The Role of Blood and Serum Proteins in the Pathogenesis of Molar-Incisor Hypomineralisation

Rami Amin Farah
BDS (Jordan), MPhil (Liverpool), PhD (Otago)

A thesis submitted for the degree of Doctorate of Clinical Dentistry-Paediatric Dentistry at the University of Otago, Dunedin, New Zealand

August 2011
Abstract

Several studies have investigated possible aetiological factors for molar-incisor hypomineralisation (MIH). Despite that, the aetiology and the pathogenesis of MIH are still not understood. With knowledge gained from the previous investigations at the Faculty of Dentistry, University of Otago, the current study built on that knowledge and aimed to investigate the possible role of serum and blood proteins in the development of MIH. The hypothesis of this study was that MIH is of traumatic origin resulting in serum and blood to leak into developing enamel and interfere with its mineralisation.

The range of the proteins present in MIH and sound enamel was assessed biochemically. Small sections of MIH and sound enamel were dissolved in 20% trichloroacetic acid that dissolved the mineral phase and precipitated the proteins. After re-suspension in sodium dodecyl sulphate (SDS), the proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Tryptic fingerprint tandem mass spectrometry (MS/MS) was performed on excised bands from the SDS-PAGE. Several new proteins were identified in sound enamel. Most of the proteins identified in MIH enamel were also present in sound enamel. The proteins unique to MIH enamel were known to play a role in tissue injury and repair, and in bleeding and coagulation. Due to high keratin and collagen contamination it was not possible to draw more conclusions.

Direct immunofluorescence (with quantum dots a fluorescent probe) on cut sections from MIH and sound enamel was used to map the distribution of serum albumin and haemoglobin in the cut sections. The albumin and haemoglobin were shown to be present in MIH enamel but not in sound enamel. A gradient of these proteins appeared to exist from
deep enamel, close to the dentine-enamel junction, to outer enamel close to the surface. The source of albumin and haemoglobin is most likely from the serum/blood reaching enamel during its formation, and not from saliva after gaining access into MIH enamel after tooth eruption. This is because salivary α-amylase could not be detected in MIH or sound enamel, ruling out the possibility of saliva being the source of albumin and haemoglobin.

To understand the distribution of the proteins in the organic matrix of MIH enamel, histopathological investigation of decalcified cut sections from sound and MIH enamel demonstrated the presence of a very dense organic matrix in MIH enamel. The matrix constricted the enamel rods, and sometimes intervened between the rods as a thick sheath.

Based on the findings of this study it was not possible to accept or reject the study hypothesis that MIH is of traumatic origin. A model, developed based on the findings of this study, suggests that serum/blood proteins leak into MIH enamel during the maturation stage of enamel development in the occlusal part of the first permanent molar. Since the cervical part would still be passing through the less sensitive secretory stage, it remains unaffected.
Acknowledgement

A graduate student is usually lucky to work with wonderful supervisors once. It was a true blessing for me to have worked with great supervisors not once, but twice during two doctorate degrees. I would like to extend the deepest debt of gratitude to my supervisors, my teachers, my mentors: Associate Professor Bernadette Drummond, Dr Brian Monk and Professor Michael Swain. They taught me from the heart, not from the book.

I would also like thank all my friends and family here in Dunedin who supported me and put up with me over the past five years. In particular, I would like to thank my friends Mr Basil Al-Amleh and his family, Dr Ahmad Ghandour and his family, Dr Momen Atieh and his family, Mr Ghassan Hamid and his family, and Mr Anuj Batra and his family, Mrs Dorothy Boyd and Mrs Alison Meldrum.

This work would not have been possible without the help of all the dentists across New Zealand who willingly donated their time and effort to help me in collecting teeth for the study.

But most importantly, I would like to thank the great children of New Zealand for donating the teeth to the study. They were not motivated by the hope of a treatment for their own problems, but by the hope that this study will help other children in the future.
Dedication

To Rana, Mino, Barhoom, Amin and Hana…

to my family, who endured with me not one, but three research degrees
# Table of Contents

Chapter I

**Introduction and Study Outline** ............................. 1

  Study outline ........................................... 2

Chapter II

**Review of the Literature, the Research Problem and Research Objectives** ............................. 3

  Definition and clinical presentation ......................... 3
  Prevalence ................................................................ 5
  Problems and management ....................................... 8
    Dental sensitivity and its control ....................... 8
    Defective enamel and its restoration .................. 9
  Planning extraction of affected MIH molars ............ 13
  Aetiology of MIH ............................................. 15
    Exposure to environmental contaminants ............... 15
    Medical conditions in the first three years of life ... 16
    The use of antibiotics in the first three years of life .... 16
    Medical problems around birth ............................ 17
  Previous research on MIH from Otago and the introduction of the research problem ......................... 18
  Research problem and objectives ............................. 23

Chapter III

**Materials and Methods: Background and General Description** ............................. 25
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample collection and ethical issues</td>
<td>25</td>
</tr>
<tr>
<td>The collected molar teeth</td>
<td>28</td>
</tr>
<tr>
<td>Protein assay</td>
<td>29</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)</td>
<td>29</td>
</tr>
<tr>
<td>Mass Spectrometry</td>
<td>29</td>
</tr>
<tr>
<td>Direct immunofluorescence</td>
<td>31</td>
</tr>
<tr>
<td>Confocal Laser Scanning Microscopy (CLSM)</td>
<td>32</td>
</tr>
<tr>
<td>Quantum dots</td>
<td>34</td>
</tr>
<tr>
<td>Chapter IV</td>
<td></td>
</tr>
<tr>
<td>Qualitative Analysis of the Protein Content of Sound and Hypomineralised Enamel</td>
<td>35</td>
</tr>
<tr>
<td>Introduction</td>
<td>35</td>
</tr>
<tr>
<td>Materials and methods</td>
<td>36</td>
</tr>
<tr>
<td>General Description</td>
<td>36</td>
</tr>
<tr>
<td>Enamel sample preparation and protein extraction</td>
<td>36</td>
</tr>
<tr>
<td>Sample preparation for MS/MS</td>
<td>36</td>
</tr>
<tr>
<td>LC-MS/MS/MS of tryptic peptides</td>
<td>37</td>
</tr>
<tr>
<td>Instrument setting for the LTQ-Orbitrap</td>
<td>37</td>
</tr>
<tr>
<td>Data analysis</td>
<td>37</td>
</tr>
<tr>
<td>Pilot Study No. 1</td>
<td>38</td>
</tr>
<tr>
<td>Enamel sample preparation and protein extraction</td>
<td>38</td>
</tr>
<tr>
<td>Pilot Study No. 2</td>
<td>38</td>
</tr>
<tr>
<td>Modified Main Study</td>
<td>39</td>
</tr>
<tr>
<td>Results</td>
<td>43</td>
</tr>
<tr>
<td>Discussion</td>
<td>48</td>
</tr>
<tr>
<td>Sound enamel proteins</td>
<td>48</td>
</tr>
<tr>
<td>MIH enamel proteins</td>
<td>51</td>
</tr>
<tr>
<td>Chapter V</td>
<td></td>
</tr>
<tr>
<td>Spatial Distribution of Albumin, Haemoglobin and α-Amylase in Sound and Hypomineralised Enamel</td>
<td>57</td>
</tr>
<tr>
<td>Chapter</td>
<td>Title</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
</tr>
<tr>
<td></td>
<td>Materials and methods</td>
</tr>
<tr>
<td></td>
<td>Direct immunofluorescence detection of albumin, haemoglobin and amylase</td>
</tr>
<tr>
<td></td>
<td>Enamel Samples</td>
</tr>
<tr>
<td></td>
<td>Enamel sample sectioning</td>
</tr>
<tr>
<td></td>
<td>Conjugating quantum dots to primary antibodies</td>
</tr>
<tr>
<td></td>
<td>Preparation of soya flour for the blocking agent</td>
</tr>
<tr>
<td></td>
<td>Reacting the conjugated primary antibodies with the enamel samples</td>
</tr>
<tr>
<td></td>
<td>Visualisation of enamel sections using CLSM</td>
</tr>
<tr>
<td></td>
<td>Dot blots</td>
</tr>
<tr>
<td></td>
<td>Dot blots to confirm the activity and specificity of the antibodies</td>
</tr>
<tr>
<td></td>
<td>Dot blots to validate DIF findings</td>
</tr>
<tr>
<td></td>
<td>Results</td>
</tr>
<tr>
<td></td>
<td>Direct immunofluorescence</td>
</tr>
<tr>
<td></td>
<td>Detection of albumin</td>
</tr>
<tr>
<td></td>
<td>Normal teeth</td>
</tr>
<tr>
<td></td>
<td>MIH molars</td>
</tr>
<tr>
<td></td>
<td>Detection of haemoglobin</td>
</tr>
<tr>
<td></td>
<td>Normal teeth</td>
</tr>
<tr>
<td></td>
<td>MIH molars</td>
</tr>
<tr>
<td></td>
<td>Detection of α-amylase</td>
</tr>
<tr>
<td></td>
<td>Dot blots</td>
</tr>
<tr>
<td></td>
<td>Antibodies reactivity and specificity</td>
</tr>
<tr>
<td></td>
<td>Validating the DIF results</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
</tr>
<tr>
<td></td>
<td>Chapter VI</td>
</tr>
<tr>
<td></td>
<td>Histopathological Study of Sound and Hypomineralised Enamel</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
</tr>
<tr>
<td>Chapters</td>
<td>Page</td>
</tr>
<tr>
<td>----------------------</td>
<td>------</td>
</tr>
<tr>
<td>Results</td>
<td>112</td>
</tr>
<tr>
<td>Discussion</td>
<td>119</td>
</tr>
<tr>
<td>Chapter VII</td>
<td></td>
</tr>
<tr>
<td>General Discussion, Conclusions and Future Research</td>
<td>123</td>
</tr>
<tr>
<td>References</td>
<td>129</td>
</tr>
<tr>
<td>Appendix 1</td>
<td></td>
</tr>
<tr>
<td>Ethical approval letter</td>
<td>147</td>
</tr>
<tr>
<td>Appendix 2</td>
<td></td>
</tr>
<tr>
<td>Instructions sheet for dentists</td>
<td>149</td>
</tr>
<tr>
<td>Appendix 3</td>
<td></td>
</tr>
<tr>
<td>Demographics sheet</td>
<td>150</td>
</tr>
<tr>
<td>Appendix 4</td>
<td></td>
</tr>
<tr>
<td>Information for participants and their legal guardians</td>
<td>151</td>
</tr>
<tr>
<td>Appendix 5</td>
<td></td>
</tr>
<tr>
<td>Information for the participating child</td>
<td>157</td>
</tr>
<tr>
<td>Appendix 6</td>
<td></td>
</tr>
<tr>
<td>Consent form</td>
<td>158</td>
</tr>
</tbody>
</table>
**List of Tables**

