr>
<td>3</td>
<td>Integrated fluorescence loss &gt;25</td>
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<table>
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<th>CODE</th>
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<tbody>
<tr>
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<td>Description</td>
</tr>
<tr>
<td>0</td>
<td>Sound</td>
</tr>
<tr>
<td>1</td>
<td>First Visual Change in Enamel (excluded in this research)</td>
</tr>
<tr>
<td>2</td>
<td>Distinct Visual Change in Enamel</td>
</tr>
<tr>
<td>3</td>
<td>Localized Enamel Breakdown (without clinical visual signs of dentinal involvement)</td>
</tr>
<tr>
<td>4</td>
<td>Underlying Dark Shadow from Dentin</td>
</tr>
<tr>
<td>5</td>
<td>Distinct Cavity with Visible Dentine (excluded in this research)</td>
</tr>
<tr>
<td>6</td>
<td>Extensive Distinct Cavity with Visible Dentine (excluded in this research)</td>
</tr>
<tr>
<td>RESTORATION</td>
<td>Restoration Applied</td>
</tr>
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</table>

<table>
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<tr>
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</tr>
<tr>
<td>2</td>
<td>Distinct Visual Change in Enamel</td>
</tr>
<tr>
<td>3</td>
<td>Localized Enamel Breakdown (without clinical visual signs of dentinal involvement)</td>
</tr>
<tr>
<td>IMPROVED</td>
<td>Lesion Improved at T2</td>
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<tr>
<td>RESTORATION</td>
<td>Restoration Applied at T2</td>
</tr>
<tr>
<td>SAME</td>
<td>Lesion Same at T1 and T2</td>
</tr>
<tr>
<td>WORSE</td>
<td>Lesion Worsened at T2</td>
</tr>
<tr>
<td>QLF IFL (%mm²)</td>
<td>Description</td>
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<td>Integrated fluorescence loss =0, i.e. sound</td>
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<tr>
<td>1</td>
<td>Integrated fluorescence loss &gt; 0 to ≤ 5</td>
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<tr>
<td>2</td>
<td>Integrated fluorescence loss &gt;5 to ≤25</td>
</tr>
<tr>
<td>3</td>
<td>Integrated fluorescence loss &gt;25</td>
</tr>
</tbody>
</table>
FIGURE 1. Example of a clinical oral photograph gallery of clinical photos from one subject captured at T1 and T2. Side-by-side comparison shows changes in lesion severity even when lesion severity is classified as ICDAS score 2 at both times.
CHAPTER 2

Quantitative light-induced fluorescence imaging

QLF images were captured using an intra-oral fluorescence camera (QLF/Clin; Inspektor Research Systems, Amsterdam, the Netherlands) and dedicated software (Inspektor pro version 3.0.0.42; Inspektor Research Systems) with video repositioning technique (Beerens et al., 2010). Photographs captured at different time points showed the tooth surface from the same angle and with the same size, except for changes due to e.g. differences in gingival swelling. Plaque removal in the clinic was performed prior to QLF photography. QLF images were judged visually for signs of decalcification, which appear as dark areas surrounded by bright green fluorescing sound tooth tissue (de Josselin de Jong et al., 1995). For all lesions detected, the integrated fluorescence loss (IFL [%mm²]) over the lesion area was determined at a 5% threshold (Al-Khateeb et al., 1998) by a single examiner (M.W.B.), who was trained and calibrated by an experienced QLF examiner (M.H.V.) on a dataset of 500 QLF images. The integrated fluorescence loss (IFL [%mm²]) over the lesion area was assessed on all surfaces where a lesion was seen on the QLF photographs. In a first step, images of surfaces in QLF photographs at T1 and T2 were aligned to adjust small repositioning errors. Then a user-defined contour was created on the QLF photograph surrounding the lesion such that the contour borders were located on healthy tooth enamel as much as possible. Contour borders not on healthy tooth tissue were excluded. The same contour was applied by an automated software algorithm to longitudinal images of the same surface (Inspektor pro version 3.0.0.42; Inspektor Research Systems). In subjects where lesion visibility at T1 was hampered as a result of swollen gingiva, the visible area of the lesion at baseline was the part of the lesion analysed at both time points. The IFL per surface was translated into QLF severity score as described in Table 1 for comparison with ICDAS and VT scores on clinical oral photographs.

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Data analysis
Frequency tables were used to visualize level of agreement between QLF, ICDAS and VT assessments.
The intra-class correlation coefficient (ICCs) for IFL values determined by QLF was assessed by Spearman rho. Inter- and intra-examiner agreements for visual assessment of clinical photographs and transitions were tested using Cohen’s Kappa. The level of significance for all tests was set at 5%. To assess intra-examiner reliability, 11 photograph galleries have been analysed at two different time points 2 weeks apart by the examiners. The inter-examiner reliability was tested using the total set of 110 photography galleries assessed by each examiner.

RESULTS
The clinical and QLF photographs of 51 subjects (24 males and 27 females) with a mean age of 15.5 (SD = 1.6 years) were included in this study. Data from 10 surfaces in one male subject were excluded due to an orthodontic invisible retainer in place in the maxilla while capturing the clinical photographs. Data from six posterior surfaces in two female patients were excluded due to insufficient lateral pictures at an angle smaller than 45°. These resulted in a total of 918 buccal surfaces in 51 subjects being assessed by QLF and on clinical photographs at debond (T1) and 1 year thereafter (T2). There were no significant gender differences in scores for QLF or clinical photographs; hence the data presented were not separated by gender.
The total number of lesions at the two time points, T1 and T2, are presented in Table 2. The sum and percentage of lesions for ICDAS and VT are included. The presence or absence of lesions was the same for ICDAS and VT. The number of affected surfaces detected on clinical photographs at T1 (433) was higher than for QLF (384). At T2 the number of lesions was reduced by 10.3% as determined by ICDAS and VT and 4.7% as determined by QLF.

Cross-tables showing how ICDAS, VT and QLF scores relate at T1 and T2 are given in Table 3. When comparing the baseline and 1 year clinical photographs of lesions with ICDAS score 2 at baseline, 131 improved towards ICDAS score 0, 271 stayed within score 2 and 27 became worse (score 3, 4 or restored). Also 38 lesions newly appeared within a 1-year timeframe.
Table 2. Number and percentage of buccal surfaces with WSL detected at T1 and T2 on clinical and QLF photographs.

<table>
<thead>
<tr>
<th>Surfaces</th>
<th>QLF</th>
<th>Clinical photographs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[N] [%]</td>
<td>[N] [%]</td>
</tr>
<tr>
<td>Affected at T1</td>
<td>384 [41.8]</td>
<td>433 [47.2]</td>
</tr>
<tr>
<td>Affected at T2</td>
<td>341 [37.1]</td>
<td>339 [36.9]</td>
</tr>
<tr>
<td>Total</td>
<td>918 [100]</td>
<td>918 [100]</td>
</tr>
</tbody>
</table>

Table 3. Cross-tables showing the comparison of ICDAS and Visual Transition scores with respective QLF scores at T1 and T2.

**ICDAS**

<table>
<thead>
<tr>
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<td>0 0</td>
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<tr>
<td>2 2</td>
<td>27</td>
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<tr>
<td>2 0</td>
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<tr>
<td>2 1</td>
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**Visual transition**

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<tr>
<td>2 Worse</td>
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<td>7</td>
</tr>
<tr>
<td>2 Improve</td>
<td>83</td>
<td>3</td>
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<tr>
<td>3 Same</td>
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<tr>
<td>3 Worse</td>
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<td>7</td>
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<tr>
<td>3 Improve</td>
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<td></td>
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Table 2. Number and percentage of buccal surfaces with WSL detected at T1 and T2 on clinical and QLF photographs.

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<tr>
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**ICDAS**

<table>
<thead>
<tr>
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<td>2 2</td>
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<tr>
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**Visual transition**

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<tbody>
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<tr>
<td>Worse</td>
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<td></td>
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<tr>
<td>Total</td>
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<td>7</td>
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</table>
In the visual transitions scores (VT), 268 improved, 109 lesions remained the same and 52 lesions worsened. According to QLF, 534 surfaces were sound (score 0) at T1, which related to an integrated fluorescence loss (IFL) of 0 % mm², while 247 lesions had IFL of > 0 and ≤ 5 % mm² and were given score 1, 103 were given score 2 (IFL > 5 to ≤ 25 % mm²). A total of 34 lesions were given score 3 (IFL > 25 % mm²). Figure 2 shows a scatter plot of the changes in integrated fluorescence loss for individual lesions from T1 to T2.

Figure 2. Scatter plot showing the IFL determined by QLF for the individual lesions/surfaces at T1 on the x-axis and T2 on the y-axis.
CHAPTER 2

Reliability

Inter-examiner reliability for ICDAS at T1 was 0.71 and at T2 was 0.73. Inter-examiner reliability for VT from T1 to T2 had an ICC of 0.72. The intra-examiner reliability (ICC) for ICDAS varied from 0.65 (T1) and 0.73 (T2) for examiner 1 to 0.66 (T1) and 0.72 (T2) for examiner 2. The intra-examiner reliabilities (ICC) for VT were 0.77 and 0.74 for examiners 1 and 2, respectively. For QLF, the intra-examiner (M.W.B) agreement was high, with an ICC of 0.93. The inter-examiner agreement with an experienced examiner (M.H.V) had an ICC of 0.87.

DISCUSSION

This study provides information on assessment of post-orthodontic buccal white spot lesions over time. The findings confirm regression of WSL after debonding of fixed orthodontic appliances and strengthen the evidence that not all lesions progress to cavities (Ferreira Zandonà et al., 2012). Monitoring post-orthodontic white spot lesions over time in a standardized way will provide information on the behaviour of these lesions and is therefore of clinical relevance. Assessment of WSL on routine digital oral photographs using ICDAS scores did not have sufficient discriminatory accuracy. The monitoring of these lesions by lesion assessment after orthodontics VT using routine digital oral photographs did provide a standardized manner to assess WSL progression or regression over time. Before considering the implications of the data of this study, it is important to consider the strengths and limitations of the study. Teeth were not dried prior to examination, so we were not able to score code 1 in clinical photographs and might therefore have underestimated the number of existing white spot lesions. ICDAS was developed for scoring in vivo and it has limitations for scoring on clinical photographs, especially for incipient lesions. In this study the continuum of caries has been described in ordinal scales for QLF as well as for ICDAS. This was done to be able to compare a continuous scale as used with QLF with an ordinal scale, as is the case with ICDAS. Alongside these limitations, this study also has a number of key strengths. Information on 918 surfaces of 51 patients was analysed. Two analysing methods were used: VT scoring and ICDAS scoring, to be able to detect and monitor over a 1-year timeframe. Photographs were used instead of in vivo assessment of the dentition with ICDAS, as commonly used. Using ICDAS does not allow side-by-side comparison and, thus, monitoring buccal white spot lesions over time.
is not accurate. Scoring ICDAS in clinical setting could be influenced by bias and time pressure. By using photographs, side-by-side comparison is possible. Data captured from clinical photographs has the advantage of assessing data longitudinally and at a convenient time, out of the clinical setting. Furthermore, QLF images enable quantification of lesion severity parameters with high precision and repeatability.

For post-orthodontic lesions, the ICDAS detection system on photographs does not have discriminatory power to assess distinct lesion changes within ICDAS score 2. Most of the post-orthodontic lesions were ICDAS score 2. The longitudinal changes in these non-cavitated lesions determine clinical treatment planning. If these lesions cavitate, they need curative treatment. If these lesions remain as an early caries decalcification, they may be treated using minimal intervention techniques. Scoring lesions by visual transition showed a higher discriminative power in lesion severity over time for lesions with ICDAS score 2.

ICDAS does not provide enough discriminative information on the behaviour of buccal lesions over time nor does QLF imaging. This has already been stated by the National Institute of Health, which stated in 2001 that current diagnostic practices are inadequate to achieve the next level of caries management in which non-cavitated lesions are identified early so that they can be managed by nonsurgical methods (Health., 2001). A study reported by Almosa et al. (Almosa et al., 2014) showed the usefulness of scoring lesions by means of ICDAS on oral photographs. This can be attributed to the fact that they included open cavities in their research.

Scoring clinical photographs and quantitative light induced fluorescence assessment both suggested regression of WSL after debonding of fixed orthodontic appliances. The visual transition evaluation suggested more discrimination of the behaviour of existing white spot lesions than found using ICDAS scores or QLF imaging.
REFERENCES


The use of Denaturing Gradient Gel Electrophoreses and conventional microbiology as indicator of white spot lesions in orthodontic patients; a cross-sectional study

Published as:
Beerens MW, ten Cate JM, van der Veen MH.
Microbial profile of dental plaque associated to white spot lesions in orthodontic patients immediately after the bracket removal.
CHAPTER 3

ABSTRACT

Objective
Denaturing Gradient Gel Electrophoresis (DGGE) is suggested to predict caries risk in young children. Such a tool would be valuable in orthodontic patients undergoing treatment with fixed appliances. In this cross-sectional study the applicability of DGGE and conventional microbiology for caries risk assessment in orthodontic patients were assessed.

Design
Dental plaque was obtained from orthodontic patients immediately prior to debonding. Presence of white spot lesions (WSL) was assessed immediately post debonding. DGGE-patterns and band counts were assessed using varying automated band detection settings and compared to visually detected bands to determine optimum settings. Optimum settings were used to compare band patterns in subjects with or without WSL. Microbiological samples were assessed for total colony forming units (CFU’s) and percentages of aciduric flora, Streptococcus mutans, Lactobacillus spp. and Candida albicans.

Results
Thirty-seven subjects were included with a mean age of 15.4 years (SD 1.6 years; 28 with WSL; 9 without WSL). Depending on computer software settings, DGGE outcomes were different. Optimum minimum profiling absolute to the most intense band of 4% showed no significant difference in band numbers for subjects with or without WSL (p = 0.845). Optimum settings for minimum profiling relative to the most intense band of 15% showed significant lower band numbers for subjects with WSL than those without (p = 0.007). No differences between groups were observed for microbiological parameters.

Conclusion
The analysis of DGGE-patterns is ambiguous. Software settings significantly affected outcomes. DGGE-patterns and band numbers, but also CFU counts were not predictive with respect to WSL formation in these orthodontic patients.
INTRODUCTION

Oral microorganisms play a key role in dental diseases such as dental caries and periodontal disease. Specific bacterial species in plaque have been associated with these oral diseases (Becker et al., 2002; Munson et al., 2004; Marsh, 2010). In caries, the main causative microorganisms are Streptococcus mutans and Lactobacilli spp (Socransky, 1979; van Houte et al., 1982; Boyar and Bowden, 1985; Tanzer et al., 2001). However, according to the current paradigms caries is not caused by a limited number of specific bacteria, but by a shift in the microbial population to a more unhealthy microbiome (Marsh, 2003; Chapman et al., 2010; Marsh, 2010; Thomas et al., 2012). Therefore, monitoring changes in the microbial composition might help guide prevention and treatment of caries (Lucchese and Gherlone, 2013). Among the methods to assess polymicrobial ecosystems, Denaturing Gel Gradient Electrophoresis (DGGE) was developed for qualitative compositional analyses (Rasiah et al., 2005; Li et al., 2007). In DGGE, band patterns and especially the number of bands detected is a measure of the bacterial diversity, while banding patterns can be used to compare similarity between specimens (figure 1). Li et al. (2007) and (Ling et al., 2010) have suggested that DGGE could predict the risk of developing early childhood caries. For patients undergoing orthodontic treatment this technique might be useful to assess the bacterial composition of plaque, and consequently the risk for dental caries. This is important as the formation of white spot lesions (WSL) occurs in 2 to 96% of these patients (Boersma et al., 2005; Chapman et al., 2010; Hadler-Olsen et al., 2012).

A method to assess caries risk must be robust and unambiguous. Therefore, to assess plaque diversity by DGGE, the software used to identify and quantitate bands must be discriminative, with a high specificity and sensitivity. An automated analysis, with objective detection of bands, is, essential to make the method a robust screening test.

The aim of this methodological study was:

1. to test the reliability of the software program Gelcompar II analysis using different automated band detection settings in comparison to manual detection, and

2. to assess the optimum setting to see if there is a difference between orthodontic patients with or without WSL and assess if DGGE has predictive value for the development of WSL during orthodontic treatment.
3. microbiological samples were assessed to see if there is a difference between patients with and without WSL for total colony forming units (CFU’s) and percentages of aciduric flora, *S. mutans*, *Lactobacillus* spp. and *C. albicans*.

**MATERIALS AND METHODS**

This blinded cross-sectional study was performed as a part of a randomised clinical trial involving orthodontic patients with WSL on the buccal surface developed during fixed appliance treatment. The study was conducted in accordance with the ethical principles of the 64th WMA Declaration of Helsinki (October 2013, Brazil) and the Medical Research Involving Human Subjects Act (WMO), approximating Good Clinical Practice (CPMP/ICH/135/95) guidelines. The medical ethical committee of the University Medical Centre of the Free University of Amsterdam, the Netherlands approved this study (MEC7/213).

**Subjects**

Subjects undergoing full fixed appliance treatment therapy were recruited at the Department of Orthodontics, Academic Centre for Dentistry Amsterdam, The Netherlands. The subjects comprised a convenience sample meeting the following inclusion criteria:
1. Treated with orthodontic fixed appliances in both arches,
2. Healthy adolescent males and females between the ages of 12 and 19 years at debonding, and

Written informed consent was obtained from all participants, and in the case of minors, written informed consent was also obtained from the parents/guardians.

Subjects at the Department of Orthodontics were advised to brush twice a day with fluoride toothpaste and during fixed appliance treatment they were advised to clean around the brackets using an interdental brush. WSL were confirmed directly after debonding. The detection of lesions was performed through quantitative light-induced fluorescence (QLF) (Inspektor Research Systems B.V., Amsterdam, The Netherlands) combined with clinical examination.
Subjects without WSL had to have no WSL on former bracketed surfaces. Subjects with WSL had to have two or more buccal WSL on former bracketed surfaces, seen without prolonged air drying as a distinct visual change in enamel ICDAS score 2. Lesions were without localized enamel breakdown and without clinical visual signs of dentinal involvement.

**Collection of plaque samples**

Plaque samples were collected immediately prior to debonding. The subjects abstained from oral hygiene for at least 12 hours prior to plaque collection. Plaque samples were collected from the gingival margin with a plastic spatula by one stroke on the buccal surfaces of the first and/or second pre-molar in the third quadrant for microbiological assessment and the fourth quadrant for DGGE assessment. The plaque samples were collected aseptically in sterile Eppendorf tubes. The plaque samples were centrifuged for 1 minute in a centrifuge (Eppendorf 5414D, Germany) at 16,100 rpm and placed on ice. One millilitre of cysteine peptone water (CPW) containing 10% glycerol was added to the plaque samples for microbiological assessment. The plaque samples for DGGE were stored in a mixture of 125 µL of TE buffer and 125 µL of 0.5 M NaOH. All plaque samples were stored at -80 °C until further processing.

**DNA isolation and DNA extraction**

DNA was extracted from the collected plaque samples using the DNA isolation Qiagen DNeasy® KIT (Qiagen, Hilden, Germany) (Muyzer and Smalla, 1998; Pham et al., 2009). Dental plaque pellets were re-suspended in 1 ml of ATL buffer and transferred to sterile Beadbeater tubes containing 0.5 grams (± 0.01 grams) of 0.1 mm glass beads. The Beadbeater tubes were processed in a Mini-Beadbeater Fast-Prep machine® (Qbiogene, Bio 101, Strasbourg, France) at a speed of 5.5 and immediately placed on ice to enhance microbial lysis of diverse Gram-positive microorganisms (de Boer et al., 2010).

A NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Waltham, MA USA) was used to detect if any contamination with e.g. proteins had occurred. For DNA the absorption is measured at 260 nm and for protein at 280 nm. The ratio of extinction is a measure of purity. For pure DNA this is ~1.80. For the samples a median was found of 1.9 (range 1.6 - 2.2) for the ratio 260nm/280nm.

**DNA isolation and DNA extraction**

DNA was extracted from the collected plaque samples using the DNA isolation Qiagen DNeasy® KIT (Qiagen, Hilden, Germany) (Muyzer and Smalla, 1998; Pham et al., 2009). Dental plaque pellets were re-suspended in 1 ml of ATL buffer and transferred to sterile Beadbeater tubes containing 0.5 grams (± 0.01 grams) of 0.1 mm glass beads. The Beadbeater tubes were processed in a Mini-Beadbeater Fast-Prep machine® (Qbiogene, Bio 101, Strasbourg, France) at a speed of 5.5 and immediately placed on ice to enhance microbial lysis of diverse Gram-positive microorganisms (de Boer et al., 2010).

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PCR (polymerase chain reaction) procedure

The V2-V3 region of the 16S ribosomal DNA was amplified using the Muyzer primers F357 (5’- CC CGC CCG CCG CCG GGG GGG GGG ACG CCT ACG GGA GGC AGC AG- 3’) and RS18 (5’- ATT ACC GGC GCT GCT GG- 3’). Amplification reactions were performed in sterile 0.2 ml Eppendorf tubes using a PCR (polymerase chain reaction) thermocycler (Biometra, Göttingen, Germany). The reaction mixture, that was prepared in the PCR cabinet, consisted of PCR Buffer containing 1 µl of each primer, 1 µl dNTP’s, 1 µl BSA, 1.5 µl MgCl2, 2.5 µl 10× Taq Buffer, 0.5 µl HotStarTaqTM DNA polymerase (Qiagen, Hilden, Germany), 1 µl template DNA and sterile Milli-Q water to a final volume of 24 µl. The positive control contained S. mutans. The cycling parameters were: 35 cycles of 94 °C, 4 min (initial denaturation); 94 °C, 0.5 min (denaturation); 54 °C, 1 min (annealing) and 72 °C, 1 min (elongation); 1 cycle of 72 °C, 5 min (final elongation) and a holding temperature of 15 °C following the final cycle. PCR products (4.0 µl) were analysed by electrophoresis and visualisation of the wells during loading. The gels were polymerised for 2 h.

Bacterial DNA extracts from the reference species (R. dentacariosa, S. mutans, L. acidophilus, V. parvulla, A. neslundii and L. plantarum) were separately PCR-amplified using the conditions described above.

DGGE Denaturing Gradient Gel Electrophoresis

DGGE was performed using the DCode universal mutation detection system (PowerPac basic BioRad™, Hercules, CA, USA). The DGGE gels were prepared and run with 1X TAE buffer diluted from a 50X TAE buffer stock (2 mol/l Tris-base, 1 mol/l acetic acid and 50 mmol/l EDTA). The denaturing gradient was formed using two 8% acrylamide (ratio acrylamide: bis-acrylamide, 37.5:1) stock solutions containing low (30%) and high (70%) concentrations of urea and formamide, increasing in the direction of electrophoresis. A 0% denaturing solution contained only 40% bis-acrylamide. The gels were polymerised after adding 60 µl of a 10% ammonium persulphate (APS) solution and 12 µl of TEMED to the 30% and 70% denaturing solutions immediately prior to pouring the gradient gel. A total of 30 µl of APS and 6 µl of TEMED were added to the 0% solution and 60 µl of gel dye. The gel dye was used to enhance the visualisation of the wells during loading. The gels were polymerised for 2 h. The samples (20 µl) were mixed with 4 µl of loading buffer. On each gel, 10 µl
of the reference species mixture (R. dentacariosa, S. mutans, L. acidophilus, V. parvula, A. nueslundii and L. plantarum) was combined with 15 µl of loading buffer and loaded into wells flanking the plaque samples. Electrophoresis was performed at a constant voltage of 200 V at 60°C for approximately 4 h. The gels were stained with 4 µl of SYBR Gold (Invitrogen, Carlsbad, CA, USA) (11) in 200 ml TAE for 30 minutes with motion using a See-Saw Rocker (Bibby Scientific, Staffordshire, UK) at a speed (oscillator) of 5-10 Hz. After staining, the gels were placed on a UV transilluminator to visualise the band patterns per sample (Muyzer et al., 1993). The visualised patterns were subsequently photographed with a digital camera (Canon Powershot G6, Tokyo, Japan). Plaque samples from 4 subjects were run in duplo on separate DGGE gels to test the repeatability of the method.

**DGGE band detection**

The DGGE gels were processed and analysed using GelCompar II software (version 6.5, Applied Maths, Sint-Martens-Latem, Belgium). Each gel contained 4 marker lanes to allow alignment of the band patterns. The same markers were used in all gels. A reference was defined from the markers on the first gel. All marker lanes on all gels were aligned using this reference to allow comparisons among gels. The banding patterns were normalised with respect to the marker lanes in all the gels.

To determine optimum automated band detection settings, bands of the reference gel were first detected visually at three separate occasions two weeks apart. Then bands were searched by automated band detection using different combinations of settings for minimum profiling, grey zone, minimal area and shoulder sensitivity. The following settings were used: minimum profiling 0 to 6% absolute and 0 to 20% relative to the most intense band; grey zone settings 0 to 3; minimal area 0, 0.1 to 0.5 and 1 to 3; shoulder sensitivity 0 to 3.

The optimum combination of settings for minimum profiling, both absolute and relative to the most intense band as well as visual detection of bands were then used on the full set of DGGE gels. According to the manual of GelCompar II, minimum profiling relative to the most intense band is advised ("Gelcompar II manual version 6.5", 2011).

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Microbiological data

The plaque samples were subjected to blind analysis with respect to group allocation (WSL or No WSL). Subject group allocation was combined with the microbiology data after the completion of all analyses. The plaque samples were sonicated (ultrasonic processor; Sonics Vibra-Cell, Newtown, CT, USA) for 2 minutes (amplitude 40, 1-s pulse duration) to disperse the cells optimally. The samples were diluted in cysteine peptone water (CPW) and 50-μl aliquots were dispensed in duplo onto agar plates using a spiral plater (EDDY JET; IUL Instruments, Barcelona, Spain). The samples were incubated anaerobically (10% H₂, 10% CO₂, and 80% N₂) at a temperature of 37 °C for 72 h on tryptic soy blood (TSB) agar (at cell dilutions of 10⁻³, 10⁻⁴, and 10⁻⁵), brain–heart infusion (BHI) agar (at pH 7 and pH 5, at cell dilutions of 10⁻³, 10⁻⁴, and 10⁻⁵), trypticase

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yeast-extract cysteine sucrose bacitracin (TYCSB) agar (at cell dilutions of $10^{-1}$, $10^{-2}$, and $10^{-3}$), Rogosa agar (at cell dilutions of $10^3$, $10^1$, and $10^0$) and Bismuth glucose glycine yeast (Biggy) agar (undiluted). BHI and TSB agar, both at pH 7, were used to obtain total numbers of colony-forming units (CFU's) per sample; where TSB agar was used as control to verify that BHI at pH 7 was an appropriate non-selective growth medium. BHI at pH 5 was used to determine aciduric flora, TYCSB agar was used to determine \( S. \) mutans, and Rogosa was used to determine \( Lactobacillus \) spp. Biggy agar (undiluted) was used to detect \( C. \) albicans.

The relative proportions of aciduric flora, \( S. \) mutans, \( Lactobacillus \) spp. and \( C. \) albicans, with respect to total counts, were compared between the groups of subjects with WSL or without WSL to overcome differences in amount of plaque sampled in subjects.

Statistical analysis

Intra-class correlation coefficients (ICC) between visually determined bands and each of the automated band detection settings were calculated to determine automated band detection settings for minimum profiling. Either, absolute or relative to the most intense band, with the highest level of agreement with visual band detection. The Mann Whitney U test was used to analyse the group medians for the band numbers in the DGGE pattern for visual band detection and the optimum automatic ‘absolute’ and ‘relative minimum profiling’ settings. To assess the similarities between whole profiles, gel images were subjected to band matching and a binary matrix of band classes was produced. The binary matrix was analysed through clustering using an unweighted pair group method with arithmetic averages (UPGMA) (Gafan et al., 2005) based on the Pearson correlation. Microbiological data was also statistical analysed using the independent samples T-test with equal variances.

RESULTS

A total of 37 subjects were included in this study (mean age 15.4 years, SD 1.6 years). A total of 14 subjects were male and 23 subjects were female. WSL on the previously bracketed surfaces were clinically confirmed in 28 subjects through QLF and clinical examination. The remaining 9 subjects were confirmed to have no WSL developed on the previously bracketed surfaces.
In order to defined the baseline similarity DMFS and bleeding on probing was assessed. The median DMFS scores were 1 (range 0 - 11) for the WSL group and 1 (range 0 - 3) for the group without WSL (p = 0.101, Mann-Whitney U). The median bleeding on probing percentages were 30% (range 10% - 80%) for the WSL group and 10% (range 0% - 30%) for the group without WSL (p < 0.001, Mann-Whitney U). No significant gender or age differences were detected in the occurrence of WSL. Therefore, the data presented below were not separated based on gender or age. The median duration of the orthodontic fixed appliance treatment was 31.9 months (range 14.6 - 155.8 months) for the WSL group and 31.9 months (range 17.4 - 39.7 months) for the group without WSL (p= 0.69, Mann-Whitney U).

Results DGGE band detection
Band patterns determined by visual detection of bands and automated band detection settings were compared for one gel comprising three marker lanes and ten sample lanes. The highest level of agreement between visual band detection and automated band detection settings was found for minimum profiling of 4% absolute to the most intense band, 0% grey zone, minimum area between 0.1% and 0.5% and shoulder sensitivity 0 (ICC p= 0.914). The highest level of agreement between visual band detection and automated band detection settings for minimum profiling relative to the most intense band (ICC p= 0.800) were found for a minimum profiling of 15% relative to the most intense band, 0% grey zone, minimum area 1% and shoulder sensitivity 0 and these settings were used for analysis of the DGGE patterns of subjects undergoing orthodontic treatment with fixed appliances.

The DGGE banding patterns of the 4 duplo samples showed a good but not perfect match with Pearson correlations varying from 0.82 to 0.94. The numbers of bands detected were the same for each of the 4 duplo comparisons, although some differences were visible by eye in the relative intensity of bands. The DGGE banding patterns and the dendrogram grouping of the individual profiles based on similarity for automated band detection with minimum profiling of 4% absolute to the most intense band, 0% grey zone, minimum area 0.1% and a shoulder sensitivity 0 are presented in Figure 1. The dendrogram is host-specific, i.e. showed large differences between the individual banding patterns for both groups, and the groups could not be

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separated based on DGGE pattern. No manual editing was applied. Some bands were not marked and detected using automated band detection in Gelcompar II. This shows the threshold of the program setting. No manual editing was applied. The number of bands detected in the DGGE pattern of subjects with or without WSL manually and at optimum ‘absolute’ (4%; minimum area 0.1%) and ‘relative’ (15%, minimum area 1%) minimum profiling settings for automated band detection are given in Table 1. For manual and automated band detection at the minimum profiling setting of 4% absolute to the most intense band, the number of bands in the WSL group was not significantly different from that in the group without WSL. For automated band detection at the minimum profiling setting of 15% relative to the most intense band the number of bands detected in the WSL group was significantly smaller than for the group without WSL (see Table 1).

**TABLE 1.** Comparison between numbers of bands detected using different profiling settings for subjects with or without WSL.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Manual</th>
<th>Automated band detection at 15% relative minimum profiling and 1% minimum area</th>
<th>Automated band detection at 4% absolute minimum profiling and 0.1% minimum area</th>
</tr>
</thead>
<tbody>
<tr>
<td>WSL</td>
<td>28</td>
<td>20 - (16 - 25)</td>
<td>13 - (9 - 17)</td>
<td>20 - (14 - 23)</td>
</tr>
<tr>
<td>No WSL</td>
<td>9</td>
<td>25 - (22 - 27)</td>
<td>18 - (16 - 22)</td>
<td>20 - (16 - 23)</td>
</tr>
<tr>
<td>p-value (Mann Whitney U)</td>
<td>0.111</td>
<td>0.007</td>
<td>0.845</td>
<td></td>
</tr>
</tbody>
</table>

Results microbiological data

Of the 37 included patients, collected microbiological data showed that of 3 samples not enough plaque was collected for cultivation. Data of one sample in the WSL group and 2 samples in the non-WSL group showed this data loss. The microbiology data, presented in Table 2, showed no significant difference between the two groups was observed in total CFU counts (p= 0.79), percentages of aciduric flora (p= 0.21), *S. mutans* (p= 0.19) and *Lactobacillus* spp. (0.49) or *C. albicans* (p= 0.36), although a trend towards a higher portion of the aciduric flora and *S. mutans* was observed in the WSL group. No correlation was found with the number of DGGE bands of a sample and the microbiological data, except for total CFU counts, for which an inverse relation was found (Spearman rho= - 0.42, p= 0.012).

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TABLE 2. Group means of microbiologic data and outcome of independent samples t-tests with equal variances assumed.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>p-value (independent samples t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total counts (CFU/surface)</td>
<td>WSL</td>
<td>28 4.23E+07</td>
<td>4.58E+07</td>
<td>0.79</td>
</tr>
<tr>
<td></td>
<td>No WSL</td>
<td>9 3.78E+07</td>
<td>3.47E+07</td>
<td></td>
</tr>
<tr>
<td>% Aciduric flora</td>
<td>WSL</td>
<td>28 40.43</td>
<td>25.27</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>No WSL</td>
<td>9 28.60</td>
<td>19.68</td>
<td></td>
</tr>
<tr>
<td>% S. mutans</td>
<td>WSL</td>
<td>28 10.00</td>
<td>13.08</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>No WSL</td>
<td>9 4.02</td>
<td>3.62</td>
<td></td>
</tr>
<tr>
<td>% Lactobacilli spp.</td>
<td>WSL</td>
<td>28 0.37</td>
<td>1.52</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>No WSL</td>
<td>9 0.01</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>% C. albicans</td>
<td>WSL</td>
<td>28 0.03</td>
<td>0.10</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>No WSL</td>
<td>9 0.00</td>
<td>0.00</td>
<td></td>
</tr>
</tbody>
</table>

DISCUSSION

In this study the automated band detection in DGGE gels proved ambiguous. The choice for minimum profiling settings either absolute or relative to the most intense band led to a very different outcome. It is concluded that DGGE has no predictive value with respect to caries risk in orthodontic patients undergoing treatment with fixed appliances. This finding is in contrast to existing literature, where DGGE was shown to have predictive value towards the development of early childhood caries (Li et al., 2007; Yang et al., 2010; Tao et al., 2013; Tao et al., 2015). However, these studies do not describe DGGE band lane detection settings in full. The choice of optimum band detection settings was not studied before. Minimum profiling settings relative to the most intense band are suggested when assessing DGGE patterns with different intensities, as is often the case when comparing clinical samples (‘Gelcompar II manual version 6.5,’ 2011). Nevertheless, in this study minimum profiling settings absolute to the most intense band had a better level of agreement with visually determined bands. Further, DGGE band patterns are best compared within one gel rather than among gels. For DGGE one is limited to a maximum of 20 specimens, including markers. The use of more samples, like in this study, implies the use of multiple gels. This issue can be overcome by aligning the gels using the lanes with mixture of reference species. The comparison of duplos from plaque samples from 4 subjects in this study, showed that a comparison among gels introduced little error.
Caries risk assessment is currently based on past caries experience or DMFS scores complemented with current oral hygiene level (Sundell et al., 2013). Additionally, a dip slide test may be used to assess levels of S. mutans and Lactobacilli spp (Krasse, 1988; Anderson et al., 1993). In orthodontic patients these risk indicators have low value (Boersma et al., 2005; van der Kaaij et al., 2015). Traditional DMFS scores do not provide information about incipient lesions. Even individuals without symptoms of caries prior to orthodontic treatment are at risk of developing WSL during fixed appliance treatment (van der Kaaij et al., 2015). Furthermore, during fixed appliance treatment, nearly all patients are ‘millionaires’ in terms of Streptococcal counts, annihilating the discriminatory value of dip slide tests (Boersma et al., 2005).