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1.</td>
<td>Summary of the published studies on the prevalence of MIH in different parts of the world.</td>
<td>7</td>
</tr>
<tr>
<td>Table 4.1.</td>
<td>Protein identified as present in sound and/or MIH enamel from the 3 MS/MS studies. Grey columns denote MIH enamel, white ones sound enamel. Light blue cells indicate proteins identified in MIH enamel samples only.</td>
<td>47</td>
</tr>
<tr>
<td>Table 4.2.</td>
<td>MS/MS results of proteins isolated from intact and fractured MIH enamel [Adapted from (Mangum et al., 2010)].</td>
<td>55</td>
</tr>
<tr>
<td>Table 5.1.</td>
<td>Specifications of the QDs used in the study.</td>
<td>63</td>
</tr>
<tr>
<td>Table 5.2.</td>
<td>Image acquisition settings.</td>
<td>68</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Outline of the research project.</td>
<td>2</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Diagrammatic representation of the components of the confocal microscope.</td>
<td>33</td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>MIH molar No.1.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>MIH molar No.2.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>MIH molar No.3.</td>
<td>40</td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>MIH molar No.4.</td>
<td>41</td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>SDS-PAGE [Adapted from (Mangum et al., 2010)]</td>
<td>54</td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>MIH molar No.1.</td>
<td>59</td>
</tr>
<tr>
<td>Figure 5.2</td>
<td>MIH molar No.2.</td>
<td>59</td>
</tr>
<tr>
<td>Figure 5.3</td>
<td>MIH molar No.3.</td>
<td>60</td>
</tr>
<tr>
<td>Figure 5.4</td>
<td>MIH molar No.4.</td>
<td>60</td>
</tr>
<tr>
<td>Figure 5.5</td>
<td>MIH molar No.5.</td>
<td>61</td>
</tr>
<tr>
<td>Figure 5.6</td>
<td>The Accutom-50 sectioning machine</td>
<td>62</td>
</tr>
<tr>
<td>Figure 5.7</td>
<td>Absorption and fluorescence emission spectra of the QDs in pH 7.2.</td>
<td>64</td>
</tr>
<tr>
<td>Figure 5.8</td>
<td>Pilot study using a green QD.</td>
<td>65</td>
</tr>
<tr>
<td>Figure 5.9</td>
<td>Diagram of dot blots.</td>
<td>70</td>
</tr>
</tbody>
</table>
Figure 5.10. Photographic and CLSM images of a sound tooth section treated with QD-anti albumin antibody. .................. 72

Figure 5.11. Photograph and CLSM image of an MIH tooth section (treated with QD-anti albumin antibody). .................. 73

Figure 5.12. Photographic and CLSM images of an MIH tooth section treated with QD-anti albumin antibody. .................. 77

Figure 5.13. Photographic and CLSM images of an MIH tooth section treated with QD-anti-albumin antibody. .................. 79

Figure 5.14. Photographic and CLSM images of a sound tooth section treated with QD-anti-Hb antibody. .................. 82

Figure 5.15. Photographic and CLSM images of an MIH tooth section treated with QD-anti Hb antibody. .................. 83

Figure 5.16. 10x magnification of the labelled area in Fig 5.15 (d). .................. 86

Figure 5.17. 20x magnification of the labelled area in Fig 5.16. .................. 87

Figure 5.18. Photographic and CLSM images of an MIH tooth section treated with QD-anti Hb antibody. .................. 88

Figure 5.19. Photographic and CLSM images of an MIH tooth section treated with QD-anti Hb antibody. .................. 90

Figure 5.20. Photographic and CLSM images of an MIH tooth section treated with QD-anti Hb antibody. .................. 93

Figure 5.21. Photographic and CLSM images of a sound tooth section treated with QD-anti amylase antibody. .................. 95

Figure 5.22. Photograph and CLSM image of an MIH tooth section treated with QD-anti amylase antibody. .................. 96

Figure 5.23. Dot blot test of antibody reactivity and specificity. .................. 99

Figure 5.24. Dot blot test for antigens extracted from teeth. .................. 100
Figure 5.25. Mineral density of a sound and an MIH tooth traced from cementum-enamel junction to cusp tip [adapted from (Farah et al., 2010c)]. .................. 103

Figure 5.26. The effect of postmortem storage on bovine teeth. .................. 107

Figure 5.27. Schematic diagram demonstrating the maturation process of enamel. .................. 108

Figure 6.1. Haematoxylin and eosin (H&E) stained histopathology image of a representative section of a sound tooth. .................. 112

Figure 6.2. H & E stained histopathological images of an MIH tooth section .................. 115

Figure 6.3. H & E histopathological image of an MIH tooth section at the cusp tip. .................. 117

Figure 6.4. H & E stained histopathological images at the cervical areas of an MIH tooth and a sound tooth. .................. 118

Figure 6.5. XMT images of cross-sections at different levels in an MIH molar [adapted from (Farah et al., 2010c)]. .................. 122

Figure 7.1. Enamel formation stages with respect to albumin. .................. 127
Chapter I

Introduction and Study Outline

Understanding the aetiology of a condition is often essential for its management. Numerous approaches, including observational, epidemiological and case control studies are used to investigate disease aetiology. Since the term molar-incisor hypomineralisation (MIH) was introduced in 2001 (Weerheijm et al., 2001b), its aetiology has been investigated using mainly observational or case control studies. Two recent systematic reviews concluded that the evidence for all causes proposed thus far is weak (Alaluusua, 2010; Crombie et al., 2009). Consequently the aetiology of MIH remains a puzzle.

The present investigation has chosen an alternative approach to understand MIH. A combination of biochemical and immunohistological methods that analysed extracted teeth has been used to test a hypothesis for the cause of MIH. The approach was possible because dental enamel is a “dead” tissue, incapable of regenerating or repairing itself because the ameloblasts which form enamel undergo apoptosis before the tooth erupts into the oral cavity. Thus, any defect introduced during enamel development remains essentially unchanged and can be studied subsequently for insight into the original insult that caused the defect. The insult can also be timed because the chronology of enamel formation is well understood.
This is a preliminary investigation, which set out to test a hypothesis which arose from a previous biochemical study in the Faculty of Dentistry at Otago University. The techniques and findings of the present study provide insight into the aetiology of MIH that will underpin further investigation of this potentially debilitating condition.

**Study outline**

After obtaining the ethical approval, extracted first permanent molars with MIH and teeth with sound enamel (extracted for orthodontic or periodontal reasons) were collected. The collected molars were subject to the series of tests as shown in Figure 1.1.

![Figure 1.1 Outline of the research project](image)

The next two chapters present a critical review of the literature on MIH, and outline the materials and methods used in this study. Three chapters then describe the research used to answer the research objectives. A final discussion chapter integrates the results of the investigation and suggests possible avenues for future research on the aetiology of MIH.
Chapter II

Review of the Literature, the Research Problem and Research Objectives

Definition and clinical presentation

Since its introduction by Weerheijm et al in 2001, the term “Molar-Incisor Hypomineralisation” (MIH) has largely replaced a group of terms used previously to describe the same condition. It is defined as ‘hypomineralisation of systemic origin of one to four permanent first molars, frequently associated with affected incisors’ (Weerheijm et al., 2001b).

This definition alone was insufficient to categorise hypomineralisation defects as MIH. For example, the definition did not include the observed demarcation characteristic of MIH defects, a feature which differentiates them from fluorosis defects. This led a working group of the European Academy of Paediatric Dentistry to introduce unified judgement
criteria for the diagnosis of MIH (Weerheijm et al., 2003). Based on these criteria and on other literature, a clinical description of MIH is as follows:

1. MIH defects are well-demarcated chalky white to yellow-brown defects that may exhibit post-eruptive breakdown (PEB) or involve atypical restorations.
2. Cervical enamel is sound, as determined by mineral density analysis and the retention of normal mechanical properties (Farah et al., 2010c; Mahoney et al., 2004).
3. Affected enamel is hypomineralised and not hypoplastic (Farah et al., 2010c; Fearne et al., 2004). Because of PEB, MIH has been mistakenly described in the past as both hypomineralised and hypoplastic.
4. The teeth most affected are the first permanent molars, with the incisors less frequently affected.
5. The distribution of the defects in the mouth is asymmetrical. Not all first permanent molars/incisors are necessarily affected.