In this study a secondary aim was to test if traditional plate counting may overcome this issue. Similar to DGGE outcome, no differences were observed between the relative amounts of aciduric flora, S. mutans, Lactobacillus spp. and C. albicans of orthodontic patients undergoing fixed appliance treatment with or without WSL.

In this study only a limited number of orthodontic patients with and without WSL on the bracketed surfaces were included. The numbers of subjects with WSL (28) and without WSL (9) were unbalanced. These numbers do reflect the high percentage of subjects with WSL during orthodontic treatment at the ACTA clinic at the time of the study.

In the WSL group the ranges for DMFS, bleeding percentage and their treatment duration extended towards higher values in comparison to the group without WSL. Bleeding percentage was discriminatory between subjects with or without WSL developed on the previously bracketed surfaces. The outcome measures of this study, DGGE band numbers and CFU counts, were not discriminatory, in assessing the presence or absence of WSL. The outcome of the study shows such a small difference between groups with or without WSL that the use of DGGE as screening test for caries risk in orthodontic patients seems unfeasible.

Although the numbers of bands detected were inconclusive, each subject showed a specific individual banding pattern. Monitoring individual changes in banding pattern and plaque diversity in time during orthodontic treatment may thus still be worthwhile to investigate as means to assess an increase in caries risk in orthodontic patients.
REFERENCES


REFERENCES


Remineralising effect of MI Paste Plus® on the regression of early caries after orthodontic fixed appliance treatment; a randomized 3-month follow-up clinical trial

Published as:
Beerens MW, van der Veen MH, van Beek H, ten Cate JM. Effects of casein phosphopeptide amorphous calcium fluoride phosphate paste on white spot lesions and dental plaque after orthodontic treatment: a 3-month follow-up.
ABSTRACT

Objectives
The effects of casein phosphopeptide amorphous calcium phosphate fluoride (CPP-ACPF) paste (MI Paste Plus®) vs. control paste on the remineralisation of white spot caries lesions and on plaque composition were tested in a double-blind prospective randomized clinical trial.

Design
Fifty-four orthodontic patients, with multiple white spot lesions observed upon the removal of fixed appliances, were followed up for 3 months. Subjects were randomly assigned to either CPP-ACPF paste (MI Paste Plus®) or control paste for home use, supplementary to their normal oral hygiene. Caries regression was assessed on quantitative light-induced fluorescence (QLF) images captured directly after debonding and 6 and 12 weeks thereafter. The total counts and proportions of aciduric bacteria, *Streptococcus mutans*, and *Lactobacillus* spp. were measured in plaque samples obtained just before debonding, and 6 and 12 weeks afterwards.

Results
A significant decrease in fluorescence loss was found with respect to baseline for both groups and no difference was found between groups. The size of the lesion area did not change significantly over time or between the groups. The percentages of aciduric bacteria and of *Streptococcus mutans* decreased from 47.4 to 38.1% and from 9.6 to 6.6%, respectively.

Conclusions
No differences were found between groups. No clinical advantages were observed for use of the CPP-ACPF paste (MI Paste Plus®) supplementary to normal oral hygiene over the time span of 3 months.
INTRODUCTION

Changes of the enamel surface as a result of decalcification, or development of so called multiple white spot lesion (WSL) is by far the most important visible iatrogenic effect of orthodontic fixed appliance treatment (Wisth and Nord, 1977; Gorelick et al., 1982; Øgaard et al., 1988a; Øgaard et al., 2004; Bishara and Ostby, 2008; Øgaard, 2008). The prevalence of WSL on at least one tooth surface of patients who undergo fixed appliance therapy has been found to vary from 4.9% (Gorelick et al., 1982) to 97% (Boersma et al., 2005). The incidence is higher in patients treated with orthodontic fixed appliance, than in non-orthodontic individuals (Zachrisson and Zachrisson, 1971). White spot lesions are defined as subsurface enamel porosities caused by an imbalance between demineralisation and remineralisation. When WSL are located on smooth surfaces they present as milky-white opacities (Bishara and Ostby, 2008). Demineralisation of enamel around brackets can be a very rapid process (Gorelick et al., 1982; Øgaard et al., 1988a) Frequent exposure to fermentable carbohydrates accelerates the rate of plaque accumulation and maturation, and leads to lower plaque pH and colonization of aciduric bacteria. Together this favours increased proportions and absolute numbers of Streptococcus mutans and Lactobacillus spp. in saliva and plaque (Scheie et al., 1984; Rosenbloom and Tinanoff, 1991; Ahn et al., 2007; Kim et al., 2010). After removal of the fixed appliance, the numbers of S. mutans and Lactobacillus spp. decrease to the amounts and proportions observed before the orthodontic treatment without additional prophylactic measures (Rosenbloom and Tinanoff, 1991). With respect to remineralisation of WSL, removing the fixed appliance as an aetiological factor contributes to a favourable balance between demineralisation and re-mineralisation. This is observed especially in combination with good oral hygiene (Willmot, 2008). Clinically, it has been observed that these WSL can disappear (Backer, 1966). Nevertheless, post-orthodontic patients still have a higher prevalence of WSL when compared with untreated age-matched controls, even 5 years after the removal of the fixed appliance (Øgaard, 1989). Removal of the appliance appears to be insufficient to completely remineralise the softened enamel. A clinical trial performed at the Academic Centre for Dentistry (ACTA) department of Orthodontics using quantitative light-induced fluorescence (QLF) showed that natural repair was limited. The majority of WSL remained unchanged, while 10% had progressed in severity 6 months after debonding.
More than 97.3% of lesions remained as permanent scars (van der Veen et al., 2007). Management of WSL should involve methods of both preventing demineralisation and encouraging the remineralisation of existing lesions. In both of these processes the efficacy of fluoride is well established. Fluoride increases the initial rate of remineralisation of early enamel lesions, and then slows down the caries process, arresting the lesion (ten Cate et al., 2008). However, there are obvious differences between the prevention of WSL during fixed appliance therapy and the curative treatment of existing WSL after debonding. Willmot (Willmot, 2008) and Øgaard (Øgaard et al., 1988b) warned against treating visible WSL on labial surfaces with concentrated fluoride agents, because this arrests both demineralisation and remineralisation in the lesion by surface hypermineralisation (ten Cate et al., 1981). These arrested lesions may persist lifelong, exhibiting a white colour, or might become yellowish or dark brown in colour as a result of the uptake of exogenous stains (Bishara and Ostby, 2008).

To enhance the natural remineralisation by saliva, bioavailable calcium and phosphate are needed. Products providing calcium and phosphate in bioavailable forms have existed since the 1980s when Reynolds et al. (Reynolds, 1987) introduced casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACP). It has been claimed that the multifactorial anticariogenic mechanism for CPP-ACP has a threefold mode of action:
1. it promotes the remineralisation of enamel lesions by maintaining a supersaturated state of the enamel minerals calcium and phosphate in plaque (Reynolds, 1998),
2. it delays the formation of biofilm (Rahiotis et al., 2008) and inhibits bacterial adhesion to the tooth surface; and
3. it acts as a buffering agent, which may prevent a reduction of pH in the oral micro-environment (Rahiotis et al., 2008).

Casein phosphopeptide amorphous calcium phosphate with fluoride (CPP-ACPF) has the same potential with the additional benefits of the added fluoride (Cross et al., 2004). Using CPP-ACPF will remineralise subsurface lesions by forming fluorapatite within the lesion (Cochrane et al., 2008). This concept has led to the production of oral hygiene supporting products, such as chewing gums and tooth creams containing CPP-ACPF, as supplement to normal daily oral hygiene procedure. The remineralisation of enamel subsurface lesions by CPP-ACP complexes has been demonstrated in numerous laboratory,
animal, and human *in situ* experimental studies (Reynolds, 1987; Shen et al., 2001; Andersson et al., 2007; Oshiro et al., 2007; Cochrane et al., 2008; Rahiotis et al., 2008; Walker et al., 2009). Two clinical trials on the remineralisation of post-orthodontic WSL by CPP-ACP cream show contrasting findings (Bailey et al., 2009; Bröchner et al., 2011). The use of CPP-ACPF has thus far only been reported in *in vitro* experiments (Zhao and Cai, 2001; Cross et al., 2004; Cochrane et al., 2008).

Therefore, the aim of this study was to investigate the effects of commercially available CPP-ACPF paste (MI Paste Plus®) *in vivo* on dental plaque and on the remineralisation of enamel WSL as assessed by QLF after the removal of fixed orthodontic appliances, in a randomized controlled clinical trial during a 3-month time period.

The null hypothesis was that the use of CPP-ACPF paste (MI Paste Plus®) *in vivo*, in addition to normal oral hygiene, does not have an effect on (i) the remineralisation of subsurface lesions and/or (ii) plaque composition (expressed as the percentage of aciduric bacteria, *S. mutans*, and *Lactobacillus* spp.) in orthodontic patients with WSL after the removal of fixed orthodontic appliances.

**MATERIAL AND METHODS**

The study was performed as a prospective, double blinded, randomized placebo controlled clinical trial. Aiming to to determine the effect of a CPP-ACPF paste vs. a control paste on plaque composition and remineralisation of enamel WSL in subjects with multiple WSL at the end of treatment with a fixed appliance. The trial was conducted during the first 3 months following appliance removal. The medical ethical committee of the University Medical Centre of the Free University Amsterdam, the Netherlands, approved this study protocol (MEC 07/213).

**Subjects**

Orthodontic patients treated with full fixed appliances in both arches, which were scheduled for debonding, were invited to participate in this study and were debonded and screened for WSL between January 2008 and August 2009 at the Department of Orthodontics, Academic Centre of Dentistry Amsterdam, the Netherlands.
The subjects fulfilled the following requirements:
1. healthy adolescent male and female subjects between 12 and 19 years of age,
2. two or more bracketed surfaces with buccal subsurface WSL, seen without prolonged air drying as a distinct visual change in enamel and/or localized enamel breakdown without clinical visual signs of dentinal involvement (Topping et al., 2009),
3. no systemic diseases,
4. no syndromic abnormalities, and
5. no proven or suspected milk protein allergy and/or sensitivity, or allergy to benzoate preservatives, as both are components of the CPP-ACPF product.

Subjects were only enrolled after signed informed consent was obtained from the patient, and, in case of minors, also the parents/guardians. None of the subjects lived in an area where the community water was fluoridated. The study group consisted of 54 participants (23 male and 31 female subjects) with a mean age of 15.5 years.

Study outline
Subjects, complying with the inclusion criteria determined by M.W.B., were then randomly assigned by M.H.V. to either the CPP-ACPF group or the control group, as determined by a computer-randomization scheme that was created and locked before the start of the study. The subjects received neutral-coloured toothpaste tubes marked A or B, which contained either CPP-ACP + sodium fluoride (0.2% w/w; 900 p.p.m.) (MI Paste Plus® 35 ml, Recaldent; GC Benelux Europe, Leuven, Belgium) or fluoride-free control paste + calcium (Ultradent 100 ml; Kruidvat NL, Renswoude, the Netherlands) for home use. Subjects were informed that they could receive either a CPP-ACPF paste or a control paste with a different form of calcium delivery. Just before debonding (T0), the subjects were screened for the presence of WSL by means of visual inspection and by use of QLF imaging. Plaque was collected buccally from the lower right first premolars at screening and 6 (T2) and 12 (T3) weeks after the start of the intervention. Immediately after debonding (T1), QLF baseline images were captured as described below. Follow-up QLF images were captured 6 (T2) and 12 (T3) weeks after debonding.
Stringent oral hygiene and product use

All participants, receiving either CPP-ACPF paste or a control paste, received the same verbal hygiene instruction by a dental hygienist. They were advised and informed how to brush properly using a fluoridated dentifrice (i.e. at least twice a day, either with a hand toothbrush or an electric toothbrush for at least 2 min) and that no additional fluoride was advised or should be applied. The referring dentists were informed that their patients were participating in the study and were asked not to administer additional fluoride during this investigation. They were further asked to contact the study investigator in the event that restorations were made on the buccal surfaces. The referring dentist was informed during this 1-year study if the investigator visually detected and/or suspected occlusal and proximal enamel/dentine caries.

The participants were instructed to use their respective paste once a day at bedtime. Verbal and written instructions were given to the patient. They were informed that the paste should be applied to the tooth surfaces using a clean, dry finger. A sufficient amount of paste was to be applied to the upper and the lower teeth. A pea-size amount for each arch was the minimum amount required. The pastes were to be kept in the mouth for as long as possible. Subjects were asked not to rinse afterwards. Compliance was checked by questions asked on each visit about the frequency of tooth brushing and application of the study paste and how often, and when these were forgotten. Furthermore, subjects were asked to bring their study paste at each visit.

QLF imaging

QLF images were captured using an intra-oral fluorescence camera (QLF/Clin; Inspektor Research Systems, Amsterdam, the Netherlands) with dedicated software (Inspektor pro version 3.0.0.42; Inspektor Research Systems). To ensure that the same area of tooth surface was analysed at each time-point, the analysis patch and surface contour were copied and then matched for size, orientation, and location, as described previously (Mattousch et al., 2007). Pixels inside the patch were considered as part of the lesion when the relative fluorescence loss exceeded a 5% threshold (Al-Khateeb et al., 1998). Images were captured after plaque removal from the buccal surfaces. Images were analysed for fluorescence loss (DF), size of lesion area (A), and integrated fluorescence loss (IFL) at the T1, T2, and T3 time-points. The images were informed during this 1-year study if the investigator visually detected and/or suspected occlusal and proximal enamel/dentine caries.

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were captured by examiners who were calibrated before the start of the investigation and who were blinded with respect to the treatment group. One QLF device was used for all measurements. All analyses were performed by a single examiner (M.W.B.), who was trained and calibrated by an experienced QLF examiner (M.H.V.) at the start of the study on a data set of 500 QLF images. In cases where lesion visibility at the baseline was hampered as a result of swollen gingiva, the visible area of the lesion at the baseline was the part of the lesion analysed at subsequent timepoints using QLF. The QLF images obtained for each subject were analysed blind with regard to treatment group; the group allocations were added to the exported data file after completion of all analyses.

Plaque processing
Plaque was sampled from the buccal surface of the lower right first premolar at T0, T2, and T3. Plaque samples were labelled with consecutive numbers that were recorded on the subjects sheets. Plaque samples were spun down in a centrifuge (Eppendorf centrifuge 5415 D; Eppendorf, Hamburg, Germany) for 30 seconds at 16,200 g, then stored at 80° C after the addition of 1 ml of cysteine–peptone water (CPW) containing 10% glycerol (as transport medium) until further processing. Plaque samples were analysed blind with respect to subject number, visit, and group allocation. Microbiology data was coupled to Subject numbers, visit numbers, and group allocation after completion of all analyses. Plaque samples were sonicated (ultrasonic processor; Sonics vibra-cell, Newtown, CT, USA) for 2 min (amplitude 40, 1-s pulse duration) to disperse the cells. The samples were diluted in CPW and 50-ml aliquots were distributed on agar plates using a spiral-plater (EDDY JET; IUL Instruments, Barcelona, Spain). The samples were incubated anaerobically (in an atmosphere of 10% H₂, 10% CO₂, 80% N₂) at a temperature of 37° C for a period of 72 h on tryptic soy blood agar (at cell dilutions of 10⁻¹, 10⁻², and 10⁻³), on brain–heart infusion (BHI) agar at a pH of 5.0 (at cell dilutions of 10⁻¹, 10⁻², and 10⁻³), on trypticase yeast-extract cystine sucrose bacitracin agar (at cell dilutions of 10⁻¹, 10⁻², and 10⁻³), and on Rogosa agar (at cell dilutions of 10⁻¹, 10⁻², and 10⁻³), to obtain the total numbers of colony-forming units (CFUs) per sample, and the proportions of aciduric bacteria, S. mutans, and Lactobacillus spp., respectively.
Power analysis
To assess the influence of CPP-ACPF on the reduction of WSL, a power analysis was conducted using G*-power 3.1.0 to determine the number of required patients (Faul et al., 2007). Based on a previous observational study at the Orthodontic Department at ACTA (van der Veen et al., 2007), a statistically significant, but clinically irrelevant, natural reduction in fluorescence loss, of 0.9 ± 0.9%, during a period of 24-weeks was found. A clinically relevant change in fluorescence loss was considered to be an average reduction of 2%, implying an effect size of 0.55. The sample size was calculated for a more conservative effect size of 0.35. For an effect size of 0.35 to be measured between the two groups, a group size of 27 was needed. To compensate for subject withdrawal, we aimed to include 30 subjects in each group. Subjects who dropped out before T2 were replaced to meet the required minimum group size of n = 27.

Data analysis
The chi-square test and the Students t-test (two-tailed) were used to determine statistically significant differences between both groups at baseline (i.e. T0 for plaque and T1 for WSL) (PASW statistics 17.0; SPSS Inc., Chicago, IL, USA). The fluorescence loss, lesion area, and corresponding integrated fluorescence loss were scored for each lesion at three time-points. The follow-up QLF images were compared with those at baseline (T1). The average fluorescence loss over all WSL, total lesion area, and corresponding IFL were calculated for each subject and then normalized to 20 surfaces by correcting for the number of missing elements and filled surfaces. Lesion progression or regression for the whole group of subjects was determined by repeated measures ANOVA, followed by Bonferroni post-hoc testing. The plaque ecology, described by the total numbers of colony forming units (CFUs) and the proportions of aciduric bacteria, S mutans, and Lactobacillus spp., was assessed at baseline (T0), and the results were compared with those obtained at the follow-up time-points using repeated-measures ANOVA, followed by a Bonferroni post-hoc test. The level of significance for all tests was set at 5%. The intraclass correlation coefficients (ICCs) were calculated to determine intraexaminer and interexaminer agreement for the training set of QLF images.

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RESULTS

Descriptive results

From a total of 184 screened participants, 65 were recruited into the study. They were randomly assigned into two groups, of CPP-ACPF product (group A; n = 35) and control (group B; n = 30). A total of ten participants dropped out between T0 and T3, seven from the CPP-ACPF group and three from the control group. The reason given for withdrawal was the time-consuming nature of the study. One further participant from the CPP-ACPF group was found to be WSL free and was removed from the study. The participants did not differ with respect to number of lesions, gender ratio, age, and fixed appliance duration compared with subjects who completed the study. The subject flow in the study is given in Fig. 1.

Fifty-four participants (23 male and 31 female; mean age ± SD, 15.5 ± 1.6 years) completed this study. The gender ratio, participant age, and duration of treatment with the fixed appliance before investigation were not statistically significantly different between the groups. A total of 424 caries-affected surfaces in a total of 1,002 elements were followed throughout this investigation (Table 1). The affected elements were distributed as follows: 14.2% central incisors; 22.6% lateral incisors; 28.3% cusps, and 34.9% premolars. The distributions were equal for the two groups.

Questions regarding frequency of brushing and product use were used to get an impression of compliance. Overall compliance in the study was considered moderate. The subjects generally brushed twice a day and used the product at night-time after brushing during the first 6 weeks of the study. Between weeks 6 and 12, the subjects forgot to brush and to use the product on average once a week, and this always occurred at night-time. The assessment of product use via returned product failed entirely because none of the subjects returned their product tubes at recall visits.
Assessed for eligibility \((n = 184)\)

Excluded \((n = 119)\)

Not meeting inclusion criteria:
- At screening < 2 WSL \((n = 98)\)
- At debond < 2 WSL \((n = 6)\)
- Caries profunda \((n = 5)\)
- To old \((n = 2)\)
- Partially fixed appliance \((n = 3)\)
- Extended fluorosis dentalis \((n = 1)\)
- Refused to participate \((n = 2)\)
- Data at screening incomplete \((n = 1)\)

Allocated to intervention group A \((n = 35)\)

Received allocated intervention \((n = 35)\)

Lost to follow-up \((n = 7)\)

Not present at visits: \((n = 6)\)
Decided not to participate any further \((n = 1)\)

Discontinued intervention due to Non-compliance \((n = 5)\)
though include

Analyzed \((n = 28)\)

Excluded from analysis \((n = 1)\)
No lesions detected

Allocated to intervention group B \((n = 30)\)

Received allocated intervention \((n = 30)\)

Lost to follow-up \((n = 3)\)

Not present at visits: \((n = 1)\)
Decided not to participate any further \((n = 2)\)

Analyzed \((n = 27)\)

Excluded from analysis \((n = 1)\)
No lesions detected

FIGURE 1. Flow of participants through the study. Group A received the CPP-ACPF product paste and Group B received the control paste.
TABLE 1. Variables investigated in this study, overall (total), and separately in the casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACPF) and control groups

<table>
<thead>
<tr>
<th>Variable investigated</th>
<th>CPP-ACPF group</th>
<th>Control group</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 27)</td>
<td>(n = 27)</td>
<td>(n = 54)</td>
</tr>
<tr>
<td>Surfaces monitored</td>
<td>511</td>
<td>491</td>
<td>1002</td>
</tr>
<tr>
<td>Surfaces affected</td>
<td>211</td>
<td>213</td>
<td>424</td>
</tr>
<tr>
<td>Gender ratio (male : female)</td>
<td>1:1.25</td>
<td>1:1.45</td>
<td>1:1.35</td>
</tr>
<tr>
<td>Age at investigation (years/months)</td>
<td>15.2 ± 1.6</td>
<td>15.8 ± 1.3</td>
<td>15.5 ± 1.5</td>
</tr>
<tr>
<td>Duration of fixed appliance treatment (years/months)</td>
<td>2.6 ± 0.8</td>
<td>2.6 ± 0.00</td>
<td>2.5 ± 0.8</td>
</tr>
</tbody>
</table>

Data are given as n or as mean ± SD

QLF results

The intra-examiner agreement for M.W.B. was high with an ICC of 0.93. The inter-examiner agreement for the experienced examiner M.H.V. had an ICC of 0.87. Descriptive data for lesion depths [ΔF (%), lesion area [A (mm²)], and IFL [IFL (% mm²)], measured at the three different time-points, T1, T2, and T3, within groups are shown in Table 2. Baseline data at T1 showed no significant differences between groups for DF, A, and IFL (P > 0.05). The significant improvement in lesion depth (DF) observed over time in both groups (P = 0.0004) was compared by repeated-measures ANOVA, but no significant difference was found between the groups. The Bonferroni post-hoc test identified a significant decrease in fluorescence loss (DF) from baseline to T3 with for both groups (P < 0.05) and no difference between groups. The lesion area (A) did not change significantly over time or between the groups. While the lesion area in the CPP-ACPF group remained essentially unchanged, the lesion area in the control group showed an initial decrease from T1 to T2, and then returned to the original value found at T1. The IFL did not change significantly over time and between the groups. In the CPP-ACPF group, the IFL was essentially unchanged over the time period studied, and in the control group some initial improvement was seen; however, three-quarters of the improvement was lost at T3.
TABLE 2. WSL regression, determined by assessment of lesion depth (DF), lesion area (A), and integrated fluorescence loss (IFL), at three different time-points (T1, T2, and T3) in the casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACPF) group and the control group.

<table>
<thead>
<tr>
<th>Group</th>
<th>CPP-ACPF</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (%)</td>
<td>8.45 ± 1.17</td>
<td>7.93 ± 1.34</td>
</tr>
<tr>
<td>A (mm²)</td>
<td>5.07 ± 5.69</td>
<td>5.09 ± 6.53</td>
</tr>
<tr>
<td>IFL (mm²)</td>
<td>56.37 ± 73.05</td>
<td>57.14 ± 86.74</td>
</tr>
<tr>
<td>T2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (%)</td>
<td>7.93 ± 1.34</td>
<td>7.52 ± 1.78*</td>
</tr>
<tr>
<td>A (mm²)</td>
<td>5.09 ± 6.53</td>
<td>5.05 ± 6.98</td>
</tr>
<tr>
<td>IFL (mm²)</td>
<td>57.14 ± 86.74</td>
<td>57.76 ± 91.73</td>
</tr>
<tr>
<td>T3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF (%)</td>
<td>7.52 ± 1.78*</td>
<td>9.10 ± 1.75</td>
</tr>
<tr>
<td>A (mm²)</td>
<td>5.05 ± 6.98</td>
<td>5.96 ± 6.38</td>
</tr>
<tr>
<td>IFL (mm²)</td>
<td>57.76 ± 91.73</td>
<td>90.81 ± 111.28</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.

*Data significantly different from T1.

Plaque results

The composition of plaque at the different time-points of this study are presented in Table 3. The numbers of CFUs observed before (i.e. at T0) and after (i.e. at T2 and T3) debonding were not significantly different between the two groups (P > 0.8). Significant decreases over time in the percentage of aciduric bacteria were found (P = 0.04). The mean percentage of aciduric bacteria decreased from 47.4% (SD) at T0 to 38.1% (SD) at T3, a reduction of nearly 10%. The reduction was significant in the CPP-ACPF group at T2 and T3, while, for the control group, the reduction in aciduric bacteria became significant at T3. No significant difference (P = 0.4) was found between the groups. The average number of S. mutans, for both groups combined, at the end of treatment with the fixed appliance (T0) comprised 9.6 ± 9.6% of the total CFUs. The average proportion of S. mutans, for both groups combined, decreased from T0 to T3 (6.6 ± 7.4%, P = 0.01). This appears to be caused by the changes seen in the control group, although the changes were not significantly different between the groups (P = 0.4). The number of Lactobacillus spp. found was low, and, in 45% of cases, below the detection limit of 400 CFUs. anova identified significant changes in the percentage of Lactobacillus spp. over time (P < 0.01), yet the changes were not consistent between groups and post-hoc testing showed a significant increase only in the control group at T2. Again, no differences were seen between the groups.

TABLE 2. WSL regression, determined by assessment of lesion depth (DF), lesion area (A), and integrated fluorescence loss (IFL), at three different time-points (T1, T2, and T3) in the casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACPF) group and the control group.

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<tr>
<td>A (mm²)</td>
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</tr>
</tbody>
</table>

Data are given as mean ± SD.

*Data significantly different from T1.
### TABLE 3. Plaque ecology, determined by total bacterial counts, and the proportions of aciduric bacteria, Streptococcus mutans, and Lactobacillus spp., of samples from the casein phosphopeptide-stabilized amorphous calcium phosphate (CPP-ACPF) group and the control group at three different time-points (T1, T2, and T3)

<table>
<thead>
<tr>
<th>Group</th>
<th>T0</th>
<th>T2</th>
<th>T3</th>
<th>T0</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total counts (10^7 CFU per sample)</strong></td>
<td>5.8 ± 4.1</td>
<td>4.1 ± 4.3</td>
<td>4.4 ± 6.0</td>
<td>3.3 ± 4.1</td>
<td>3.9 ± 4.3</td>
<td>5.2 ± 6.0</td>
</tr>
<tr>
<td><strong>Aciduric bacteria (%)</strong></td>
<td>52.5 ± 30.4</td>
<td>45.7 ± 28.4</td>
<td>44.7 ± 26.0</td>
<td>42.3 ± 30.4</td>
<td>41.6 ± 28.4</td>
<td>31.5 ± 26.0</td>
</tr>
<tr>
<td><strong>Streptococcus mutans (%)</strong></td>
<td>9.4 ± 9.6</td>
<td>4.8 ± 5.9</td>
<td>9.0 ± 7.4</td>
<td>9.7 ± 9.6</td>
<td>4.9 ± 5.9</td>
<td>4.2 ± 7.4</td>
</tr>
<tr>
<td><strong>Lactobacillus spp. (%)</strong></td>
<td>0.2 ± 0.8</td>
<td>0.1 ± 1.1</td>
<td>0.1 ± 0.9</td>
<td>0.1 ± 0.8</td>
<td>0.4 ± 1.1</td>
<td>0.2 ± 0.9</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD. Bacterial counts are expressed as a percentage of the total counts per sample obtained at each time point.

*Data significantly different from T0.

### DISCUSSION

The null hypothesis could not be rejected. The use of CPP-ACPF paste (MI Paste Plus®) in vivo, in addition to normal oral hygiene, does not have an effect on:

1. remineralisation of subsurface lesions, and/or
2. plaque composition, expressed as the percentages of aciduric bacteria, S. mutans, and Lactobacillus spp. in orthodontic patients with WSL after the removal of fixed orthodontic appliances.

This contradicts the findings reported by Bailey et al. (Bailey et al., 2009), but supports the findings of Bröchner et al. (Bröchner et al., 2011) who studied the effects of a remineralisation cream containing CPP-ACP in the enhanced regression of postorthodontic WSL and the effect on plaque composition. Twelve weeks after debonding, the lesions showed significant improvement with respect to fluorescence loss, as monitored by QLF. Nevertheless, lesion regression after debonding was not seen to the extent expected. The time span of this study (12 weeks) may be too short to detect active lesion regression. Bröchner. (Bröchner et al., 2011) reported a reduction in lesion area of 58% after only 4 weeks. However, the lesions investigated by Bröchner et al. (Bröchner et al., 2011) were extremely small (0.19 mm²) in comparison to the lesions studied in our study, which were (0.79 mm²) at baseline. The fact...
that lesions are not easily remineralised may require a different approach. New, upcoming techniques for lesion infiltration seem promising and provide an immediate aesthetic improvement (Paris et al., 2010). The efficacy of these products still needs to be proven in long-term clinical studies to ensure that they do indeed protect the WSL from further demineralisation (Paris and Meyer-Luecke, 2010). Another option would be to first open up the lesions by acid-etching before remineralisation treatment. Only an in situ study with very early decalcifications was reported (Al-Khateeb et al., 2000). Given the fact that fixed appliances form a retention site for plaque, it was assumed that the amount of plaque sampled would be higher at the end of treatment with fixed appliance than after debonding. The fixed appliances hampered plaque sampling, resulting in similar amounts of plaque being collected before and after debonding. Also, the numbers of CFU counts per sample were similar at all time-points. Thus, in contrast to the expected decrease, the results showed no significant changes.

Furthermore, it was expected that the proportions of aciduric bacteria, *S. mutans*, and Lactobacillus spp. would have been high at the end of treatment with the fixed appliance and to decrease after the appliance was debonded. This was confirmed, as we found a high proportion of aciduric bacteria at T0 and a significant decrease at 6 and 12 weeks after debonding (T2 and T3, respectively). The use of CPP-ACP paste did not show an additional beneficial effect. Also, the proportion of *S. mutans* was extremely high just before debonding. Based on the literature it was assumed that we would observe a greater reduction in the proportion of *S. mutans* in the CPP-ACP group than in the control group, as a result of binding of the CPP-ACP nano-complexes to *S. mutans* (Schupbach et al., 1996; Rose, 2000). This assumption was not supported by the results from our study. The proportion of *S. mutans* may simply be so high that a longer period is needed to reach the expected and desired reduction.

The proportion of *Lactobacillus* spp. was low. In contrast to the reported correlation between *S. mutans* and *Lactobacillus* spp. with caries (Boersma et al., 2005; Sanpei et al., 2010), *Lactobacillus* spp. were frequently not detected at all just before debonding. In many cases the Rogosa agar was heavily colonized with *S. mutans* rather than with *Lactobacillus* spp. Although Rogosa agar favours the growth of *Lactobacillus* spp. over other species, other bacteria may still grow when abundant. When comparing our results with those from that lesions are not easily remineralised may require a different approach. New, upcoming techniques for lesion infiltration seem promising and provide an immediate aesthetic improvement (Paris et al., 2010). The efficacy of these products still needs to be proven in long-term clinical studies to ensure that they do indeed protect the WSL from further demineralisation (Paris and Meyer-Luecke, 2010). Another option would be to first open up the lesions by acid-etching before remineralisation treatment. Only an in situ study with very early decalcifications was reported (Al-Khateeb et al., 2000). Given the fact that fixed appliances form a retention site for plaque, it was assumed that the amount of plaque sampled would be higher at the end of treatment with fixed appliance than after debonding. The fixed appliances hampered plaque sampling, resulting in similar amounts of plaque being collected before and after debonding. Also, the numbers of CFU counts per sample were similar at all time-points. Thus, in contrast to the expected decrease, the results showed no significant changes.

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the literature (Boersma et al., 2005; Sanpei et al., 2010) it was noted, first, that these studies isolated *S. mutans* and *Lactobacillus* spp. from saliva, and, second, that a dip-slide test or a test strip was used. The use of such tests may not be valid in cases where *S. mutans* outnumbers *Lactobacillus* spp. to such an extent that the Rogosa agar on the CPP-ACFP strip displays *S. mutans* rather than *Lactobacillus* spp. In such tests the colony morphology typically is not checked. The finding, that the numbers of both aciduric bacteria and *S. mutans* decrease slowly after debonding (Table 3), indicates that the composition of plaque becomes less cariogenic after debonding. A longer follow-up period, however, seems necessary to evaluate if a healthy plaque ecology is re-established.

Using QLF as an assessment method is, as described by Bröchner, a reflectional method to clinical scores (Boersma et al., 2005; Ferreira Zandona et al., 2010). With the use of QLF important additional data can be obtained concerning depth and precise area measurement. QLF has been accepted as a detection method in longitudinal observation (Stookey, 2004; Ferreira Zandona et al., 2010). The QLF technique has been applied in several controlled clinical trials, with the consistent observation that it is capable of monitoring and quantifying changes in the mineral content and size of clinically visible non-cavitated WSL and can therefore be used to assess the impact of reversal of the caries process (Stookey, 2004; Ferreira Zandona et al., 2010). The benefits of the QLF technique were apparent, especially in a longitudinal *in vivo* observation of demineralisation and remineralisation processes (Ferreira Zandona et al., 2010). The additional use of International Caries Detection and Assessment System (ICDAS) could help to make data more comparable among studies, but with lesions only classified by ICDAS scores 2 and 3, the scale was considered too crude in comparison with the continuous scale of QLF.

As in any clinical trial, compliance is an important issue. In this study the subjects were adolescents, a population where sometimes compliance is difficult to achieve. The use of the paste supplementary to tooth brushing twice a day was considered to be difficult to maintain by many subjects, and brushing and application of paste were forgotten, on average, once per week during the last 6 weeks of the study. The taste of the CPP-ACFP is quite different from that of ordinary fluoridated toothpastes commercially available in the Netherlands. While noncompliance because of disliking the taste of the CPP-ACPF paste was expected, only two subjects complained about the taste, but continued the study nevertheless. The high number of seven dropouts in
the CPP-ACPF group out of a total of 10 suggests that taste may have been a reason for ceasing to participate in the study, although the reason given was always the time-consuming nature of the participation.

In conclusion, this randomized controlled clinical trial showed that there were no significant differences between the commercially available CPP-ACPF paste (MI Paste Plus®) and the fluoride-free control paste on the remineralisation of enamel WSL and plaque composition. This was found during a 3-month follow-up of orthodontic patients, immediately after debonding of fixed appliances. In both groups, limited changes in fluorescence loss were found 12 weeks after debonding, but a decrease in the percentage of aciduric bacteria and S. mutans in the plaque was detected over time.
REFERENCES


Long-term remineralising effect of MI Paste Plus® on the regression of early caries after orthodontic fixed appliance treatment; a randomized 12-month follow-up clinical trial

Published as:
ABSTRACT

Background
Casein-phosphopeptide-amorphous-calcium-fluoride-phosphate (CPP-ACPF) can remineralise subsurface lesions of enamel, so called white spot lesions (WSL). It is the active ingredient of MI Paste Plus®. The long-term remineralisation efficacy is unknown.

Objective
To evaluate the long-term effect of MI Paste Plus® versus a control paste on remineralisation of enamel after fixed orthodontic treatment over a 12-month period.

Design
This trial was designed as a prospective, double-blinded, placebo-controlled, randomised clinical trial.

Methods
Patients with subsurface lesions scheduled for removal of the appliance were included. They applied either MI Paste Plus® or control paste once a day at bedtime for 12 months, complementary to normal oral hygiene.

Main outcome measures
Changes in enamel lesions (primary outcome), fluorescence loss and lesion area were determined by quantitative light-induced fluorescence (QLF). Secondary outcomes were microbial composition (by conventional plating with petri dishes), and acidogenicity of plaque (by capillary ion analysis (CIA)), and lesion changes scored visually on clinical photographs.