In addition to the above criteria, it is noted that the number of affected incisors correlates with the number and severity of affected molars (Cho et al., 2008; Fteita et al., 2006; Jalevik et al., 2001a; Soviero et al., 2009; Weerheijm, 2003; Wogelius et al., 2008), and that the upper incisors are more commonly affected than lower incisors (Cho et al., 2008; Fteita et al., 2006; Jasulaityte et al., 2008; Lygidakis et al., 2008; Weerheijm et al., 2001a; Weerheijm, 2003; Wogelius et al., 2008). No differences have been found in the distribution of mild, moderate and severe defects among children with MIH (Jalevik et al., 2001a), or in MIH prevalence between males and females (Fteita et al., 2006; Jalevik et al., 2001a; Jasulaityte et al., 2007; Jasulaityte et al., 2008; Leppaniemi et al., 2001; Muratbegovic et al., 2007; Preusser et al., 2007; Soviero et al., 2009).
Prevalence

Since the term MIH was introduced in 2001, its prevalence has mainly been described in European populations. There have also been reports from Asia, Australasia, Africa and South America, but none from North America. Mahoney and Morrison (2009) reported a prevalence of 14.9% in 522 eight year old children from Wainuiomata, New Zealand with no statistically significant differences detected among different ethnic groups. This is the only published report about MIH prevalence in New Zealand. Table 2.1 summarises the published studies on the prevalence of MIH with regard to study location, sample size, examination settings, and MIH prevalence. The few studies in the table predating the 2001 definition of MIH have been included because Jalevik (2010) has indicated that those studies undoubtedly referred to MIH. One paper was omitted because of its small sample size and unsatisfactory recruitment method (Balmer et al., 2005).

The reported prevalence of MIH ranges between 2.8% and 40.2%, with a median of about 14%. Jalevik (2010) has concluded that most papers reported in Table 2.1 have methodological problems. For example, about one third of the surveys did not indicate the number of examiners or the calibration procedure used. Some studies were conducted in the dental chair while others were crude field surveys. In some cases the teeth examined were dried while in others the teeth were wet. Few studies excluded defects that were less than 2 mm in size.

Without a known cause for MIH, real inter-country differences in prevalence cannot be excluded. More likely, however, are the effects of confounding factors. These include differences between examiners, the use of different inclusion and exclusion criteria, and variation in the conditions and settings under which dental examinations were carried out. Surprisingly, while the examination of dried teeth is expected to give a higher reported prevalence of MIH than wet teeth, Table 2.1 shows that both methods report comparable prevalence.
In these studies, the mean number of MIH teeth is between 1.9 and 5.6 per affected patient (Arrow, 2008; Jalevik and Noren, 2000; Jasulaityte et al., 2008; Lygidakis et al., 2008; Muratbegovic et al., 2007; Wogelius et al., 2008). The mean number of affected molars is between 1.5 and 3.4 per patient (Cho et al., 2008; Fteita et al., 2006; Jasulaityte et al., 2008; Lygidakis et al., 2008).
<table>
<thead>
<tr>
<th>Study</th>
<th>Location</th>
<th>Sample Size</th>
<th>Examination Method</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Cho et al., 2008)</td>
<td>Hong Kong</td>
<td>2635</td>
<td>In dental clinics</td>
<td>2.8%</td>
</tr>
<tr>
<td>(Fteita et al., 2006)</td>
<td>Libya</td>
<td>378</td>
<td>Using a lamp, in schools, teeth dried</td>
<td>2.9%</td>
</tr>
<tr>
<td>(Kukleva et al., 2008)</td>
<td>Bulgaria</td>
<td>2960</td>
<td>Using a lamp, wet teeth</td>
<td>3.58%</td>
</tr>
<tr>
<td>(Dietrich et al., 2003)</td>
<td>Germany</td>
<td>2,408</td>
<td>Using a lamp, in schools, wet teeth</td>
<td>5.6%</td>
</tr>
<tr>
<td>(Preusser et al., 2007)</td>
<td>Germany</td>
<td>1,022</td>
<td>Using a lamp, in schools, wet teeth</td>
<td>5.9%</td>
</tr>
<tr>
<td>(Kuscu et al., 2009)</td>
<td>Turkey</td>
<td>153</td>
<td>Using a lamp, in schools, wet teeth</td>
<td>9.2%</td>
</tr>
<tr>
<td>(Weerheijm et al., 2001a)</td>
<td>The Netherlands</td>
<td>497</td>
<td>At schools</td>
<td>10%</td>
</tr>
<tr>
<td>(Lygidakis et al., 2008)</td>
<td>Greece</td>
<td>3,518</td>
<td>Using dental light in a dental chair</td>
<td>10.2%</td>
</tr>
<tr>
<td>(Muratbegovic et al., 2007)</td>
<td>Bosnia and Herzegovina</td>
<td>560</td>
<td>Not reported</td>
<td>12.3%</td>
</tr>
<tr>
<td>(Calderara et al., 2005)</td>
<td>Italy</td>
<td>227</td>
<td>At schools</td>
<td>13.7%</td>
</tr>
<tr>
<td>(Kemoli, 2008)</td>
<td>Kenya</td>
<td>3,591</td>
<td>At schools</td>
<td>13.7%</td>
</tr>
<tr>
<td>(Jasulaityte et al., 2008)</td>
<td>The Netherlands</td>
<td>442</td>
<td>Using a dental light, dry teeth</td>
<td>14.3%</td>
</tr>
<tr>
<td>(Zagdwon et al., 2002)</td>
<td>The UK</td>
<td>307</td>
<td>At schools</td>
<td>14.5%</td>
</tr>
<tr>
<td>(Pasareanu et al., 2006)</td>
<td>Romania</td>
<td>681</td>
<td>Wet teeth</td>
<td>14.5%</td>
</tr>
<tr>
<td>(Jasulaityte et al., 2007)</td>
<td>Lithuania</td>
<td>1,277</td>
<td>Using a lamp, in schools, wet teeth</td>
<td>14.9%</td>
</tr>
<tr>
<td>(Kuscu et al., 2008)</td>
<td>Turkey</td>
<td>147</td>
<td>Using a dental light, dry teeth</td>
<td>14.9%</td>
</tr>
<tr>
<td>(Mahoney and Morrison, 2009)</td>
<td>New Zealand</td>
<td>522</td>
<td>At schools</td>
<td>14.9%</td>
</tr>
<tr>
<td>(Koch et al., 1987)</td>
<td>Sweden</td>
<td>2252</td>
<td>Using a lamp and mirror, in schools, wet teeth</td>
<td>15.4%</td>
</tr>
<tr>
<td>(Alaluusua et al., 1996a)</td>
<td>Finland</td>
<td>102</td>
<td>No reported</td>
<td>16.6%</td>
</tr>
<tr>
<td>(Jalevik et al., 2001a)</td>
<td>Sweden</td>
<td>516</td>
<td>Using a lamp, in schools, wet teeth</td>
<td>18.4%</td>
</tr>
<tr>
<td>(Ghanim et al., 2011a)</td>
<td>Iraq</td>
<td>823</td>
<td>At schools</td>
<td>18.6%</td>
</tr>
<tr>
<td>(Leppaniemi et al., 2001)</td>
<td>Finland</td>
<td>488</td>
<td>Using a dental light in a dental chair, wet teeth</td>
<td>19.3%</td>
</tr>
<tr>
<td>(Da Costa Silva et al., 2010)</td>
<td>Brazil</td>
<td>918</td>
<td>At schools</td>
<td>19.8%</td>
</tr>
<tr>
<td>(Savisit et al., 2008)</td>
<td>Thailand</td>
<td>479</td>
<td>Not reported</td>
<td>20.3%</td>
</tr>
<tr>
<td>(Arrow, 2008)</td>
<td>Australia</td>
<td>511</td>
<td>Using a dental light, dry teeth</td>
<td>22%</td>
</tr>
<tr>
<td>(Alaluusua et al., 1996a)</td>
<td>Finland</td>
<td>97</td>
<td>Not reported</td>
<td>24.7%</td>
</tr>
<tr>
<td>(Wogelius et al., 2008)</td>
<td>Denmark</td>
<td>745</td>
<td>In dental clinics, wet</td>
<td>37.3–48.4%</td>
</tr>
<tr>
<td>(Soviero et al., 2009)</td>
<td>Brazil</td>
<td>292</td>
<td>Using a lamp, in schools, wet teeth</td>
<td>40.2%</td>
</tr>
</tbody>
</table>

**Table 2.1.** Summary of the published studies on the prevalence of MIH in different parts of the world.
Problems and management

MIH presents significant problems for both the patient and the clinician. Three separate surveys carried out by the European Academy of Paediatric Dentistry, the Australian and New Zealand Society of Paediatric Dentistry and a group of dental academics from Iraq concluded that MIH is a complicated clinical problem for the dentist to manage (Crombie et al., 2008; Ghanim et al., 2011b; Weerheijm and Mejare, 2003).

Dental sensitivity and its control

MIH causes heightened sensitivity to thermal and mechanical stimuli (Jalevik and Klingberg, 2002; Weerheijm, 2003; Willmott et al., 2008). Once erupted, the affected first permanent molars make eating, drinking and tooth brushing difficult. The increased porosity of MIH enamel (Jalevik and Noren, 2000), which may be combined with the exposure of dentine due to enamel breakdown and the presence of wide dentinal tubules at young age cause increased sensitivity to external stimuli (Heijls et al., 2007; Rodd et al., 2007). Inflammatory changes in the pulpal tissue of MIH molars (Rodd et al., 2007) result in heightened sensitivity (Narhi et al., 1994; Orchardson and Peacock, 1994), and can cause difficulty in achieving effective local anaesthesia (Fayle, 2003). Some clinicians recommend supplementary sedation or relative analgesia when local anaesthesia is ineffective (Mathu-Muju and Wright, 2006; Shargill and Hutton, 2007; William et al., 2006b). In addition, systemic pain relief may be needed to target pulpal pain effectively. Although unfounded by clinical research, some clinicians prefer the use of a surgical dose of paracetamol before a potentially-painful dental procedure.