Randomization
Participants [age = 15.5 years (SD = 1.6)] were randomly assigned to either the MI Paste Plus® or the control group, as determined by a computer-randomization scheme, created and locked before the start of the study. Participants received neutral-coloured concealed toothpaste tubes marked A or B.
Blinding
The patients and the observers were blinded with respect to the content of tube A or B.

Results
A total of 51 patients were analysed: MI Paste Plus® (n = 25) versus control group (n = 26) (14 patients of the original 65 patients dropped out). There was no significant difference between the groups over time for all the used outcome measures. There was a significant improvement in enamel lesions (fluorescence loss) over time in both groups (P < 0.001 and P < 0.001), with no differences between groups.

Limitations
Being an in vivo study, non-compliance of the subjects could have influenced the result.

Conclusion
The additional use of MI Paste Plus® in patients with subsurface enamel lesions after orthodontic fixed appliance treatment did not improve these lesions during the 1 year following debonding.

Registration
This trial is registered at the medical ethical committee of the VU Medical Centre in Amsterdam (NL.199226.029.07).
INTRODUCTION

Enamel subsurface lesions, so-called white spot lesions (WSL), can form rapidly around orthodontic brackets. These WSL are vulnerable to ongoing demineralisation (Gorelick et al., 1982; Mizrahi, 1983; Øgaard et al., 1988; Lovrov et al., 2007). Individuals with elevated levels of acidogenic bacteria in saliva and plaque are at high risk for the development of WSL (Scheie et al., 1984; Rosenbloom and Tinanoff, 1991; Ahn et al., 2007; Kim et al., 2010).

A product, MI Paste Plus® (Tooth Mousse Plus®), was developed to improve remineralisation. This product contains 900 p.p.m. fluoride with added calcium and phosphate, in a composition ideal for depositing fluorapatite into enamel (Reynolds, 1997; Cross et al., 2004; Reynolds, 2008). A crucial component of the product is the milk-derived protein casein phosphopeptide (CPP), which stabilizes amorphous calcium phosphate (ACP). This is converted to fluorapatite deposited in enamel by the available fluoride (Cross et al., 2004; Cochrane and Reynolds, 2012).

The efficacy of CPP-ACPF was demonstrated in vitro both for the prevention and for the regression of incipient lesions (Cochrane et al., 2008; Reynolds et al., 2008). However, there is a lack of reliable evidence of the efficacy of CPPACPF for the treatment of post-orthodontic WSL in vivo (Chen et al., 2013; Raphael and Blinkhorn, 2015). Also the long-term effect of this remineralising agent is unclear (Li et al., 2014).

In this prospective double-blinded randomized placebo-controlled superiority trial, we assessed the long-term (12 months) remineralisation effect of MI Paste Plus® on existing WSL immediately after fixed orthodontic appliance treatment in vivo, to be used in addition to normal oral hygiene. The primary outcome assessed by quantitative light-induced fluorescence (QLF) is fluorescence loss and lesion area. Secondary outcome was based on microbial composition by conventional plating and acidogenicity of plaque by capillary ion analysis (CIA). Additionally, lesion changes were assessed visually on clinical oral photographs.
The null hypothesis to be tested was that the *in vivo* use of commercially available MI Paste Plus®, in addition to normal oral hygiene, does not have an effect on:

1) The remineralisation of WSL over time,
2) Plaque composition assessed as total counts of colony-forming units (CFUs) and percentage of aciduric bacteria, *Streptococcus mutans*, *Lactobacillus* spp., and *Candida albicans*, as well as plaque acidogenicity over time, and
3) The visual changes in WSL over time in patients during 1 year after the removal of fixed orthodontic appliances.

**MATERIALS AND METHODS**

The medical ethical committee of the University Medical Centre of the Free University Amsterdam, The Netherlands, approved this study protocol (NL.199226.029.07). Treatment of WSL in former orthodontic patients was assessed directly post-debonding until 12 months thereafter. This study determined the long-term effect of MI Paste Plus® versus a control paste on caries lesion extent and microbial parameters.

**Trial design**

This trial was performed as a prospective, double-blinded, randomized, placebo-controlled superiority trial. The allocation of subjects followed a randomization scheme with stratification for gender. This resulted in an allocation ratio of 6:7 for MI Paste Plus® and control paste. No changes to the original protocol were made during or after the trial. It was initially intended to also assess microbial diversity by Denaturing Gradient Gel Electrophoresis (DGGE). Due to improvements in molecular biology techniques used (Alcaraz et al., 2012; Schulze-Schweifing et al., 2014; Beerens et al., 2017) as well as limitations experienced with DGGE (Beerens et al., 2017), this method was not utilized.

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Participants
Eligible subjects had been treated with orthodontic full fixed appliances in both arches at the Department of Orthodontics of ACTA. Subjects were enrolled after debonding and signing informed consent. All participants fulfilled the following requirements:
1) Healthy adolescent males or females between 12 and 19 years of age;
2) Two or more buccal WSL on former bracketed surfaces, seen without prolonged air drying as a distinct visual change in enamel and/or localized enamel breakdown without clinical visual signs of dentinal involvement [International Caries Detection and Assessment System (ICDAS) code 2];
3) No systemic diseases or syndromic abnormalities and
4) No proven or suspected milk protein allergy and/or sensitivity, or allergy to benzoate preservatives, as both are components of the MI Paste Plus® product.
Eligible subjects were invited to participate in this study and were screened by M.W.B. for WSL directly after debonding. The study group consisted of 65 participants: 28 male and 37 female subjects with a mean age of 15.5 years (SD = 1.6).

Study settings
This single center trial took place at the Department of Orthodontics, Academic Centre for Dentistry Amsterdam, The Netherlands from January 2008 to August 2010. Amsterdam is the capital of The Netherlands with a population of 756 000 at the time in 2009, having 156 000 children between the ages of 0 – 19 years (Centraal Bureau voor de Statistiek, 2009). There is a broad range in socioeconomic status for children undergoing orthodontic treatment, as orthodontics is mostly accessible for all children until the age of 18, as a result of the social health service structure. In Amsterdam, the community tap water is not fluoridated.

Randomization, intervention procedure, and blinding
Participants, complying with the inclusion criteria as determined by M.W.B., were randomly assigned by M.H.V. to group A (MI Paste Plus®) or B (the control group), as determined by a computer-randomization scheme, created and locked before the start of the study. Participant allocation was kept separate
Participants were instructed to use their respective paste once a day at bedtime after tooth brushing. Participants received verbal and written instructions on product use and oral hygiene procedures by a dental hygienist. They were advised how to brush properly using a normal toothpaste (i.e. at least twice a day, either with a hand toothbrush or an electric toothbrush for at least 2 minutes). No additional fluoride was to be applied. Participants were informed to apply at least a pea-size amount to the tooth surfaces in each arch using a clean, dry finger and keep the study product in the mouth for as long as possible. Participants were instructed not to rinse afterwards. Compliance was checked by questions regarding product use asked at each visit. Furthermore, participants were asked to bring their study paste to each visit. Prior to each study visit, they were asked to refrain from tooth brushing from the evening before the visit and from eating and drinking 2 hours prior to the visit. Each visit started with plaque sampling. After plaque sampling, the tooth surfaces were cleaned and polished for adequate viewing of WSL in the QLF and digital oral photographs.

The participants’ dentists were informed of their patients’ participation and were asked not to administer additional fluoride during this investigation. They were further asked to contact the study investigator if restorations were made on the buccal surfaces.

Subjects were informed that they would receive either the MI Paste Plus® paste or the control paste with a different form of calcium delivery. The patients and the observer were blinded with respect to the content of tube A or B. Examiners M.W.B., F.B., and MHV were also blinded.
Study procedure and outcomes

Plaque for microbial composition and acidogenicity was sampled before debonding (T0) and 6 weeks (T2), 3 months (T3), 6 and 12 months (T4, T5) after debonding. QLF photographs were taken after debonding (T1) and at 6 weeks (T2), 3 and 6 months (T3, T4), and 12 months after debonding (T5). Finally, clinical oral photographs were taken at T1 and T5. WSL severity as assessed by QLF was the primary outcome measure. Microbial composition, as determined by conventional plating and acidogenicity of plaque, was secondary outcome measures. Additionally, WSL changes were visually assessed on digital oral photographs.

Quantitative light-induced fluorescence

QLF images were captured using an intra-oral fluorescence camera (QLF/Clin; Inspektor Research Systems, Amsterdam, The Netherlands) with a dedicated software (Inspektor pro version 3.0.0.42; Inspektor Research Systems) as described by Beerens (Beerens et al., 2010). Images were assessed for fluorescence loss ($\Delta F$ [%]), lesion area ($A$ [mm$^2$]), and integrated fluorescence loss (IFL) ($\Delta F \times A$ [% x mm$^2$]).

Plaque processing

Plaque was sampled from the buccal surface of the lower right first or second premolar for microbial composition. Also, plaque was sampled from the buccal surface of the upper right and left first or second premolar for acidity of plaque, before and after sucrose pulse, respectively. Plaque samples were analysed blind with respect to subject number, visit, and group allocation. Microbial composition was determined by the total numbers of CFUs (counts/sample), and the proportions of acidic bacteria [% bacteria count/total count], S. mutans [% bacteria count/total count], Lactobacillus spp. [% bacteria count/total count], and the fungus C. albicans [% fungal count/total count] as described by Beerens (Beerens et al., 2010). The acidogenicity of plaque was analysed by means of capillary ion electrophoresis (Waters’ trade name: Capillary Ion Analysis, CIA [μmol acid/mg protein]) (Koopman et al., 2016). Calibration curves were made for each component separately. As internal standard, oxalate was included in all samples. To normalize the samples, the protein concentration of all samples was determined (Bradford, 1976).
Clinical oral photographs

For each subject and time point, pictures were collated in a photography gallery comprising four pictures per patient (Figure 1) and printed at high quality to examen and compare. These photographs were assessed by two calibrated examiners (F.B.) and (M.W.B.). Photographs were analysed in random order for subject and time using the International Caries Detection and Assessment System (ICDAS) criteria (Cai et al., 2003). The ICDAS code 1 (First Visual Change in Enamel, seen only after prolonged air drying) was not used due to the fact that no air drying was applied before photographs were made. The examiners assessed separately, and in case lesions were scored differently, a consensus was reached.

FIGURE 1. Example of a clinical oral photo gallery of clinical photos from one subject captured at T1.
Sample size

To assess the influence of casein phosphopeptide-amorphous calcium fluoride phosphate (CPP-ACPF) on the reduction of WSL, a power analysis was conducted as described by Beerens. (Beerens et al., 2010). Based on a previous observational study at the Orthodontic Department at ACTA (Boersma et al., 2005). A statistically significant, but clinically irrelevant, natural reduction in fluorescence loss, of 0.9 % (SD = 0.9 %) was found, during a 24 week time period. A clinically relevant change in fluorescence loss was considered to be an average reduction of 2 %, implying an effect size of 0.55. The sample size was calculated for a more conservative effect size of 0.35. For an effect size of 0.35 with a power of 0.9 to be measured between the two groups, a group size of 27 was needed (G*-power 3.1.0, ANOVA for repeated measures, between factors). Although orthodontic patients, in general, are seen at 4- to 6-week intervals during the active phase of treatment, during the retention phase, subjects often do not show up for their scheduled appointments. At the department of orthodontics at ACTA, this level of no shows is relatively high. To compensate for subject withdrawal, it was aimed to include 30 subjects in each group. Subjects who dropped out before T2 were replaced to meet the required minimum group size of 27.

Interim analysis

The 3-month data from this study were reported in December 2010 (Beerens et al., 2010). The trial was not stopped earlier than planned.

Data analysis

Statistical analysis was performed with SPSS (PASW statistics 21.0; SPSS Inc., Chicago, IL, USA). Change of enamel lesions, assessed by QLF, was the primary outcome measure. The average fluorescence loss for all WSL, total lesion area, and IFL were calculated for each subject and then normalized to 20 surfaces corrected for the number of missing and filled surfaces during the trial. Student’s (two-tailed) t-test was used to determine differences between both groups at baseline and follow-up time points. Lesion and microbial changes in time per subject were determined by repeated-measures ANOVA, followed by pairwise comparisons with Bonferroni correction. Visual lesion changes were assessed with clinical photographs as secondary outcome. The Mann–Whitney
U-test was used to determine differences between both groups at debonding and 1-year post-debonding. The intraclass correlation coefficients (ICCs) were calculated to determine intra- and inter-examiner agreement. Intra- and inter-examiner agreements for the QLF images were high (ICC = 0.93 M.W.B. intra; ICC = 0.87 M.W.B. with experienced examiner MHV).

Intra- and inter-examiner agreements for clinical photographs using ICDAS were calculated for examiner 1 (F.B.) and examiner 2 (M.W.B.): ICC = 0.65 (T1) and 0.73 (T5) F.B. intra; ICC = 0.66 (T1) and 0.72 (T5) M.W.B. intra; ICC = 0.71 (T1) and 0.73 (T5) F.B. with M.W.B..

The level of significance for all tests was set at 5%. Subjects with missing interim data were included. Data were supplemented by the average of the previous and following data point by assessing the means.

RESULTS

Eligible participants were recruited from January 2008 to August 2009. From the 184 screened participants, 65 were enrolled in the study and randomly assigned into two groups: the MI Paste Plus® group (group A; n = 35) and the control group (group B; n = 30). All participants received intended treatment. Inclusion of participants stopped when at least 30 subjects were enrolled in each group and seen at the 6-week visit.

A flow diagram, from enrolment and group allocation to study conclusion, is shown in Figure 2. A total of 14 patients dropped out between T0 and T5, 10 from the MI Paste Plus® group and 4 from the control group. Reasons given for withdrawal were the time-consuming nature of the study or a shift of patient’s priority. Furthermore, exclusion after randomization occurred for one participant from the MI Paste Plus® group where WSL were detected prior to debonding, but who was found to be WSL free after debonding.

Fifty-one participants (27 males and 24 females; mean age ± SD, 15.32 ± 1.6 years) completed the study. A total of 25 participants were analysed in the MI Paste Plus® group versus 26 in the control group. In Table 1, an overview of baseline data is given per intervention. No significant differences were found with respect to gender ratio, age of the participants, treatment duration, the number of decayed, missing (due to caries) and filled surfaces of permanent teeth (DMFS), and bleeding on probing. Electric- and manual-brushing methods were distributed similar over the groups at baseline.

U-test was used to determine differences between both groups at debonding and 1-year post-debonding. The intraclass correlation coefficients (ICCs) were calculated to determine intra- and inter-examiner agreement. Intra- and inter-examiner agreements for the QLF images were high (ICC = 0.93 M.W.B. intra; ICC = 0.87 M.W.B. with experienced examiner MHV).

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Assessed for eligibility (n = 184)

Enrolment (n = 65)

Randomization

Allocated to intervention Group A (n = 35)
Received allocated intervention (n = 35)

Lost to follow-up (n = 10)
Not present at visit (n = 7)
Decided not to participate any further (n = 3)

Discontinued intervention due to Non-compliance (n = 6) though included

Analyzed (n = 25)
Excluded from analysis (n = 1)
No lesions detected

Analysis

Allocated to intervention Group B (n = 30)
Received allocated intervention (n = 30)

Lost to follow-up (n = 4)
Not present at visit (n = 1)
Decided not to participate any further (n = 3)

Analyzed (n = 26)
Excluded from analysis (n = 0)
No lesions detected

Excluded (n = 119)
Not meeting inclusion criteria:
- At screening < 2 WSL (n = 98)
- Caries profunda (n = 5)
- To old of age (n = 2)
- Partially MB appliance (n = 3)
- Caries profunda (n = 5)
- Systemic disease (n = 1)
- Refused to participate (n = 2)
- Data at screening incomplete (n = 1)

Not enrolled at visit (n = 7)
Decided not to participate any further (n = 3)

Lost to follow-up (n = 4)
Not present at visit (n = 1)
Decided not to participate any further (n = 3)

Discontinued intervention due to Non-compliance (n = 6) though included

Analyzed (n = 26)
Excluded from analysis (n = 1)
No lesions detected

Analysis

Excluded (n = 1)
No lesions detected

FIGURE 2. Flow of participants through the study. Group A received the MI Paste Plus® product paste and Group B received the control paste.
However, brushing methods during 1-year follow-up were frequently changed and often used alternated. A total of 403 caries-affected surfaces in 942 elements were followed up throughout the investigation. The affected elements were distributed as follows: 14.3% central incisors, 22.8% lateral incisors, 29.1% cusps, and 33.8% premolars. This distribution was similar for the two groups. Overall compliance in the study was moderate. Questions regarding frequency of brushing and product use revealed that, during the first 6 weeks of the study, the subjects generally brushed twice a day and used the product at night-time after brushing. Between weeks 6 and 12 months, the subjects forgot to brush and to use the product on average once a week, and this always occurred at night-time. Assessment of product use via returned product failed because none of the subjects returned their product tubes at recall visits, despite our request.

**TABLE 1.** Baseline data, showing no statistical differences between the groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MI Paste Plus® (A)</th>
<th>Control (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allocated to intervention group</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Gender Ratio M:V (%male)</td>
<td>16:19 (45.7%)</td>
<td>12:18 (40.0%)</td>
</tr>
<tr>
<td>Mean participant age (years)</td>
<td>15y8m</td>
<td>15y3m</td>
</tr>
<tr>
<td>Fixed appliances (FA) duration</td>
<td>2y5m</td>
<td>2y3m</td>
</tr>
<tr>
<td>DMFS</td>
<td>2.09</td>
<td>2.07</td>
</tr>
<tr>
<td>Bleeding on probing (%)</td>
<td>36</td>
<td>33</td>
</tr>
</tbody>
</table>

Lesion changes assessed by QLF

Arresting or reduction of extent of enamel lesions, by QLF, was the primary outcome regarding effectiveness of MI Paste Plus® (Table 2). No significant differences between the groups were found at baseline (T1) for lesion area (A), lesion depth (ΔF), and IFL (t-test independent groups, P > 0.05). Repeated-measures ANOVA showed no significant changes in lesion area (A) over time or between the groups.