Control of hypersensitivity in MIH molars requires that the child use toothpaste containing at least 1,000 ppm fluoride (Lygidakis, 2010; Lygidakis et al., 2010; Willmott et al., 2008). Similarly, professional topical application of neutral fluoride products is believed to help further mineralise the enamel and reduce sensitivity (Hansen, 1992; Mathu-Muju and
Wright, 2006; William et al., 2006b; Willmott et al., 2008). Evidence for the value of topical fluoride is, however, anecdotal (Lygidakis, 2010; Lygidakis et al., 2010). The use of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) to help ‘mineralise’ the surface/subsurface layer has also been suggested (Lygidakis, 2010; Lygidakis et al., 2010; On, 2008; William et al., 2006b; Willmott et al., 2008). An in situ study on the use of CPP-ACP for MIH management found evidence of increased calcium in surface enamel after three years of CPP-ACP application (Baroni and Marchionni, 2010). However, clinical studies have yet to show CPP-ACP is effective in reducing sensitivity.

**Defective enamel and its restoration**

Independent of the type of restorative material used, the form and position of MIH restorations often differ significantly from caries restorations. This is because MIH defects frequently involve the slopes of the cusps, an abnormal location for carious lesions. The resultant restorations were referred to as ‘atypical restorations’ in the 2003 diagnostic criteria for MIH (Weerheijm et al., 2003). A common feature of restorative materials used for MIH is high failure rates, evident from the need for repeated restorations (Chawla et al., 2008; Jalevik and Klingberg, 2002).

A retrospective assessment of the treatment records of first permanent molars in 36 MIH patients and 36 matched controls (followed up for 4.5 years) showed that DMFS was significantly higher in the MIH group (Kotsanos et al., 2005). Children from the MIH group had more restorative treatments, individual teeth had more surfaces treated and the treatments were more invasive. MIH children had three times as many replacement fillings than the control group. In the MIH group amalgam restorations required the most re-treatments (61%) while 25% of composite fillings needed re-treatment. Of 24 stainless steel crowns placed, none required re-treatment. William et al (2006b) suggested that the atypical outline of the defective enamel makes adhesive restorations a better option than amalgam. This opinion is shared by others (Fayle, 2003; Willmott et al., 2008). However, the composite resin used for cuspal replacement in carious teeth suffers significantly from polymerisation shrinkage, often
resulting in microleakage and deflection of the remaining cusps (Summit, 2006). Although several methods have been proposed to counter the effect of the polymerisation shrinkage, including ramped curing light and layering the composite resin, the use of composite resin for cuspal replacement in molars is not advisable. Its use should preferably be restricted to instances where the defective enamel is well demarcated, confined to one or two surfaces and when cuspal replacement is not indicated (Fayle, 2003). Within these limitations, composite resin restorations have shown high success rates over an extended period (Lygidakis et al., 2003).

Despite having a higher success rate than amalgam, the poor etching profile of MIH enamel has raised concerns about the use of resin composites (Jalevik et al., 2005; Mahoney, 2005). William et al (2006a) compared the microshear bond strength of composite resin to MIH and sound enamel in vitro. They found a significant reduction in the bond strength to MIH enamel. Like the above mentioned studies, shallower etch patterns in MIH (likely due to the protein coating of the rods and their crystals) enamel may reduce micro-mechanical bonding to composite resin. However, the weaker part of the system was the MIH enamel itself. Defective enamel under the composite fractured (resulting in cohesive failure of the system) rather than the composite restoration detaching. This may have led some experts to recommend complete removal of defective enamel before placement of restorations (William et al., 2006b; Willmott et al., 2008).

The results of the William et al (2006a) study should, however, be considered with caution. Microshear bond strength is primarily a failure initiation test. It predominantly focuses the forces to the weakest point of a system and does not reflect the gradual degradation and fatigue failure of the system that would normally occur in the oral cavity. The considerable scatter in their results adds uncertainty about the validity of their interpretation. In addition, the reduction in the mechanical properties of MIH enamel represents a wide range (Mahoney et al., 2004a, Mahoney et al., 2004b), and generalisations need to be made with care.
In large defects, it is inappropriate to depend on composite resin to support the residual tooth structure, especially when compromised enamel remains. The general agreement that adhesive restorations, unlike amalgam, support tooth structure may not be always correct (Joynt et al., 1987; Summit, 2006). A study of 10,869 posterior teeth restored with amalgam or composite resin restorations with at least one cusp present, showed that the prevalence of cuspal fracture was comparable for both restoration types (Wahl et al., 2004).

Glass ionomer cement (GIC) has been suggested as an alternative to composite resin restorations for moderate defects. While composite resin restoration margins need to be placed in sound enamel, chemical bonding of GIC to enamel allows restoration margins to be placed in affected but still hard enamel (Fayle, 2003; Mahoney, 2001; William et al., 2006a; William et al., 2006b). However, GIC restorations in MIH molars have shown significantly higher failure rates than either amalgam or composite resin restorations (Mejare et al., 2005). Due to the compromised performance of GIC after medium-term use, it should only be used as a temporary restoration in cases when the tooth is indicated for timed extraction or when a more definitive treatment is planned for the near future (Fayle, 2003; Mahoney, 2001). Resin-modified GIC may be a better option for interim restorations than regular GIC because of its ease of handling and greater wear resistance (William et al., 2006b).

The finding of increased organic content in MIH enamel (Farah et al., 2010b; Mangum et al., 2010) is consistent with the recommendation that sodium hypochlorite (NaOCl) be used to increase the bonding strength by denaturing/deproteinising MIH enamel (Fayle, 2003; Mahoney, 2001; Mathu-Muju and Wright, 2006; William et al., 2006b). This recommendation was primarily based on two papers which claimed enhanced bonding of composite resin to hypocalcified amelogenesis imperfecta teeth after treatment with 5% NaOCl (Saroglu et al., 2006; Venezie et al., 1994). In contrast, an in vitro study reported that the treatment of hypomaturation amelogenesis imperfecta enamel with NaOCl caused increased disorganisation in the enamel, with some samples completely disintegrating (Wright et al., 1992). Although the in vitro study used a higher concentration of NaOCl over an extended period, evidence supporting the use of NaOCl is incomplete (one of the studies
was a case report). Further research is needed before this method should be recommended for routine clinical use.

As a broad recommendation, intracoronal restorations with composite resins may be used for small defects. It may be advisable to remove all defective enamel and to place cavity walls in sound enamel (Shargill and Hutton, 2007). Extending the composite resin restoration so it bonds to the cervical and other normal enamel may also be good practice. Alternatively, GIC may be placed as an intermediate layer between the tooth structure and the resin to benefit from its chemical bonding to enamel (Lygidakis, 2010). Extracoronal restorations may be a better option for moderate sized defects, with higher success rates expected. This option includes stainless steel crowns and cast onlay restorations, as both give comparable success rates (Zagdwon et al., 2003). Stainless steel crowns probably involve easier tooth preparation techniques. Several studies have described the use of stainless steel crowns on permanent molars (Croll and Castaldi, 1978; Croll et al., 1981; Croll, 1999; 2000; Randall, 2002; Seale, 2002). Interdental placement of orthodontic separating elastics a week before preparing the tooth for a stainless steel crown helps to open the contacts and reduces tooth preparation (Fayle, 2003). On the other hand, onlays can provide more definitive restorations but are initially more expensive. Their potential for success and a decreased need for restoration replacement probably make them cost-effective over the dentition lifetime. For large defects with extensive post-eruptive breakdown, planned extraction of affected molars should be considered. The next section discusses this issue in more detail.

It is uncommon for MIH in anterior teeth to cause aesthetic problems that require restorative treatment. If needed, composite resin restorations/veneers are useful in the intermediate term. If aesthetic appearance is an issue, this can usually be resolved using a proprietary microabrasion system, possibly followed by bleaching, depending on the age of the patient. Porcelain veneers are generally used in late adolescence once gingival margins have stabilised (William et al., 2006b).

Icon (DMG Dental Products, Hamburg) is a new material that depends on unfilled resin infiltration into demineralised/early carious enamel was introduced recently (Paris et al.,
2007). It seems to offer good and stable management of early carious lesions around orthodontic brackets, but fails to improve the aesthetics of incisors affected by developmental defects (Felix Whorle, personal communication). The high protein content of developmental enamel defects may hinder acid dissolution of the affected area and infiltration by the resin. Also, where enamel is defective, unfilled resin may be insufficient to mimic the appearance and translucency of normal enamel.

Planning extraction of affected MIH molars

Timed extraction related to dentition development can be used to manage MIH molars. It has been suggested for moderately to severely affected first permanent molars (Mejare et al., 2005; Shargill and Hutton, 2007). The high failure rate of the different restorative techniques suggests that timed extractions should be considered to avoid a lifetime burden of repeat restorative care. The choice between restorative treatment and extraction depends on many factors including the dental age of the patient, the number and severity of the defects, occlusion and potential orthodontic consequences, the opinion of the patient and their parents and the cooperation of the patient.