Lesion changes assessed by QLF

Arresting or reduction of extent of enamel lesions, by QLF, was the primary outcome regarding effectiveness of MI Paste Plus® (Table 2). No significant differences between the groups were found at baseline (T1) for lesion area (A), lesion depth (ΔF), and IFL (t-test independent groups, P > 0.05). Repeated-measures ANOVA showed no significant changes in lesion area (A) over time or between the groups.
### TABLE 2. Caries regression, determined by assessment of lesion area (A), fluorescence loss (∆F), and integrated fluorescence loss (IFL) at five different time points (in the MI Paste Plus® group and the control group. (T1 = directly after debonding, T2 = 6 weeks, T3 = 3 months, T4 = 6 months, and T5 = 12 months after debonding)

<table>
<thead>
<tr>
<th></th>
<th>MI Paste Plus®, N = 25</th>
<th>Control, N = 26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
</tr>
<tr>
<td>lesion size (A [mm²])</td>
<td>4.65 ± 5.66</td>
<td>4.57 ± 6.88</td>
</tr>
<tr>
<td>Fluorescence loss (delta F [%])</td>
<td>-8.07 ± 1.39</td>
<td>-7.57 ± 1.72</td>
</tr>
<tr>
<td>Integrated fluorescence loss (delta F × A [% × mm²])</td>
<td>-44.63 ± 68.11</td>
<td>-47.06 ± 86.43</td>
</tr>
</tbody>
</table>

Data are given as mean ± SD.  
*Data significant different from baseline.  
**Data significant different from T2.
A repeated-measures ANOVA with a Greenhouse–Geisser correction showed a significant improvement in fluorescence loss (ΔF) over time [F(3.014, 31.346) = 17.155, P < 0.001; for MI Paste Plus® group, F (2.659, 19.705) = 11.533, P < 0.001; and for the control group F (3.097, 14.120) = 6.757, P < 0.001], but no significant differences between the two groups. Multiple comparisons with baseline using Bonferroni correction showed significant differences of the fluorescence loss (ΔF) in the MI Paste Plus® group from baseline to T4 (P = 0.021) and T5 (P < 0.001) and from T2 to T5 (P = 0.002) and the control group from baseline to T5 (P = 0.002). Repeated-measures ANOVA showed no significant changes in the IFL over time or between the groups. In both groups, a trend of improvement in IFL was seen.

Microbial composition of plaque

Microbial composition of plaque (secondary outcome) described by CFUs (counts/sample), proportions of acidic bacteria [% bacteria count/total count], S. mutans [% bacteria count/total count], Lactobacillus spp. [% bacteria count/total count], and the fungus C. albicans [% fungi count/total count] is given in Table 3.

At T0 (baseline for microbial composition), no significant differences between groups were found (P > 0.05). Five participants in the control group were excluded from the analysis due to missing data at T5 (1 year). Of these five participants no plaque was present on the surface in four cases. In one case, the sample was lost.

Total CFUs did not change significantly over time and were not different between the groups (repeated-measures ANOVA, P > 0.05). Repeated-measures ANOVA with a Greenhouse-Geisser correction showed a significant reduction in percentage of acidic bacteria over time in the MI Paste Plus® but not in the control group (MI Paste Plus® group F (2.700, 2829.398) = 2.916, P < 0.047; control group F (2.763, 3149.550) = 1.853, P > 0.05). No significant differences between the groups (P > 0.05) were found. Multiple comparisons with baseline using Bonferroni correction showed significant differences in the reduction in percentage of acidic bacteria from baseline to T5 in the MI Paste Plus® group (Table 3). A similar trend was seen in the control group, although not significant over time.

Repeated-measures ANOVA with Greenhouse–Geisser correction showed no significant changes in the the reduction in percentage of S. mutans over time in the MI Paste Plus® group from baseline to T4 (2.700, 2829.398) = 2.916, P < 0.047; control group F (2.763, 3149.550) = 1.853, P > 0.05). No significant differences between the groups (P > 0.05) were found. Multiple comparisons with baseline using Bonferroni correction showed significant differences in the reduction in percentage of aciduric bacteria from baseline to T5 in the MI Paste Plus® group (Table 3). A similar trend was seen in the control group, although not significant over time.

Repeated-measures ANOVA with Greenhouse–Geisser correction showed no significant changes in the the reduction in percentage of aciduric bacteria from baseline to T5 in the MI Paste Plus® group (Table 3). A similar trend was seen in the control group, although not significant over time.
for the MI Paste Plus® group, but significant changes in time were found for the control group (MI Paste Plus® group F(3.048, 229.581)= 1.728, P > 0.05; control group F(2.347, 625.465)= 3.236, P = 0.039). No significant differences between the groups (P > 0.05) were found. The reduction in percentage of Lactobacillus spp. and C. albicans did not change significantly over time or between the groups (repeated-measures ANOVA with Bonferroni correction, P > 0.05).

### TABLE 3. Microbial composition, determined by total bacterial counts, the proportions of aciduric bacteria, Streptococcus mutans spp., Lactobacillus spp. and Candida Albicans, at five different time points in the MI Paste Plus® group and the control group. (T0= before debonding, T2= 6 weeks, T3= 3 months, T4= 6 months, and T5= 12 months after debonding)

<table>
<thead>
<tr>
<th></th>
<th>MI Paste Plus®, N = 25</th>
<th>T0</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony-forming units (CFUs) (10^7) counts/sample</td>
<td>5.4 ± 6.3</td>
<td>4.3 ± 4.8</td>
<td>4.3 ± 4.4</td>
<td>4.2 ± 5.2</td>
<td>5.5 ± 4.3</td>
<td></td>
</tr>
<tr>
<td>Aciduric bacteria (% bacteria count/total count)</td>
<td>53.2 ± 33.5</td>
<td>47.2 ± 31.7</td>
<td>46.4 ± 27.8</td>
<td>32.9 ± 27.1</td>
<td>29.3 ± 19.2</td>
<td></td>
</tr>
<tr>
<td>Streptococcus mutans (% bacteria count/total count)</td>
<td>9.8 ± 14.1</td>
<td>3.9 ± 7.4</td>
<td>8.6 ± 10.5</td>
<td>4.7 ± 9.6</td>
<td>8.6 ± 13.6</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus spp. (% bacteria count/total count)</td>
<td>0.2 ± 0.5</td>
<td>0.0 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td>0.0 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>Candida Albicans (% fungi count/total count)</td>
<td>1.0 ± 2.2</td>
<td>0.5 ± 1.9</td>
<td>0.7 ± 1.8</td>
<td>0.2 ± 0.9</td>
<td>1.0 ± 4.4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Control, N = 26</th>
<th>T0</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony-forming units (CFUs) (10^7) counts/sample</td>
<td>3.4 ± 3.2</td>
<td>3.6 ± 3.6</td>
<td>5.1 ± 4.6</td>
<td>8.8 ± 2.7</td>
<td>6.0 ± 9.5</td>
<td></td>
</tr>
<tr>
<td>Aciduric bacteria (% bacteria count/total count)</td>
<td>49.2 ± 49.4</td>
<td>48.0 ± 38.4</td>
<td>32.2 ± 25.3</td>
<td>34.4 ± 27.6</td>
<td>31.3 ± 21.2</td>
<td></td>
</tr>
<tr>
<td>Streptococcus mutans (% bacteria count/total count)</td>
<td>12.2 ± 19.5</td>
<td>4.3 ± 7.9</td>
<td>4.2 ± 9.2</td>
<td>10.8 ± 16.5</td>
<td>5.9 ± 9.0</td>
<td></td>
</tr>
<tr>
<td>Lactobacillus spp. (% bacteria count/total count)</td>
<td>0.1 ± 0.2</td>
<td>0.4 ± 1.8</td>
<td>0.3 ± 0.9</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td></td>
</tr>
<tr>
<td>Candida Albicans (% fungi count/total count)</td>
<td>5.7 ± 15.2</td>
<td>11.5 ± 57.2</td>
<td>2.0 ± 7.6</td>
<td>0.9 ± 2.0</td>
<td>0.2 ± 1.0</td>
<td></td>
</tr>
</tbody>
</table>

Bacterial counts are expressed as a percentage of the total counts per sample obtained at each time point.
Data are given as mean ± SD.
*Data significantly different from baseline.
Acidogenicity of plaque

Acidogenicity of plaque (secondary outcome) was determined as the amount [µmol acid/mg protein] of formate, succinate, acetate, lactate, propionate, butyrate, and phosphate in resting plaque and after 10 minutes of sucrose rinse (Supplementary Figure S).

No significant differences between the groups were found for any acid at baseline. Phosphate was significantly lower in the MI Paste Plus® group in comparison with the control group at baseline (MI Paste Plus® = 0.40, SD = 0.21; control group = 0.57, SD = 0.34, P = 0.04).

No significant differences in acid and phosphate composition of resting plaque or after sucrose pulse were seen in time or between the groups (repeated-measures ANOVA, P > 0.05).

Lesion changes assessed by clinical oral photographs

Changes in enamel lesions between T1 and T5 (secondary outcome) assessed on the clinical photographs (Figure 1) are shown in Table 4.

Three participants were excluded, one in the MI Paste Plus® and two in the control group. These participants had an incomplete photograph gallery at T5.

No significant differences were found between the groups at baseline (Mann-Whitney U; mean U = 79 980, z = –4.54, P = 0.001), showing less visible lesions in the MI Paste Plus® group than in the control group. Most of the surfaces were scored 0 for both the MI Paste Plus® and the control group at T1.

There was no significant difference between the groups over time. Lesions that scored 2 essentially did not change over time. One lesion was given an ICDAS score of 3 on the photograph gallery. This lesion was assessed as ICDAS score 2 clinically. The lesion in the control group that scored 3 at T1 and 0 at T5 has been restored with a filling and appeared undetectable. The lesions that scored 0 at T1 and 2 at T5 are presumably lesions that appeared after gingiva reduction.
SUPPLEMENTARY FIGURE S. Graph A,B,C and D: Acidity of plaque, determined by the amount of formate, succinate, acetate, lactate, phosphate, propionate, butyrate at five different time-points (T0, T2, T3, T4 and T5) in the MI Paste Plus® group and the control group before and after sucrose pulse. Plaque acidities are expressed as µmol acid per milligram protein obtained at each time-point.
TABLE 4. Enamel change, determined by International Caries Detection and Assessment System (ICDAS) at the time points of debond, blinded assessed at baseline (T1) and 12 months (T5) thereafter, in the MI Paste Plus® group and the control group.

<table>
<thead>
<tr>
<th>MI Paste Plus® n = 24</th>
<th>ICDAS score at T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICDAS score at T1</td>
<td>0 2 3 5 Total T1</td>
</tr>
<tr>
<td>0</td>
<td>229 21</td>
</tr>
<tr>
<td>2</td>
<td>87 102 3</td>
</tr>
<tr>
<td>3</td>
<td>1 2 3</td>
</tr>
<tr>
<td>Total T5</td>
<td>316 123 4 1 444</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control n = 24</th>
<th>ICDAS score at T5</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICDAS score at T1</td>
<td>0 2 3 5 Total T1</td>
</tr>
<tr>
<td>0</td>
<td>199 29</td>
</tr>
<tr>
<td>2</td>
<td>66 108 21</td>
</tr>
<tr>
<td>3</td>
<td>1 1 1</td>
</tr>
<tr>
<td>Total T5</td>
<td>266 138 22</td>
</tr>
</tbody>
</table>

Missing data n = 3. Data are given as amount counted at the two different time points.
*This lesion has been restored and was scored 0 at T5.

Adverse effect
There was no harm experienced by the participants influencing their general health. However, in the MI Paste Plus® group, a total of five patients had the assumption that their teeth gradually discoloured to a more yellow tone. These findings were considered incidental. No objective measures were used to test this possibly adverse effect; however, it was observed on the digital photographs of several patients in the MI Paste Plus® group.

OVERALL CONCLUSION
The use of MI Paste Plus® in orthodontic patients with subsurface enamel lesions (WSL) did not improve these lesions over the 1-year period. This was evaluated by QLF imaging, microbiological composition and acidogenicity, as well as by digital oral photographs.

The plaque composition, regarding bacterial counts, the proportions of aciduric bacteria,
S. mutans spp., Lactobacillus spp., and C. albicans, showed no change to a more healthier composition, observed for both groups. MI Paste Plus® did not have an effect on the visual changes of WSL on the long term, when assessed on photographs. Lesions remained visible over time.

**DISCUSSION**

**Key findings**

This study is the first to address the efficacy of MMP for the treatment of post-orthodontic WSL in vivo during 1 year following debonding. A lack of positive evidence was found to support the effectiveness of MI Paste Plus® as a remineralising agent, to be effective for the treatment of post-orthodontic WSL. This outcome was confirmed by several independent detection methods, which strengthens this conclusion.

**Explanation**

MI Paste Plus® does not have a positive effect on WSL improvement seen by QLF imaging or optical assessment nor does it have a neutralizing effect on the bacterial oral flora. Regardless of the application of the product or control, lesions tended to improve after removing orthodontic fixed appliance. Similarly, removing the orthodontic fixed appliance had a positive effect on the composition and acidity of the bacteria on the long term, which was not affected by either product.

**Comparing these findings with other studies**

Although the efficacy of CPP-ACPF for the prevention and regression of incipient lesions has been demonstrated in vitro (Cochrane et al., 2008; Reynolds et al., 2008), there is a lack of reliable evidence for the treatment of post-orthodontic WSL in vivo (Chen et al., 2013; Raphael and Blinkhorn, 2015) and the long-term effect of this remineralising agent is unclear (Li et al., 2014). This study is the first to address these aspects. In vitro (Reynolds, 1997; Cochrane et al., 2008) and in situ studies (Cai et al., 2003; Morgan et al., 2008; Reynolds et al., 2008) have demonstrated that CPP-ACP may promote the remineralisation of subsurface enamel lesions. These findings are summarized in a meta-analysis for in vitro and in situ studies regarding the effect of CPP-ACP as a caries-preventive agent.

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(Bailey et al., 2009). When evaluating in vivo studies, Chen (Chen et al., 2013) reported a lack of reliable evidence to support remineralising agents for the treatment of post-orthodontic WSL. A systematic review described by Li (Li et al., 2014), reported the same conclusion, although, in this systematic research, the effect of CPP-ACPF was assessed for orthodontic and non-orthodontic lesions. Our findings contradict the findings of Bailey (Bailey et al., 2009) and Bröchner (Bröchner et al., 2011) who reported a positive effect of casein supplements after only 12 and 4 weeks, respectively. Bailey concluded a positive effect within 12 weeks although no statistical differences were found using ICDAS code 2. Their conclusion was based on visual assessment of lesion activity or inactivity. Bröchner reported a reduction in lesion area of 58 % after 4 weeks. However, the lesions investigated were very small (0.19 mm²). One may debate clinical relevancy. Andersson (Andersson et al., 2007) compared the effects of CPP-ACP with fluoride mouthwashes on the regression of WSL and concluded that both regimens could promote regression of WSL after debonding of fixed orthodontic appliances, though the visual evaluation suggested an aesthetically more favourable outcome of the CPP-ACP.

Strength and limitations of this study

The study was performed in a general population of teenagers in Amsterdam, The Netherlands. The Netherlands is part of Western Europe and has no water fluoridation. Therefore, water fluoridation did not affect the outcome of this study. WSL developed during orthodontic treatment appear more rapidly and are more porous than WSL in non-orthodontic patients. As a result, the findings of this study are only applicable to WSL developed during orthodontic treatment. The efficacy of this remineralisation agent on WSL after orthodontic treatment with full fixed appliances was not influenced by background levels of fluoride. As for all randomized clinical trials, non-compliance of the subject could have influenced the result. The assessment of product use via returned product failed because none of the subjects returned their product tubes at recall visits. Also, we did not use an application tray, for example, a removable clear retainer to improve the cream to stay in place. Though by not using an application tray, saliva could now also influence possible remineralisation. One of the possible limitations influencing the results of the study is the preservation of CPP-ACPF in MI Paste Plus®. This could be the explanation for

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the positive results found in vitro and in situ and contradicts the findings of in vivo study results.

The question can also be raised if there was a similarity of intervention. As there might be a taste difference between the two products. Cross contamination is not to be expected as no siblings were included. In this study 27 participants per group was aimed for, as was assessed as the effect size. Unfortunately, due to drop out, it became lower resulting in 25 to 26 per group. The used power was 0.9. If using the power of 0.8, at least 20 participants should have been included. So, it is still acceptable to draw conclusions. The effect found is so small that, though statistically significant, it is still not clinically relevant.

Implications
The use of MI Paste Plus® in patients with subsurface enamel lesions after orthodontic fixed appliance treatment does not show an additional superior improvement of these lesions on the long term as measured by means of QLF imaging, microbiological composition and its acidity, as well as by digital oral photographs. This suggests that there is no clinical evidence to support that MI Paste Plus® is a remineralisation agent because it is not effective to improve post-orthodontic subsurface lesions.

REGISTRATION
This trial is registered in The Netherlands at Amsterdam Free University of the University Medical Centre medical ethical committee under number NL.199226.029.07.
GC Benelux, Leuven, Belgium provided free supplies of MI Paste Plus® used in the study. None of these authors or study received personnel or consulting payments or any other form of personal benefit from GC Benelux.

TRIAL PROTOCOL
Full details of the trial protocol NL.199226.029.07 are available on request.
REFERENCES


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 GENERAL DISCUSSION

Orthodontics is very popular for restoring not only function, but also facial aesthetics. Especially in high income countries, orthodontic treatment with fixed appliances is frequently applied (Ren et al., 2014). A downside of these fixed appliances is that they impede effective plaque removal. Poor dental hygiene procedures combined with a high-frequency carbohydrate diet, lead to colonisation of aciduric bacteria. This may contribute to the occurrence of dental caries (Øgaard et al., 1988). Although the caries prevalence and severity has declined over the last four decades, especially in high income countries (Frencken et al., 2017), the growing demand for orthodontic treatment and a high occurrence of oral biofilm-related complications, orthodontic treatment might become a public health threat (Ren et al., 2014).