A retrospective study of 76 patients with MIH examined the outcomes of treatment at 18 years of age (Mejare et al., 2005). Comprehensive records, including photographs of the affected teeth at the first visit, allowed the researchers to confirm MIH diagnosis and investigate the outcome of each type of treatment. They were able to establish the influence of the severity and number of affected teeth on the outcomes of treatment. A statistically significant correlation was found between the number of severely affected molars (with post-eruptive breakdown) and the number of extractions. Most of the MIH molars that were not extracted underwent restorative treatment, with 48% of these restorations judged unacceptable at age 18, while space closure was deemed to be acceptable in 87% of the patients with extracted molars. First permanent molar extraction was not shown to affect the sagittal relationships of the patients. However, 34% of the patients for whom one or more
first permanent molars were extracted also underwent orthodontic treatment. It was not clear whether the extractions were the main reason for the orthodontic treatment. Interestingly, acceptable space closure after extraction was similar in patients who received orthodontic treatment and those who did not. Mejare et al (2005) therefore recommended extracting molars with severe MIH defects, since extractions gave good results, good patient satisfaction, and avoided frequent restoration failure. Jalevic and Moller (2007) found that in almost all the cases where first permanent molars were extracted at the optimum age of 8.5 to 9.5 years, second permanent molars achieved a favourable position in the arch, with good contacts with the second premolars and good occlusion with the opposing teeth.

Despite these positive outcomes, first permanent molars are not the orthodontist’s tooth of choice for extraction when crowding is present. Their posterior location in the arch means that the time for moving anterior teeth may be longer, and anchorage is more difficult to achieve. In addition, evidence for the preferred time of extraction to optimise the movement of the second molars is relatively weak (Williams and Gowans, 2003). It is generally agreed that the optimum age for extraction is when the furcation of the roots of the second permanent molars (particularly the lower molars) are starting to calcify, which is usually around 8.5 to 9.5 years of age (Williams and Gowans, 2003). At this age, the mesial drift potential for the second permanent molar is highest and the distal drift of the second premolar is believed to be minimal. A wait for the optimum age for extractions may mean that the affected molars will need some interim restorative or even endodontic care. Mejare et al (2005) showed that most MIH molars planned for extraction required several visits before extraction as the molars required temporization. In conclusion, timed extraction should be considered as a possible part of the long term management of MIH. With appropriate occlusion and proper planning it can have a highly acceptable outcome.
Aetiology of MIH

The indication in the definition of MIH by Weerheijm et al (2001b) that its origin is systemic appears speculative because solid evidence for its aetiology is lacking. Two recent reviews concluded that there is insufficient support for any one of the aetiological factors proposed (Alaluusua, 2010; Crombie et al., 2009). The consensus from several studies exploring the aetiology of MIH is that all factors investigated lack valid support (Arrow, 2008; Crombie et al., 2009; Jalevik et al., 2001b; Mathu-Muju and Wright, 2006; Muratbegovic et al., 2007; Preusser et al., 2007; Whatling and Fearne, 2008; Willmott et al., 2008).

The aetiological factors suggested for MIH (modified from (Crombie et al., 2009)) include:

Exposure to environmental contaminants

The polychlorinated biphenyls (PCBs) and dioxins (Alaluusua et al., 1996b; Jan and Vrbic, 2000; Jan et al., 2007) are common environmental contaminants that may be taken up by breastfeeding infants. Animal studies, predominantly from Finland, suggested that tooth development was sensitive to PCBs and dioxins (Alaluusua et al., 1993). Further studies suggested an association between prolonged breast feeding and enamel mineralization defects in healthy children (Alaluusua et al., 1996a). However, these researchers recently retracted this suggestion and concluded that at the current levels of PCBs and dioxins measured in the placenta and breast milk no association with MIH could be concluded (Alaluusua and Lukinmaa, 2006; Laisi et al., 2008). Three other studies also found no correlation between breastfeeding and enamel defects (Beentjes et al., 2002; Dietrich et al., 2003; Jalevik et al., 2001b). A study from Turkey found the prevalence of MIH in children from areas polluted with dioxins and from areas clear of this pollutant were both around 9% (Kuscu et al., 2009).
Medical conditions in the first three years of life

Jalevik et al (2001b) reported that upper and lower respiratory infections in early infancy may increase the risk of MIH. Similarly, Tapias-Ledesma et al (2003) reviewed the medical records of 48 children with enamel defects in their first permanent molars and 149 children without enamel defects and found a strong correlation between urinary tract infections and enamel defects. In another matched control study, Lygidakis et al (2008) reported that 33.9% of children with MIH (from a group of 360 children) suffered from childhood illnesses in the first year of life, compared with only 12.5% in the matched control group. The reported illnesses included upper and lower respiratory tract infections, asthma and urinary tract infections. Beentjes et al (2002) concluded that MIH was associated with otitis media, pneumonia, and high fever in early childhood.

In contrast, a study from Bosnia and Herzegovina (Muratbegovic et al., 2007) compared matched groups of 69 twelve-year old children with and without MIH. No significant correlation between MIH and common childhood illnesses was found. Dietrich et al (2003) reached a similar conclusion. Whatling and Fearne (2008) found that among all the common childhood illnesses, only chicken pox showed a significant correlation with the occurrence of MIH.

The use of antibiotics in the first three years of life

The suggestion that antibiotics use may be related to MIH was based on a survey of 141 children, twenty three of whom had MIH (Laisi et al., 2009). MIH was found to be more common among children who had taken amoxicillin or erythromycin in the first year of life. The possibility of other confounding factors, such as a relationship with the illness or the fever, was not assessed. It was also noted that amoxicillin was introduced in Sweden after MIH was first reported in that country in the early 1970s (Koch et al., 1987). Several other
studies have found no association between MIH and treatment with any antibiotic class (Jalevik et al., 2001b; Muratbegovic et al., 2007; Tapias-Ledesma et al., 2003; Whatling and Fearne, 2008).

Medical problems around birth

An association between perinatal problems and enamel defects in the primary dentition is well documented (Aine et al., 2000; Drummond et al., 1992; Fadavi et al., 1992; Lai et al., 1997; Naidoo et al., 2005; Rugg-Gunn et al., 1997; Seow, 1996). For MIH this association is not clear. Some studies reported an association between MIH and medical problems around birth but others did not.

In a study of 109 children in the UK, Whatling and Fearne (2008) found that MIH was significantly more common in children whose mothers had medical problems during pregnancy. In a matched control study, Lygidakis et al (2008) reviewed the medical records of 360 MIH children in their first year of life, as well as the mothers’ medical histories in the last trimester of pregnancy. They found perinatal problems in 88% of the children with MIH, compared with 19% in the matched control group.

A recent Australian survey (Arrow, 2009) found children who had required medical care during the neonatal period were more affected by MIH than children who were healthy during infancy (84% vs. 69%). This correlation applied to illnesses occurring in the first 12 months of life, while subsequent illness had no significant effect on the occurrence of MIH. A recent matched control study from Sweden (Brogardh-Roth et al., 2011) found that MIH was more common in children born preterm (38%) than in the control group (16%). On the other hand, other studies have failed to find such a connection (Beentjes et al., 2002; Jalevik et al., 2001b; Muratbegovic et al., 2007).
Previous research on MIH from Otago and the introduction of the research problem

Several research projects on the structure, biomechanics, biochemistry, aetiology and management of MIH have been conducted at the University of Otago since 2006. The first publication from that work demonstrated a significant correlation between the mechanical properties of MIH enamel and its laser-induced fluorescence (LF) measured using the DIAGNOdent pen (KaVo, Biberach, Germany) (Farah et al., 2008). The high LF readings from MIH enamel defects were comparable with those of deep dentinal caries with a high load of bacteria, as DIAGNOdent readings are reported to reflect bacterial load. Polymerase chain reaction (PCR) with universal bacterial primers was then used to detect bacteria in cut enamel pieces from MIH enamel, sound enamel and carious enamel and dentine. Bacterial DNA was detected in carious dental tissues but not in sound or MIH enamel (Farah, 2009). It was concluded that in the absence of dental caries the laser fluorescence of enamel measured by DIAGNOdent may indicate the degree of hypomineralisation (Farah et al., 2008; Li et al., 2003). DIAGNOdent sensitivity may be high but its specificity is likely to be weak. The device may be detecting other organic fluorophores (e.g. intrinsic enamel proteins) rather than bacterial metabolites (Bader and Shugars, 2004).

The inability to detect bacteria in MIH enamel using PCR contrasts with the findings of Fagrell et al (2008), who argued that the increased sensitivity of MIH molars is caused by bacterial penetration into the underlying dentine. Although they found a few bacteria in dentine, they gave no information on the presence of post-eruptive breakdown, whether the teeth were symptomatic, the percentage of samples containing bacteria, or how contamination during sample preparation was prevented. In addition, the high LF readings from MIH enamel would have corresponded to a high bacterial load similar to that in dentinal caries, and not only few bacteria as demonstrated in their results. In contrast, Heijs et al (2007) did not report the presence of bacteria in a scanning electron microscopy (SEM) study of MIH enamel and dentin.
A plausible explanation for the sensitivity of MIH enamel is its increased porosity, a characteristic shown by polarised light microscopy (Jalevik and Noren, 2000). The increased porosity may allow external stimuli other than bacterial irritation, such as acids or chemical/thermal stimuli, to reach the dentine (Addy et al., 2000; Bissada, 1994; Heijs et al., 2007; Rodd et al., 2007). Increased MIH enamel porosity also implies a reduction in mineral density. The mineral density (MD) of MIH enamel was measured using x-ray microtomography (XMT) for ten sound and ten MIH teeth (Farah et al., 2010c). The average MD of MIH enamel was 19% lower than sound enamel. XMT also provided a qualitative finding that the MIH defects followed the incremental lines of enamel formation. Cuspal areas were usually mildly affected and cervical enamel was always normal.