Early dental caries lesions, so called white spot lesions (WSL) occur especially at the gingival margins and at the bracket-adhesive enamel junction (van der Veen et al., 2007). The management of these WSL is of clinical importance, also for aesthetic reasons (Murphy et al., 2007).

Risk factors and risk assessment

Risk factors for caries that have been reported are: Young age at the start of the treatment, inadequate oral hygiene before the beginning of the treatment and during the treatment, inappropriate diet (high frequency of fermentable carbohydrates in food and beverages), history of recent carious lesions or a high number of decayed missing and filled surfaces (DMFS) and duration of treatment (Chapman et al., 2010; Khalaf, 2014). Poor oral hygiene seems to pose the highest risk in developing WSL (Khalaf, 2014).

Poor oral hygiene not only increases the amount of biofilm but also the prevalence of cariogenic bacteria, such as Streptococcus mutans and Lactobacillus spp. (Al Mulla et al., 2009). By comparing patients with and without WSL by determining characteristics of the dental biofilm, a caries risk profile may be assessed. This thesis concludes, however, that microbiological composition shows a high inter-individual variation between patients with and without orthodontic WSL. This was assessed by two methods: Conventional microbiological plating and DGGE. Therefore, differences in microbiological ecology do not have a predictive value for caries risk assessment.

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CHAPTER 6

Monitoring WSL over time

Orthodontic treatment with fixed appliances is a risk factor for developing WSL (Øgaard et al., 1988). Key to prevention and management of WSL is finding a monitoring method for WSL formation and severity. Lesion assessment is defined as the method of determining whether or not caries is present and to characterise or monitor a lesion, once it has been detected (Gomez, 2015). The conventional method for caries assessment is visual inspection. The International Caries Detection and Assessment System (ICDAS) is the current standard in visual inspection (Ismail et al., 2007). The technique discriminates various stages of disease on an ordinal scale, from the first visual change in the enamel to frank caries lesions involving the pulp. The ICDAS scoring system was shown to be an accurate and reproducible method to detect early lesions and also to detect changes during longitudinal follow-up (Ferreira Zandona et al., 2010). However, clinical lesions monitoring by ICDAS scoring on smooth surfaces did not prove to be an accurate method (Guedes et al., 2014).

In the past years, quantitative (objective) methods for detecting and monitoring caries lesions that assess caries severity on a continuous scale have been introduced (ten Bosch and Angmar-Mansson, 2000). Quantitative light induced fluorescence (QLF) is such a potential tool for the detection of early caries lesions and for monitoring preventive interventions (Gomez, 2015). This method was used for the monitoring of WSL in this thesis. However, QLF is not yet available in clinical practices as most orthodontists have a photo camera for documenting treatment outcomes on clinical oral photographs. The use of clinical oral photographs to monitor WSL in time was studied in this thesis. The findings show that visual comparison of photographs taken in time provides discriminatory power to reveal changes in WSL. The use of ICDAS assessment on these clinical oral photographs did not have sufficient discriminatory power. Monitoring WSL by comparing time series of clinical oral photographs side-by side is useful for clinical decision making in the management of WSL. An internationally accepted assessment method for monitoring WSL over time should be agreed upon.

Preventive measures during orthodontic treatment

To prevent development of WSL, orthodontists should judge each patient’s caries risk before and during treatment, given that maintaining adequate oral

Preventive measures during orthodontic treatment

To prevent development of WSL, orthodontists should judge each patient’s caries risk before and during treatment, given that maintaining adequate oral
hygiene during orthodontic treatment is difficult. Additional measures may be needed when self-care needs to be enforced. Efforts to prevent WSL should be taken jointly by orthodontists, dentists, dental hygienists, the patients, and their parents/caretakers.

Based on scientific evidence, fluoride has a beneficial effect for the prevention of WSL (Benson et al., 2013). In case of good compliance (Geiger et al., 1988), daily home use of fluoride mouth rinses in addition to brushing with fluoride toothpaste can reduce the occurrence and severity of WSL (Benson et al., 2013). A type of treatment not requiring patients compliance is the application of high concentration fluoride varnishes; this reduces the formation of WSL and decreases WSL depth during orthodontic treatment (Stecksen-Blicks et al., 2007). The suggested interval of in-office fluoride varnish application varies from every six weeks to twice a year depending on the caries risk of the patient. The agent is advised for moderate to high risk patients. Unfortunately, a clinical guideline based on the available scientific evidence is lacking.

**Treatment of existing white spot lesions**

When prevention has failed, and WSL have formed, effort should be made to prevent further caries decay. Removing the fixed appliances results in less stagnation areas for plaque biofilm accumulation and results in better self-cleaning by the oral musculature and saliva.

Treatment of white spot lesions can be specified for four phases after bracket removal:

1) Natural remineralisation,
2) Camouflage,
3) Micro abrasion, and finally
4) Restorative treatment.

The first phase is called secondary prevention and consists of control and non-invasive care methods to facilitate remineralisation of existing active WSL. During this phase, the first few months after debonding, a natural regression of WSL often occurs. Active WSL have a better prognosis for recovery when hypermineralisation of the outer surface has not occurred yet. Therefore, high doses of fluoride must not be used to prevent hypermineralisation from occurring. Remineralisation products should enhance remineralisation without blocking the surface layer. CPP-ACPF products have been advocated...
as remineralisation enhancing agents. However, the commercially available MI Paste Plus®, with CPP-ACP as active ingredient, did not show this intended effect as was concluded in this thesis. After natural remineralisation has been given time to occur, a second step is to camouflage the inactive WSL by external bleaching. Inactive WSL are visible as permanent scars and vulnerable to staining (Sonesson et al., 2017). External bleaching may camouflage such WSL (Knösel et al., 2007). External bleaching, however, increases caries susceptibility in patients that have poor oral hygiene (Fliatz and Hicks, 1996).

If bleaching is not conclusive or satisfying to the patient, acid micro-abrasion (Ardu et al., 2007) or micro abrasion with pumice powder (Akin and Basciftci, 2012) will provide aesthetical improvement. Micro-abrasion aims to remove the hypermineralised surface layer. Resin infiltration (Icon technique) (Knösel et al., 2007; Senestraro et al., 2013) may be considered combined with micro abrasion (Abdelaziz et al., 2016). The Icon technique provides an immediate improved esthetical appearance of the WSL compared with untreated lesions. A downside of resin is that this material may discolor over time (Ceci et al., 2017). The last phase is restorative treatment, by applying resin fillings or even veneers. During the retention phase, directly after orthodontic bracket removal, it is appropriate for the orthodontist to control the first phase. Secondary steps and beyond should be left to the dentist.

Future perspective
In general, fluoride is effective in reducing enamel caries decay (Feyerskov and Kidd, 2008). The prevention of WSL is considered crucial. Despite available scientific evidence to prevent WSL with fluoride, clinical guidelines have not been formulated until now. Orthodontists and other dental health professionals should formulate and accept guidelines in their treatment strategies for orthodontic patients having WSL. Also the social health reimbursement system should include preventive measures in their tariff rates to prevent WSL during orthodontic treatment (Kerbusch et al., 2010).

If preventive measures are not effective and WSL appear, promoting remineralisation should be the preferred approach for active WSL that have
not already been remineralised. Applying high doses of fluoride at this stage will only remineralise the outer layer and will preserve the porosities (and whiteness) of the lesion, increasing the risk of staining.

Unfortunately, minimal invasive treatment strategies to promote remineralisation of existing WSL have not been proven *in vivo* until now. Further research should focus on this specific tool in caries management. Future research may look into ways to positively influence biofilm factors (a healthy microbial ecology of dental plaque) and to restore lesions with calcium and phosphate ions forming structures that simulate unaffected enamel. Novel materials that aim to remineralise lesions or affect the biofilm should be tested in their commercially available form. For this, *in vitro* and *in situ* models provide the potential effectiveness of a product available to patients, but confirmation should be obtained from *in vivo* studies. Looking into the application method of a product, in-office use is preferred, as compliance might have an influence during *in vivo* research. To overcome this significant problem, future product evaluation should focus on in-office application forms so that the true effect of the product can be measured without the detrimental effects of non-compliance.
CONCLUSIONS

1. MI Paste Plus®, a remineralisation agent, does not show its intended effect in patients who have white spot lesions (WSL) after orthodontic treatment with full fixed appliances.
2. WSL tend to regress over time but do not disappear after removal of the appliances.
3. After bracket removal the microbial composition gradually changes towards a more healthy composition. This is a gradual change that does not occur immediately after bracket removal.
4. The ICDAS scoring system on clinical oral photographs does not have enough discriminatory power to address changes in WSL over time.
5. Comparing respective clinical oral photographs taken over time provides discriminatory power in assessing changes in WSL severity. This method of monitoring WSL over time is useful for clinical decision making in the management of WSL.
6. Quantitative light induced fluorescence (QLF) is confirmed as a useful outcome measure in detecting and monitoring WSL over time.
7. The DGGE-banding pattern software, Gelcompare-II is susceptible to bias when used with the provided settings.
8. Denaturing Gradient Gel Electrophoresis (DGGE) and analysis of microbial composition does not have a predictive value for caries risk assessment of the formation of WSL in orthodontic patients treated with full fixed appliances.
9. Conventional microbiological plating, analysing bacteria counts, percentage of aciduric flora, *S. Mutans*, *Lactobacillus* spp. and *Candida Albicans* is not predictive for caries risk assessment of the formation of WSL in orthodontic patients treated with full fixed appliances.
10. This thesis describes the first randomised control trial assessing the effectiveness of MI Paste Plus® *in vivo* for the treatment of WSL after orthodontic fixed appliance on the long term (12 months) and stipulates the importance of combining independent outcome measures in research.
REFERENCES


SUMMARY (ENG)

Chapter 1
General introduction of the thesis
This thesis describes the efficacy of a remineralisation agent called MI Paste Plus® for the remineralisation of early caries decay or so called white spot lesions (WSL) after orthodontic treatment with fixed appliances. Orthodontic fixed appliances often result in the formation and progression of WSL. Limited access of saliva and inadequate oral hygiene procedures allow undisturbed and prolonged contact between plaque and the bracketed tooth surface, which predisposes the respective site to being caries susceptible.

While the strategies in the primary prevention of WSL are nowadays evidence based, our knowledge about control and atraumatic care of existing WSL, the secondary prevention, is limited.

Treatment of existing WSL that have been formed during orthodontic treatment should prevent hypermineralisation of the surface. This hypermineralised layer inhibits the ion movement to the subsurface and prevents mineralisation in the deeper layer. This results in the maintaining of the whiteness of the lesions. Therefore we have studied a minimal intervention strategy based on CPP-ACPF technology. This agent provides bioavailable calcium and phosphate into saliva and plaque enabling to drive remineralisation within the deeper layer of the WSL. MI Paste Plus® is a commercially available remineralisation agent, based on this technology, claiming to remineralise WSL.

We have used a combination of several independent outcome measures, which could be informative to assess caries risk and severity.

In this thesis a single centre prospective double blind randomised placebo controlled trial is described. This trial was conducted at the Department of Orthodontics, Academic Centre of Dentistry Amsterdam, the Netherlands from January 2008 to August 2010. A 12 months evaluation and the use of several independent detection methods strengthens the conclusions drawn.
Chapter 2

White spot lesions after orthodontic treatment assessed by clinical photographs and by quantitative light-induced fluorescence imaging: a retrospective study.

Standardised monitoring of the severity of WSL over time is useful for clinical decision making in the management of WSL. This retrospective study evaluates and uses standardized monitoring methods to assess changes in WSL in post-treatment orthodontic patients over a 12 months period. Two methods were compared:
1. Routinely taken clinical oral photographs were analysed by both scoring visual changes and International Caries Detection and Assessment System (ICDAS)-criteria; and
2. QLF images analysed by computer software.

Fifty-one subjects were included and photographs taken with either method were recorded directly after debonding (T1) and one year later (T2). The oral photographs from both time points were assessed independently by ICDAS and by comparing the clinical oral photographs taken over time to assess visual transition (VT). The QLF images were categorized based on the integrated fluorescence loss at the two time points (T1 and T2).

A total of 918 formerly bracketed surfaces were analysed. ICDAS scoring on oral photographs detected 433 lesions while QLF imaging detected 384 lesions. The oral photographs and QLF images both revealed improvement of the WSL over the 12 month period.

If a lesion was detected, an ICDAS score 2 was scored most often at both points in time. Changes were seen but the ICDAS scoring system was not sensitive enough to reveal the changes in WSL over time, in other words ICDAS assessment on clinical oral photographs did not have sufficient discriminatory power to reveal changes in WSL. Contrarily, comparing respective clinical oral photographs taken over time provides discriminatory power in assessing changes in WSL severity. This method of monitoring WSL over time is useful for clinical decision making in the management of WSL.
Chapter 3
The use of Denaturing Gradient Gel Electrophoreses and conventional microbiology as indicator of white spot lesions in orthodontic patients; a cross-sectional study.

Predicting caries risk in patients undergoing treatment with fixed appliances would be valuable with respect to WSL formation. Denaturing Gradient Gel Electrophoresis (DGGE) and microbial composition have been suggested to predict caries risk in young children. In the current study the predictive value of these two methods was assessed in patients with full fixed appliances. Dental plaque samples of patients with and without WSL were mutually compared. Dental plaque was obtained from 37 patients immediately prior to bracket removal. These samples were analysed with Gelcompar-II software applied on Denaturing Gradient Gel Electrophoresis (DGGE) patterns; the software was adjusted by varying the automated band detection settings and the findings were compared to visually detected bands to find optimum settings. Secondly, the plaque samples were analysed by a conventional microbiology plating method to assess numbers of total colony forming units (CFU’s), percentages of aciduric flora, Streptococcus mutans, Lactobacillus spp. and Candida albicans, respectively. The presence of WSL was determined immediately after bracket removal, which showed that 28 patients had WSL and only nine were WSL free. By changing software settings, the number of bands detected was altered. Band numbers identified for subjects with or without WSL were either significant or not significant depending on the settings of the software. No differences between groups were observed for the microbiological parameters (aciduric flora, S. mutans, Lactobacillus spp. and C. albicans).

We conclude that Gelcompar-II software settings significantly affect outcomes. DGGE and conventional microbiological parameters for total colony forming units (CFU’s) and percentages of aciduric flora, S. mutans, Lactobacillus spp. and C. albicans cannot predict risk of the formation of WSL in these orthodontic patients.

Chapter 3
The use of Denaturing Gradient Gel Electrophoreses and conventional microbiology as indicator of white spot lesions in orthodontic patients; a cross-sectional study.

Predicting caries risk in patients undergoing treatment with fixed appliances would be valuable with respect to WSL formation. Denaturing Gradient Gel Electrophoresis (DGGE) and microbial composition have been suggested to predict caries risk in young children. In the current study the predictive value of these two methods was assessed in patients with full fixed appliances. Dental plaque samples of patients with and without WSL were mutually compared. Dental plaque was obtained from 37 patients immediately prior to bracket removal. These samples were analysed with Gelcompar-II software applied on Denaturing Gradient Gel Electrophoresis (DGGE) patterns; the software was adjusted by varying the automated band detection settings and the findings were compared to visually detected bands to find optimum settings. Secondly, the plaque samples were analysed by a conventional microbiology plating method to assess numbers of total colony forming units (CFU’s), percentages of aciduric flora, Streptococcus mutans, Lactobacillus spp. and Candida albicans, respectively. The presence of WSL was determined immediately after bracket removal, which showed that 28 patients had WSL and only nine were WSL free. By changing software settings, the number of bands detected was altered. Band numbers identified for subjects with or without WSL were either significant or not significant depending on the settings of the software. No differences between groups were observed for the microbiological parameters (aciduric flora, S. mutans, Lactobacillus spp. and C. albicans).

We conclude that Gelcompar-II software settings significantly affect outcomes. DGGE and conventional microbiological parameters for total colony forming units (CFU’s) and percentages of aciduric flora, S. mutans, Lactobacillus spp. and C. albicans cannot predict risk of the formation of WSL in these orthodontic patients.
Chapter 4
Remineralising effect of MI Paste Plus® on the regression of early caries after orthodontic fixed appliance treatment; a randomized 3-month follow-up clinical trial.

The effectiveness of MI Paste Plus® as remineralising agent for WSL is analysed for a period of 3 months, by two outcome measures:
1. QLF imaging to assess changes in WSL severity, and
2. Conventional microbiology to determine changes in plaque composition.

This chapter describes the results of a double-blind prospective randomised controlled trial.
Fifty-four orthodontic patients, with multiple white spot lesions observed after the removal of fixed appliances, were followed up for 3 months. Included subjects were randomly assigned to either MI Paste Plus® paste or to a placebo paste, as control, to be used supplementary to their normal oral hygiene. Caries regression was assessed on quantitative light-induced fluorescence (QLF) images captured directly after debonding and 6 and 12 weeks thereafter. The total counts and proportions of aciduric bacteria, S. mutans, and Lactobacillus spp. were determined in plaque samples obtained at three time points, just before debonding and 6 and 12 weeks later.

Results showed a significant decrease in fluorescence loss compared to baseline values for both groups, with no difference found between these groups. The size of the lesion area did not change significantly over time, neither between the groups. The percentages of aciduric bacteria and of S. mutans also decreased. Again no differences were found between the groups. No clinical advantages are found for the use of MI Paste Plus® paste supplementary to normal oral hygiene over the time span of 12 weeks.
Chapter 5

Long-term remineralising effect of MI Paste Plus® on the regression of early caries after orthodontic fixed appliance treatment; a randomized 12-month follow-up clinical trial.

The long term effects of MI Paste Plus® as a remineralising agent were analysed for a period of 12 months, by assessing several outcome methods:
1. QLF Imaging,
2. Conventional microbiological assessment,
3. Acidogenicity of plaque determined by capillary ion analysis (CIA), and
4. Assessment of ICDAS-scores on clinical photographs.

This chapter describes a double-blind prospective randomised controlled trial. Fifty-one orthodontic patients, with multiple white spot lesions observed after the removal of fixed appliances were followed for a period of 12 months. The MI Paste Plus® group consisted of 25 patients, while the control group consisted of 26 patients.