Reduced mineral density of hard tissues usually results in poorer mechanical properties (Spears, 1997). Mahoney et al (2004) showed that the hardness and elastic modulus of affected areas in MIH enamel were reduced by up to 80% compared with normal enamel. Similarly, our nanoindentation study found the hardness and elastic modulus of MIH enamel were up to 70% less than that of sound enamel (Farah et al., 2008; Farah, 2009). The distribution of the reduced mechanical properties along the MIH enamel matched the distribution of the reduced MD as measured by XMT. In addition, the severity of the defects, as identified by their MD, correlated strongly with the colour of the enamel and its LF (Farah et al., 2010a). The darker the colour of the defect, the more hypomineralised it was. This finding may enable the dentist to determine the severity of MIH defects based on their colour and LF.

Reduced MD and elevated protein content have been suggested as reasons for the reduced mechanical properties of the defective enamel (Mahoney et al., 2004; Xie et al., 2008b). In 2010 we assessed the quantity and quality of the protein content of MIH enamel (Farah et al., 2010b). A similar study subsequently confirmed our findings (Mangum et al., 2010). We used the Lowry assay (Lowry et al., 1951) to quantify the protein content and SDS polyacrylamide gel electrophoresis (SDS-PAGE) to separate the constituent proteins. The identity of protein bands in the 50-70 kDa range was assessed by tandem mass spectrometry of tryptic fingerprints. Brown enamel showed a 15 to 21 fold higher protein content than
sound enamel, while the protein content of both chalky white and yellow enamel were approximately 8 times higher than sound glossy enamel. Hypomineralised amelogenesis imperfecta (AI) has been shown to have a similar, up to a 20-fold, increase in organic content over sound enamel (Wright et al., 1997). Despite this similarity, MIH and hypomineralised AI are significantly different. In AI, the increased protein content is mainly intrinsic enamel proteins like amelogenins, while in MIH the increased protein is due to serum proteins, mainly serum albumin. Serum albumin is the most abundant protein in MIH enamel (Farah et al., 2010b) but is absent in hypomaturative types of AI (Wright et al., 1997). Furthermore, AI results in generalised defects that affect all or most of the dentition while MIH is associated only with first permanent molars, permanent incisors and sometimes second primary molars. Finally the appearance of MIH enamel under SEM is different from that of AI enamel (Farah, 2009). Although the basic prismatic enamel architecture can be seen in MIH enamel, the borders between the prisms are usually not clear and in some areas the enamel appears featureless.

These differences in the types of proteins detected, combined with the clinical and histological appearances, probably indicate separate causes for MIH and AI. Hypomineralisation defects, by definition, occur during the mineralisation stage of enamel development. In AI, these defects occur when the degradation of the extracellular matrix proteins cannot be performed properly. This is either because of faulty proteolytic enzymes, as in the case of a defect in the enamelysin or kallikrein-4 genes, or because of mutations in the enamel matrix proteins rendering them unidentifiable to the enamel proteases, e.g. genetic defects in the amelogenin gene AMELX (Barron et al., 2010). The inheritance of mutations in AMELX and the enamel proteases show that AI is a hereditary disorder, unlike MIH.

While there are distinct differences between MIH and AI, MIH defects are similar to defects of enamel that has sustained trauma during the mineralisation stage of development. Trauma to developing enamel in the maturation stage results in an area of clinically demarcated opaque or yellow-brown enamel like that seen in MIH (Diab and Elbadrawy, 2000; Suckling, 1980; Suckling et al., 1989; Von Arx, 1993). Histologically, the SEM images are also very similar (Suckling et al., 1989). In fact, Suckling et al (1989) classified MIH
defects and traumatic defects in the same group based on their clinical and histological appearances.

Blunt instrument trauma delivered to developing enamel in sheep during the maturation stage resulted in opacities at both the site of the direct trauma and at remote sites. The remote trauma was likely caused by transmitted pressure that forced cells against the bony wall of the crypt (Suckling, 1980). This distribution of enamel defects can sometimes be seen in MIH molars. It has also been shown that excess serum albumin is found in enamel sustaining trauma during the mineralisation phase. Tarjan et al (2002) physically insulted the developing incisor tooth germ in rats. This insult allowed blood and albumin to penetrate past the detached ameloblasts and enter the developing enamel.

This information suggests the hypothesis that MIH defects may be due to trauma. Since the occlusal part of the first permanent molars starts mineralising around birth time, this places the possible trauma close to the time of birth. The nature of that trauma, whether pathological or physical, is not known. A wide range of illnesses (pathological trauma) can increase ‘permeability’ to blood proteins. This is demonstrated in epidermolysis bullosa junctionalis, where weakness in the hemidesmosomes between the ameloblasts allows serum albumin to leak into developing enamel. This results in opaque, yellow-brown defects with excess amounts of serum albumin (Kirkham et al., 2000). This type of pathological trauma is, however, generalised and affects the whole surface of all the teeth and more than just the first permanent molars and the incisors. Acute inflammatory reactions localised to the areas of the first permanent molars and incisors can also result in increased “permeability” to serum proteins. Generalised systemic illnesses have been shown to cause enamel defects. These may include inborn errors of metabolism, neonatal disturbances, infectious diseases, neurological disturbances, endocrinopathies, nutritional deficiencies, nephropathies, enteropathies, liver diseases and intoxication (Pindborg, 1982). It is of particular note that high fever (pyrexia) causes leakage of blood into developing enamel in rats (Kreshover and Clough, 1953). However, epidemiological studies have yet to identify a connection between MIH and any particular systemic illness or fever (Crombie et al., 2009). Furthermore, if a systemic illness
is indeed the cause of MIH, why is MIH not seen in other teeth since systemic illnesses can affect children at any age?

The suggestion that MIH is caused by a physical trauma to developing enamel of the first permanent molars seems unlikely due to the posterior position of the molars in the oral cavity, and the absence of deciduous predecessors. What kind of physical trauma could result in the clinical picture seen in MIH, where first permanent molars are affected asymmetrically with or without defects in the incisors? This distribution, and the chronological development of the dentition, suggests the possibility that pressure from passing through the birth canal or pressure from the obstetrician’s fingers or forceps may be that traumatic insult. Vascular pressure during birth is often seen as capillary haemorrhage in the face, retina conjunctiva and meningies at the time of birth (Hughes et al., 1999; Williams et al., 1993; Levin et al., 1980; Baum and Bulpit, 1970). This occurs when the head is exposed to atmospheric pressure while the body is under increased pressure due to maternal expulsive efforts. The resiliency of the facial bones in infants is evident in many newborns whose noses are somewhat flattened by the pressure exerted on the face during delivery. Such pressure on resilient bones may be transmitted to the teeth with parts of the enamel in the maturation stage of development, i.e. the occlusal parts of the first permanent molars. Pressure or other physical trauma during delivery, may be distributed around the teeth from all directions, resulting in circumferential types of defect, or the pressure may be more local, resulting in demarcated areas in affected molars. Both circumferential and localised defects are seen in MIH.
Research problem and objectives

Despite the above arguments about the causes of MIH, the source of albumin found in MIH enamel can only be speculated about. Other possible sources of this protein include saliva, bleeding gingiva during eruption and/or crevicular fluid. In other words, if blood is present within MIH enamel, it will have entered during enamel formation (as the result of traumatic insult), or after enamel formation is complete (either during tooth eruption or from gingival bleeding). Blood is unlikely to enter into enamel during eruption as haemorrhage does not normally occur at that time (Bhaskar, 1991). It is also unlikely that blood from gingival bleeding will enter into hypomineralised enamel. External proteins do not penetrate already demineralised enamel (or hypomineralised enamel in the case of MIH) and can only gain access to enamel during the dynamic process of demineralisation/remineralisation (Robinson et al., 1998b). Serum proteins that enter demineralising enamel have a specific pattern of distribution, and they only penetrate specific zones in the demineralised (hypomineralised in MIH) area and not the entire lesion (Shore et al., 2000). The penetration will also be limited as exposure to blood will be brief.

The purpose of this investigation was to better understand the protein content of MIH enamel and its origin, and to investigate the role of a specific part of MIH enamel proteins (namely serum/blood proteins) in the development of MIH.

To address the research problem, the objectives of this investigation were:

- To identify, as comprehensively as possible, the range of proteins from MIH and sound enamel using tandem mass spectrometry
- To determine the presence, distribution and possible gradient of two blood/serum marker proteins (haemoglobin and albumin) in MIH and sound enamel using direct immunofluorescence and confocal laser scanning microscopy. It was hoped that mapping the distribution of these proteins would shed light on their origin.
To determine the presence, distribution and possible gradient of the salivary marker protein α-amylase in MIH and sound enamel using direct immunofluorescence and confocal laser scanning microscopy. Salivary proteins were expected to penetrate MIH enamel poorly.

To determine the spatial distribution of the organic part of MIH and sound enamel using decalcified histopathological sections to help understand the distribution of the detected proteins.
Chapter III

Materials and Methods:
Background and General Description

This chapter provides a general description of the materials and methods used in the current research project. Details specific to each experiment are described in appropriate depth in the remaining chapters.

Sample collection and ethical issues

Extracted MIH first permanent molars were collected from several centres in New Zealand for this in vitro study. The extractions were performed as part of appropriate dental management plan for each patient. Before collection of the molars, relevant ethical issues were considered. In New Zealand, as in most parts of the world, research on human or animal participants, their tissues and materials, or personal information requires that ethical approval
is obtained through the proper channels and that the participant donating tissues also gives informed consent. Privacy, cultural, personal and health aspects usually need to be considered. The bodies responsible for granting ethical approval are the Health and Disability Ethics Committees of the New Zealand Ministry of Health under the provisions of the Health Information Privacy Code 1994. Since the project required the collection of extracted molars and personal health information from the participants through their dentists, both aspects required ethical approval.

The essential requirements of an informed consent are detailed in the Operational Standards for Ethics Committees 2006 published by the Ministry of Health (Ministry of Health of New Zealand, 2006). These include:

- Adequate information should be provided, in a manner clear to participants (including the use of an interpreter if needed), before any consent is given.
- The consent should be voluntary and without any undue influence.
- In cases involving children, in addition to the child’s assent, consent must be given by a person with appropriate legal authority (a parent or legal guardian).

Specific cultural issues apply in New Zealand, with special recognition given to Māori culture and the principles of the Treaty of Waitangi. The University of Otago has a policy on research consultation with Māori that operates under the umbrella of the Treaty of Waitangi and a memorandum of understanding with Ngai Tahu. This consultation was carried out before applying for ethical approval.

The current project continues an aspect of a project that started in 2006. It was realised early in the original project that the Otago region, including the city of Dunedin, would not provide the number of extracted MIH molars needed for the study. It was decided to approach dental centres located in different District Health Boards (DHBs) to provide extracted MIH molars. This required ethical approval from the New Zealand Multi-Region Ethics Committee.
The research project was approved in March 2007 (ethical approval number MEC/06/12/177) for a period of two years (Appendix 1). This approval was extended in 2009 (for two more years) to cover the current study. Paediatric dentists, oral surgeons, orthodontists, and dentists with special interest in paediatric dentistry (who usually manage children with MIH) were approached for help in obtaining extracted MIH molars. A detailed description of the clinical presentation of MIH was sent to each of these dentists. As all the extracted molars were obtained by paediatric dentists and dentists with special interest/experience in paediatric dentistry, it was trusted that the teeth were correctly identified as MIH molars and not affected by other types of enamel defects. The clinical appearance of the extracted teeth and the lack of presence of affected teeth other than the first permanent molars and incisors confirmed the MIH diagnosis.

A second more detailed letter followed with specific instructions for obtaining the informed consent, collecting patient demographic data and transport of the extracted teeth. Dentists were instructed to send first permanent molars with MIH only if children were willing to give up the extracted teeth. A $10 book voucher was sent as a gesture of gratitude to each participating child. Dentists who agreed to help in collecting the teeth were sent a package, approved by the ethics committee, containing the following items:

1. A letter providing specific instructions for the dentist (Appendix 2)
2. A questionnaire that would provide patient demographic information and assess whether the patient had other hypomineralised teeth (Appendix 3)
3. A tightly-sealed container containing thymol crystals for tooth disinfection. The dentist was requested to add saline or distilled water to provide a medium in which the extracted teeth would be sent to the researcher.
4. A tightly-sealed padded bag in which the sealed container would be enclosed.
5. An information sheet for the parents/guardians (Appendix 4)
6. A simplified information sheet for the participating children (Appendix 5)
7. A consent form to be filled and signed by the parents/guardians and returned with the teeth to the researcher (Appendix 6)
New Zealand Post was consulted and gave approval for sending extracted teeth in the tightly sealed containers.

Participating dentists were reminded frequently of the study by letter, email or direct contact. Dentists who sent extracted teeth were sent another package. Teeth received by the investigator were each assigned a special code (which linked each tooth to the patient from whom they were collected). The teeth were immediately refrigerated at 4°C until they were tested. Apart from the special code, the tooth containers had no reference to the patient’s name or address. The demographic details and the filled consent forms were stored in a locked cabinet that could be only accessed by the researcher and main supervisor.

The collected molar teeth

Upon receipt of an extracted molar, it was cleaned of blood and soft tissue under running water using a tooth brush and a curette. The teeth were then refrigerated in double distilled water with thymol crystals. In order to minimize the effects on the biochemical properties of the teeth, no other disinfectants were used.

Prior to testing, the five surfaces of each tooth were photographed using a Nikon D80 digital camera (Nikon Inc, USA) fitted with a Tamrom SP Di AF 90mm 1:2.8 Macro 1:1 macro lens (Tamron, Commack, USA) and a Sigma EM-140 DG ring flash (Sigma Corporation of America, Ronkonkoma, USA). Defects on the teeth ranged from posteruptive breakdown to localised, well demarcated white-opaque or yellow-brown defects on the slopes of the cusps, the marginal ridges, and/or the occlusal surfaces. None of the MIH molars showed hypomineralisation in the cervical third. In the absence of post-eruptive breakdown, the enamel was usually smooth and without pitting.
Protein assay

*Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*

In non-denaturing polyacrylamide gel electrophoresis, proteins are separated based on their charge and mass/size. Proteins move in an electric field according to their net charge. At the same time, molecular size-dependent interactions between proteins and the gel matrix slow down the movement according to the size of the protein.

In SDS-PAGE, proteins are treated with the negatively charged detergent sodium dodecyl sulphate (SDS) before electrophoresis. This detergent binds tightly, denatures and approximately equalises the charge density of each protein. This allows the electrophoretic separation of most proteins according to their mass. By comparing relative migration of the sample proteins with a group of proteins of known molecular weight in an adjacent lane, the molecular weight of each sample protein can be estimated (Lacks et al., 1979).

Once protein bands have been separated in the SDS-PAGE, they can be detected using various methods. The most common method is staining with Coomassie Brilliant Blue stain. This dye remains bound to proteins in the gel when non-bound background stain is removed by destaining in a mixture of methanol and acetic acid. More sensitive methods of protein band detection include silver staining, the use of fluorescent protein binding dyes and autoradiography of radiolabeled proteins (Campbell, 1995).

*Mass Spectrometry*

In the present study, mass spectrometry (MS) analysis of tryptic fingerprints was used to identify enamel proteins. In a typical MS procedure, the sample (mixture of protein fragments) is loaded into the mass spectrometer, and then ionised by one of several methods,
resulting in the formation of charged particles. These particles are then passed through a magnetic or electric field into a detector. Based on their relative motion, the mass-to-charge ratio (m/z) of the particles is calculated (Sparkman, 2000).

The mass spectrometer consists of an ionization source, a mass analyser and a detector. Once the ion source has converted particles or molecules of the sample into ions (usually cations), they become easier to move and manipulate than neutral molecules. The ionization can be effected in several ways. The ions can be generated by bombarding gaseous sample molecules with a beam of electrons. Alternatively, chemical ionization produces ions by proton transfer from a large excess of ionized reagent gas yielding a positive ion. A third method is called desorption ionization (e.g. field desorption, electrospray and Matrix Assisted Laser Desorption/Ionisation (MALDI)). During this processes the molecules are evaporated from a surface and ionised. For example, in MALDI, the impact of high energy photons leads to both sample evaporation and ionization (Dass, 2007; Domon and Aebersold, 2006).

In the analyser the ions are sorted and separated according to their m/z ratio by applying electromagnetic fields. The most widely used analysers are magnetic sectors, quadrupole mass filters, quadrupole ion traps, Orbitrap, Fourier transform ion cyclotron resonance spectrometers, and time-of-flight mass analysers. The mass spectrometer used in the present study was a linear quadrupole ion trap (LTQ)-Orbitrap XL hybrid mass spectrometer. The separated ions were detected electronically and the resulting information stored and analysed in a computer.

In the present study, MS was used for qualitative analysis of enamel proteins. It employed enzymatically digesting excised SDS-PAGE protein bands into peptides using trypsin. Liquid chromatography (LC) was then used to fractionate the peptide digest, which were then introduced directly into the mass spectrometer through electrospray ionization (EI). The masses of the individual peptides were measured and these masses then in silico (computer simulation) compared to a database of known protein sequences (UniProt/SWISS-PROT) using a search engine (Mascot). The search engine’s function is to translate the known genome of the human into proteins, then cuts the proteins at sites theoretically cleaved by trypsin, and calculate the masses of the peptides from each protein. Comparison of the
masses of the peptides of the digested proteins to the theoretical peptide masses of human proteins identifies best matches based on a complex statistical analysis. When a significant number of digested peptides match the predicted masses for a particular protein, this protein is identified as likely to be present in the sample (Hardouin, 2007; Shadforth et al., 2005).

Where a large number of proteins are present in one sample, the spectrum of resultant peptides might significantly complicate the database search and possibly mis-identify proteins in the mixture. The use of MS/MS protein processing can overcome this problem by extracting protein sequence information for individual protein fragments (Clauser et al., 1999). MS/MS (also known as Tandem MS) involves multiple steps of MS. A hybrid mass spectrometer is used for MS/MS.

**Direct immunofluorescence**

Immunofluorescence microscopy is used to detect the distribution and relative abundance of a known antigen (proteins in the present study). Since individual proteins or their antigen-antibody complexes are usually not readily visualised in the microscope, the fluorescence of a suitable dye covalently bonded to the antibody is detected using a fluorescence microscope or by confocal microscopy. The immunofluorescence of antigen-antibody-fluorescent dye complexes was used here to trace the distribution of haemoglobin, albumin, and amylase in the organic matrix of enamel. In comparative studies, the fluorescence of the antigen-antibody complex should provide a relative measure of the amounts of antigen present at each position in the sample. The basic concepts of fluorescence and the use of confocal microscopy are explained in subsequent sections.

Two types of immunofluorescence are available: direct (or primary) immunofluorescence (DIF) in which one primary antibody (conjugated to the fluorescent dye) specific to the target protein is used, and indirect (secondary) immunofluorescence (IDIF) in which the dye is instead conjugated to a species-specific secondary antibody against the primary antibody.
DIF and IDIF have advantages and disadvantages. IDIF is more commonly used, and although it requires more steps and a longer time to perform, it is cheaper, can amplify signals and is more flexible in large-scale studies because of the use of species-specific secondary antibodies. Because the present study envisaged triple labeling using quantum dots instead of fluorescent dyes, DIF was employed.