There was a significant improvement in WSL severity (fluorescence loss) over time in both groups, with no differences between groups. A significant reduction in aciduric bacteria over time was seen for the MI Paste Plus® group, but not in the control group. The reduction in aciduric bacteria was not significantly different between the groups. No significant changes in S. mutans over time for the MI Paste Plus® group were found, contrary to a significant reduction of S. mutans in time was found for the control group. The reduction in S. mutans was not significantly different between the groups. No significant differences in acidogenicity of plaque were seen in time or between the groups. ICDAS assessment showed the lesions to be unchanged in both groups.

The use of MI Paste Plus® after orthodontic fixed appliance treatment in patients with subsurface enamel lesions did not improve these lesions during the one year following debonding.
Chapter 6

In conclusion

In this thesis, research is being described that aims at secondary prevention of white spot lesions (WSL), i.e., the remineralisation of these lesions. Also methods to monitor existing WSL in time and methods to predict risk of the formation of WSL are analysed.

As described in this thesis, the monitoring of WSL severity by visual comparison of clinical oral photographs taken over time provides discriminatory power in assessing changes in WSL severity. ICDAS assessment on clinical oral photographs did not have enough discriminatory power to reveal changes in WSL. The risk for formation of WSL cannot be predicted by DGGE, nor by conventional microbiological parameters. MI Paste Plus®, a remineralisation agent, does not show its intended effect in patients who have WSL after orthodontic treatment with full fixed appliance. The intended effect was not seen in a 12 weeks period nor a 12 months period after appliance removal. Further research on the secondary prevention of WSL, should focus on the transport of calcium and phosphate ions into the deeper layer of the lesion and prevent hypermineralisation of the lesions. The primary prevention of WSL is considered crucial. Despite available scientific evidence in the prevention of WSL with fluoride, clinical guidelines have not been formulated until now.
Hoofdstuk 1
Algemene inleiding

Dit proefschrift beschrijft onderzoek naar de effectiviteit van een remineralisatiepasta, genaamd MI Paste Plus®, voor het herstel van witte vleklaesies (White Spot Lesions - afgekort WSL zoals hierna in de tekst gebruikt). WSL zijn beginnende ontkalking van cariës, veelal ontstaan tijdens de orthodontische behandeling met vaste apparatuur (de slotjesbeugel). Door deze slotjesbeugel is adequate mondhygiëne aanzienlijk lastiger, waardoor tandplaque op de moeilijk bereikbare plaatsen blijft zitten. Ook vloeit er minder speeksel langs het tandoppervlak om tandplaque op een natuurlijke wijze te kunnen afvoeren. Deze plaque kan WSL veroorzaken in het tandglazuur.

De maatregelen om WSL primair te voorkomen tijdens de orthodontische behandeling zijn wetenschappelijk onderbouwd. Daarentegen is onze kennis over de secundaire preventie beperkt. Dat zijn strategieën om WSL, die zijn ontstaan, op een minimaal-invasieve manier te behandelen en zo te herstellen. Tijdens de behandeling is het belangrijk dat de bovenste laag van de ontkalking niet hypermineraliseert, waardoor de diepere lagen niet meer kunnen mineraliseren. Dit ontstaat als de buitenste laag herstelt en daardoor de dooldoorlaatbaarheid van ionen naar de diepere gedeelten wordt gehinderd. Door deze hypermineralisatie behoudt de WSL zijn karakteristiek witte kleur.

Om remineralisatie tot in de diepere laag van de laesie te verkrijgen, is een minimaal invasieve remineralisatiestrategie onderzocht die in dit proefschrift wordt beschreven. Deze strategie is gebaseerd op de CPP-ACPF techniek. Via deze techniek worden vrij beschikbare calcium- en fosfaat-afnemingen in speeksel en tandplaque aangeboden om remineralisatie in de diepere laag van de WSL te bewerkstelligen. Mi Paste Plus® is een voor de patiënt commercieel verkrijgbaar product. Dit product is gebaseerd op deze techniek en claimt WSL te remineraliseren.

Voor het in dit proefschrift beschreven onderzoek zijn verschillende onafhankelijke meetinstrumenten toegepast om te beoordelen of deze gebruikt kunnen worden voor de bepaling van het cariës risico en de ernst van de cariës.

In dit proefschrift wordt een gerandomiseerd klinisch onderzoek (RCT) beschreven dat heeft plaatsgevonden in één onderzoekscentrum en
dubbelblind en placebo gecontroleerd is uitgevoerd. Het had als doel om de effectiviteit van MI Paste Plus® te bepalen. Het onderzoek is uitgevoerd in de periode januari 2008 - augustus 2010 op de afdeling Orthodontie van het Academisch Centrum Tandheelkunde te Amsterdam (ACTA). Het gebruik van verschillende onafhankelijke meet-instrumenten en de evaluatie over een langere termijn van 12 maanden, bekrachtigen de uitkomst van dit onderzoek.

**Hoofdstuk 2**

Witte vleklaesies na orthodontische behandeling bepaald aan de hand van klinische foto’s en door kwantitatieve licht-geïnduceerde fluorescentiebeeldvorming (QLF); een retrospectieve studie.

Het monitoren van de ernst van WSL in de tijd op een gestandaardiseerde manier is noodzakelijk voor klinische besluitvorming bij het beheersen van WSL. Dit retrospectieve onderzoek evalueert het gebruik van gestandaardiseerde meetmethoden in de tijd. Veranderingen van WSL na de orthodontische behandeling werden gevolgd over een periode van 12 maanden. Twee methoden zijn vergeleken:

1. Intra-orale klinische foto’s zijn geanalyseerd door zowel visueel waarneembare veranderingen vast te leggen als met behulp van het ICDAS scoringssysteem (Internationaal Caries Detectie en Assessment Systeem),

   en

2. QLF afbeeldingen geanalyseerd middels computer software.

Gegevens van 51 proefpersonen werden geïncludeerd in het onderzoek. Van deze proefpersonen werden foto’s genomen met beide methoden, direct na het verwijderen van de beugel (T1) en een jaar later (T2). De klinische foto’s werden op beide tijdstippen onafhankelijk beoordeeld met gebruik van het ICDAS scoringssysteem. Daarnaast werden de foto’s van patiënten in de tijd vergeleken door visueel waarneembare veranderingen (VT) te beoordelen. QLF foto’s werden onderzocht en gecategoriseerd op het fluorescentieverlies op de twee tijdstippen (T1 en T2).

In totaal werden 918 tandoppervlakken onderzocht die voorheen voorzien waren van een slotje. Op klinische foto’s werden 433 laesies gedetecteerd, bij het gebruik van ICDAS, terwijl op de QLF afbeeldingen 384 laesies gedetecteerd
werden. Met beide technieken was een verbetering van de WSL te zien gedurende de 12 maanden. Als een laesie aanwezig was in het tandoppervlak, werd ICDAS-score 2 het vaakst gescroond op beide tijdstippen. Veranderingen werden wel waargenomen in de tijd, maar het gebruik van het ICDAS scorringsysteem op klinische foto’s is onvoldoende gevoelig om veranderingen van WSL nauwkeurig te volgen. Met andere woorden, het gebruik van het ICDAS scorringsysteem op klinische foto’s heeft onvoldoende onderscheidend vermogen om veranderingen in WSL aan te tonen. Daarentegen biedt het visueel vergelijken van in de tijd genomen klinische foto’s voldoende onderscheidend vermogen voor het beoordelen van veranderingen in de ernst van WSL in de tijd. Geconcludeerd kan worden dat het monitoren van de ernst van WSL in de tijd door middel van visuele beoordeling op klinische foto’s nuttig is voor klinische besluitvorming bij het beheersen van WSL.

**Hoofdstuk 3**

Het gebruik van Denaturing Gradient Gel Electrophoresis (DGGE) en conventionele microbiologie als een indicator voor witte vleklaesies; Een cross-sectioneel onderzoek.

Het kunnen voorspellen van het cariërsrisico bij patiënten die een behandeling met vaste apparatuur ondergaan, kan nuttig zijn bij een inschatting of WSL zullen ontstaan. Denaturing Gradient Gel Electrophoresis (DGGE), een techniek om bacteriefragmenten te scheiden en onderzoek naar de microbiële samenstelling blijken beide een voorspellende waarde te hebben om het cariërsrisico bij jonge kinderen te beperken. Deze methoden zouden nuttig zijn bij de voorspelling van het cariërsrisico bij orthodontie patiënten. In deze studie werd de voorspellende waarde van deze twee methoden beoordeeld bij patiënten met volledige behandeling met vaste apparatuur (de slotjesbeugel). Monsters van tandplaque van patiënten met en zonder WSL werden onderling vergeleken. Tandplaque werd verkregen van 37 patiënten direct voorafgaand aan het verwijderen van de vaste apparatuur. Deze monsters werden gebruikmakend van Gellcompare-II-software op DGGE patronen geanalyseerd. De software werd aangepast door de geautomatiseerde banddetectie-instellingen te variëren. Deze werden vervolgens vergeleken met andere monsters van patiënten met WSL en zonder WSL. Daarentegen biedt het visueel vergelijken van in de tijd genomen klinische foto’s voldoende onderscheidend vermogen voor het beoordelen van veranderingen in de ernst van WSL in de tijd. Geconcludeerd kan worden dat het monitoren van de ernst van WSL in de tijd door middel van visuele beoordeling op klinische foto’s nuttig is voor klinische besluitvorming bij het beheersen van WSL.
met de visuele detectie van banden, om zo de optimale instelling te vinden. Daarnaast werden de plaquemonsters geanalyseerd met behulp van een conventionele microbiologische platingtechniek (incubatie op petrischalen) voor het bepalen van aantallen kolonievormende eenheden (CFU’s) als maat voor de hoeveelheid bacteriën, en de percentages van zurenvormende flora, *Streptococcus mutans*, *Lactobacillus* spp. en *Candida albicans*. De aanwezigheid van (WSL) werd onmiddellijk na verwijdering van de vaste apparatuur bepaald. Hierbij bleek dat van de 37 patiënten, 28 patiënten WSL hadden en slechts negen WSL-vrij waren.

De software-instellingen bleken bepalend voor het aantal detecteerbare DGGE-banden. De wijziging van de software instelling bepaalde of er een significant verschil was tussen de patiënten met en zonder WSL. Met de conventionele microbiële plating werden er geen verschillen tussen groepen waargenomen voor de microbiologische parameters (zuren vormende flora, *S. mutans*, *Lactobacillus* spp. en *C. albicans*).

Concluderend, de instellingen van Gelcompar-II software bepalen of resultaten significant verschillend zijn. DGGE en conventionele microbiologische parameters voor aantallen kolonievormende eenheden (CFU’s) en percentages van zurenvormende flora, *S. mutans*, *Lactobacillus* spp. en *C. albicans* kunnen het risico op de vorming van WSL bij deze orthodontische patiënten niet voorspellen.

### Hoofdstuk 4

**Het effect van MI Paste Plus® op de remineralisatie van witte vleklaesies en tandplaque na de orthodontische behandeling met vaste apparatuur; een gerandomiseerd klinisch onderzoek met een follow-up van 3 maanden.**

De effecten van MI Paste Plus® als remineraliserend middel voor WSL werden gedurende een periode van 3 maanden geanalyseerd middels twee onderzoeksmethoden:

1. Kwantitatieve licht-geïnduceerde fluorescentie (QLF) beeldvorming om veranderingen in de mate van WSL te beoordelen, en
2. Conventionele microbiologie om veranderingen in de samenstelling van de plaque te bepalen.
Hoofdstuk 6
Concluderend
In dit proefschrift wordt onderzoek beschreven dat zich richt op herstel van witte vleklaesies (WSL), dit betreft de secundaire preventie. Daarnaast wordt gekeken naar methoden om bestaande WSL te monitoren en methoden om het risico van het ontstaan van WSL vast te stellen.

In dit proefschrift is aangetoond dat WSL kunnen worden gemonitord
door visuele vergelijking van, klinische foto’s in de tijd, waar het ICDAS scoringssysteem op klinische foto’s onvoldoende onderscheidend vermogen heeft. Het risico op het ontstaan van WSL tijdens de behandeling met vaste apparatuur kunnen niet voorspeld worden door middel van DGGE noch door conventionele microbiologische parameters. Het gebruik van MI Paste Plus® als remineralisatie-pasta heeft geen aanvullend effect op de remineralisatie van bestaande WSL bij een normale mondhygiëneprocedure, niet over een korte periode (12 weken) en ook niet over een lange periode (12 maanden). Onderzoek zal zich verder moeten richten op het remineraliseren van bestaande WSL (de secundaire preventie), door calcium- en fosfaationen in de diepere lagen van de WSL te brengen zonder de bovenste laag te hypermineraliseren. Er is wetenschappelijke onderbouwing voor de primaire preventie van WSL die omgezet zou moeten worden tot een richtlijn.
List of Publications and Author Contributions

Dankwoord

Curriculum vitae auctoris
LIST OF PUBLICATIONS

Long-term remineralising effect of MI Paste Plus® on Regression of Early Caries after Orthodontic Fixed Appliance Treatment; A 12-month Follow-up Randomized Controlled Trial.

M.W. Beerens, J.M. ten Cate, M.J. Buijs, M.H. van der Veen.

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M.W. Beerens, F. Boekitwetan, M.H. van der Veen, J.M. ten Cate.

Effects of casein phosphopeptide amorphous calcium fluoride phosphate paste on white spot lesions and dental plaque after orthodontic treatment: a 3-month follow-up.

M.W. Beerens, M.H. van der Veen, H. van Beek, J.M. ten Cate.
AUTHOR CONTRIBUTIONS

of the published manuscripts in this thesis.

Authors and their initials:
M. (Moniek) W. Beerens MB
M. (Monique) H. van der Veen MvdV
J. (Bob) M. ten Cate JtC
F. (Florence) Boekitwetan-Lim FB
M. (Mark) J. Buijs MJB
H. (Herman) van Beek HvB

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Authors:
M.W. Beerens, F. Boekitwetan, M.H. van der Veen, J.M. ten Cate.
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Conceived and designed the study: MvdV, MB, FB
Performed the study: MB, FB,
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Critically revised the manuscript: JtC

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Author contributions:
Conceived and designed the study:  MvdV, MB
Performed the study:    MB, MvdV
Analysed the data:    MB, MvdV
Drafted the manuscript:   MvdV, MB
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Chapter 4

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Authors:
M.W. Beerens, M.H. van der Veen, H. van Beek, J.M. ten Cate.
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Author contributions:
Conceived and designed the study: MvdV, JtC
Performed the study: MB, MvdV
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Monique H. van der Veen is a co-inventor on several patents relating to quantitative light-induced fluorescence. The authors declare that otherwise there is no conflict of interest pertaining to the data presented in this article. The authors alone are responsible for the content and writing of the paper.

Chapter 5

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Long-term remineralising effect of MI Paste Plus® on Regression of Early Caries after Orthodontic Fixed Appliance Treatment; A 12-month Follow-up Randomized Controlled Trial.
Authors: M.W. Beerens, J.M. ten Cate, M.J. Buijs, M.H. van der Veen.

Author contributions:
Conceived and designed the study: MvdV, JtC
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Tot slot mijn grote trots, Floris Radius. Nu 2 jaar oud. Jij bent mijn meesterwerk!
MONIEK BEERENS was born in Jakarta, Indonesia, on May 13th 1981. She received her high school diploma from Public High School RSG ‘t Slingerbosch in Harderwijk, the Netherlands in 2000. In her last year of high school she, together with her high school friend, received an honourable reference as part of the Van Melsen Award of the Radboud University of Nijmegen, a programme for novel research projects conducted by high school students. In the same year she started her professional education at the Department of Dentistry and Oral Hygiene of the Faculty of Medical Sciences at the University of Groningen, RUG in The Netherlands.

In 2005 she graduated with a Degree in General Dentistry. Her thesis was based on research conducted on the *atraumatic restorative treatment* (ART) approach for primary teeth in school children in Belém, Brasil. She was granted this research opportunity by the Department of Paediatric Dentistry of the Academic Centre for Dentistry Amsterdam (ACTA) in the Netherlands. After working as a dentist for one year she started a full time Post-Graduate Orthodontic Specialisation at the Department of Orthodontics of ACTA where she graduated in 2010. During her specialisation she performed in a randomised controlled trial, a research collaboration between the Departments of Orthodontics and Preventive Dentistry of ACTA. Following her specialisation she combined working as an orthodontist with preparing research publications that resulted in this thesis.

She and her partner Erik Radius have a son, Floris (2016).
White Spot Lesions after Orthodontic Fixed Appliance Treatment

The effectiveness of MI Paste Plus® as a remineralising agent
a randomised controlled trial

Moniek Willemien Beerens

1. MI Paste Plus®, a remineralisation agent, does not show its intended effect in patients who have white spot lesions (WSL) after orthodontic treatment with full fixed appliances.

2. WSL tend to regress over time but do not disappear after removal of the appliances.

3. After bracket removal the microbial composition gradually changes towards a more healthy composition. This is a gradual change that does not occur immediately after bracket removal.

4. The ICDAS scoring system on clinical oral photographs does not have enough discriminatory power to address changes in WSL over time.

5. Comparing respective clinical oral photographs taken over time provides discriminatory power in assessing changes in WSL severity. This method of monitoring WSL over time is useful for clinical decision making in the management of WSL.

6. Quantitative light induced fluorescence (QLF) is confirmed as a useful outcome measure in detecting and monitoring WSL over time.

7. The DGGE-banding pattern software, Gelcompar II, is susceptible to bias when used with the provided settings.

8. Denaturing Gradient Gel Electrophoresis (DGGE) and analysis of microbial composition does not have a predictive value for caries risk assessment of the formation of WSL in orthodontic patients treated with full fixed appliances.

9. Conventional microbiological plating, analysing bacteria counts, percentage of aciduric flora, S. Mutans, Lactobacillus spp. and C. Albicans is not predictive for caries risk assessment of the formation of WSL in orthodontic patients treated with full fixed appliances.

10. This thesis describes the first randomised control trial assessing the effectiveness of MI Paste Plus® in vivo for the treatment of WSL after orthodontic fixed appliance on the long term (12 months) and stipulates the importance of combining independent outcome measures in research.