Confocal Laser Scanning Microscopy (CLSM)

Since its commercial introduction in the 1990s, confocal microscopy has become an important approach for biomedicine because it has numerous capabilities that are not available through conventional optical microscopy.

Fluorescence microscopy depends on the properties of fluorophores like organic dyes and quantum dots. The absorption of light at certain wavelengths (excitation) by the fluorophore results in the emission of light at a specific (lower energy) wavelength. Fluorescence microscopy uses this property to detect and localise molecules, organelles, cells or proteins to which a fluorophore is bound. For example, the presence and distribution of a certain antigen in a specimen can be detected using fluorescent-tagged antibodies specific to that antigen that have bound to the specimen. The specimen is then visualised using the fluorescence or confocal microscope.

The confocal microscope is essentially an epifluorescence microscope, in which the light source illuminates the specimen from the same side and not from the opposite side (trans-fluorescence). As explained in Figure 3.1, the epifluorescence microscope utilises a dichromatic mirror that reflects light shorter than a certain wavelength (violet light from the laser light source in Figure 3.1). The laser light excites the specimen which in turn emits light (red light in Figure 3.1) that passes through the dichromatic mirror. The highest intensity of the excitation light is at its focal point on the specimen. However, the other parts of the sample receive some of the laser light and they fluoresce (blue light in Figure 3.1), causing
background noise. This problem is overcome by incorporating a pinhole in the path of the emitted light at the focal point of the objective lens so that only light from the specimen’s focal point passes through. Because the focal point of the objective lens forms the image where the pinhole is, these two points are known as “conjugate” or “confocal”; hence the name of the microscope (Claxton et al., 2006; Prasad et al., 2007).

![Diagram](Image)

**Figure 3.1.** Diagrammatic representation of the components of the confocal microscope. In CSLM the specimen is illuminated point by point. The exciting laser beam is scanned across the entire specimen, and light emitted at the focal points is reconstructed into an image using computer software.
Quantum dots

Quantum dots (QDs) are nanocrystals of comparable size to proteins. QDs are made of semiconductor material, e.g. selenides or sulfides of metals like cadmium or zinc. Their ~10-50 atoms diameter core is coated with a zinc sulphide semiconductor shell which enhances optical properties and stability. They are fluorophores with intrinsic brightness and photostabilities that are several fold higher than conventional organic dye fluorophores. The shell is covered with an organic polymer layer that confers solubility in water and allows conjugation to target molecules such as immunoglobulins (Deerinck, 2008).

QDs have broad absorption spectra and narrow emission spectra, giving minimal overlap between colours/spectra. In addition, different QDs can be equally excited using a single wavelength, making them excellent tools for multiple labeling of single specimens (Chan et al., 2002). The wavelength emitted by a QD depends on its size and not on the material from which it is made. This is referred to as tuneability. Quantum dots are therefore “tuned” during production to emit a desired colour. Smaller QDs emit light at the blue end of the spectrum while larger QDs emit light closer to the red end.

QDs are more versatile than conventional fluorophores because they can also be used in electron microscopy. QD fluorescence is durable and can withstand the chemical treatments and high temperatures required during sample processing for electron microscopy. QDs are sufficiently electron dense to be visualized in the electron microscope in the 60-keV to 400-keV accelerating voltage range (Deerinck, 2008).

In the present investigation QDs conjugated to primary antibodies against human serum albumin, human haemoglobin and salivary amylase were used to map the presence/distribution of those protein antigens in normal and defective enamel.
Chapter IV

Qualitative Analysis of the Protein Content of Sound and Hypomineralised Enamel

Introduction

Farah et al. (2010b) estimated the protein content of MIH and sound enamel using a miniaturized version of the method of Lowry et al (1951). MIH enamel showed an 8–21-fold higher protein content than sound enamel. Samples of protein extracted from MIH and sound enamel were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the component protein bands identified using tryptic fingerprint tandem mass spectrometry (MS/MS) of excised gel pieces. The results indicated abundance in MIH enamel of serum proteins, particularly serum albumin. The study was incomplete because only protein bands estimated by SDS-PAGE to be 50-70 kDa in size were analysed by MS/MS.
The aim of this study was to provide a comprehensive compendium of the proteins present in MIH and sound enamel by using MS/MS. Two pilot studies were undertaken to provide improved methodology.

**Materials and methods**

**General Description**

*Enamel sample preparation and protein extraction*

These procedures were carried out essentially according to Farah et al (2010b). A sterile high-speed handpiece and bur were used under sterile saline irrigation to cut small pieces of enamel from teeth. The enamel pieces were then crushed into coarse powder (in a small metal dish using a large sterile dental amalgam condenser) and weighed. TCA (990 µl of 20%) and the protease inhibitor phenylmethylsulfonylfluoride (PMSF, 10 µl of 100 mM in isopropanol) were added to the powdered enamel and the mixture incubated at room temperature overnight. The enamel powder was usually completely dissolved by the next day. The sample was then centrifuged (13,200 rpm, 10 min at 4°C) and the pellet suspended in 50 µl of 1% sodium dodecyl sulphate (SDS). The sample was analysed by MS/MS at the Centre for Protein Research, University of Otago.

*Sample preparation for MS/MS*

The protein sample was digested with trypsin (DigestPro Msi, Intavis AG, Cologne, Germany) using a robotic workstation and a protocol based on the method of Shevchenko et al. (1996). Eluted peptides were dried using a centrifugal concentrator.
**LC-MS/MS of tryptic peptides**

Peptide samples resolubilised in 8µl 1% [v/v] acetonitrile in 0.2% [v/v] formic acid were injected into an Ultimate 3000 nano-flow LC-System (Dionex Co,CA) that was in-line coupled to the nano-electrospray source of a LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Scientific, San Jose, CA). Peptides were separated using an in-house packed emitter-tip C-18 column (8-9cm long, 75 um internal diameter fused silca tubing) developed using a 1-80% [v/v] acetonitrile gradient in 0.2% [v/v] formic acid at a flow rate of 200-300 nl/min.

**Instrument setting for the LTQ-Orbitrap**

Full MS in a mass range between m/z 300-2000 was performed in the Orbitrap mass analyser with a resolution of 60,000 at m/z 400 and an AGC target of 2e5. The preview mode for FTMS master scan was enabled to generate precursor mass lists. The strongest 5 signals were selected for collision induced dissociation (CID)-MS/MS in the LTQ ion trap at a normalised collision energy of 35% using an AGC target of 1e5 and two microscans. Dynamic exclusion was enabled with 2 repeat counts during 30 sec and an exclusion period of 180 sec. The exclusion mass width was set to 0.01.

**Data analysis**

For protein identification MS/MS data were searched against the SWISS-PROT primary sequence database (downloaded April 2009) using the Mascot search engine (http://www.matrixscience.com). The search was enabled for full tryptic peptides with up to 3 missed cleavage sites. Carboxyamidomethyl cysteine, oxidized methionine, and pyroglutamate were included as variable modifications. The precursor mass tolerance threshold was 10 ppm and the maximum fragment mass error 0.8 Da. The identifications were filtered with a significance threshold of p < 0.05 and ions score cut-off of 35. The
number of significant peptide identifications (and the corresponding sequence coverage) was then extracted for the proteins identified.

**Pilot Study No. 1**

*Enamel sample preparation and protein extraction*

Enamel (0.006 g) from a single healthy tooth, unaffected by MIH, was treated with 20% trichloroacetic acid (TCA) to dissolve the mineral phase and precipitate the proteins in samples for proteomic analysis (Farah et al, 2010b). It should however be noted that TCA may not precipitate some of the smaller and/or hydrophobic peptides. The salt concentration of the sample in this pilot study was too high after tryptic digestion and rapidly blocked both the trap and analytical columns.

**Pilot Study No. 2**

The protocol from the first pilot study was modified to reduce mineral contamination as follows:

1. Half of the pellets of precipitated protein obtained by TCA treatment of enamel samples were washed three times with TCA to remove residual mineral. The other half of the pellets acted as a control and was not washed with TCA.
2. The pellets dissolved in SDS were separated by SDS-PAGE (Laemmli, 1970).
3. Gloves, head cap and face shield were used during enamel preparation and protein extraction to minimise keratin and collagen contamination.

Enamel from four MIH molars and five sound teeth were used in this pilot study. The final weights of the enamel samples before dissolving in 20% TCA were:

- MIH-1 (control): 0.009 g
- MIH-2 (triple washed with TCA): 0.007 g
- Sound-1 (control): 0.011 g
- Sound-2 (triple washed with TCA): 0.008 g

Modified Main Study

The results of the pilot studies led to the following method of sample preparation.

Four MIH molars from three individuals and two sound teeth were selected for this study. The four MIH molars are shown in figures 4.1 – 4.4. The teeth were stored at 4º C in distilled water containing thymol crystals until they were used.

Figure 4.1. MIH molar No.1. The distal and distobuccal surfaces showed post-eruptive breakdown (PEB), and were used in the MIH-PEB sample. The intact mesial and mesiolingual parts were used in the MIH-intact sample. (a) buccal, (b) mesial, (c) lingual, (d) distal, (e) occlusal surfaces.
Figure 4.2. MIH molar No.2. The buccal surface showed PEB and was used in the MIH-PEB sample. (a) buccal, (b) mesial, (c) lingual, (d) distal, (e) occlusal surfaces.

Figure 4.3. MIH molar No.3. Intact MIH enamel was used in the MIH-intact sample. (a) buccal, (b) mesial, (c) palatal, (d) distal, (e) occlusal surfaces
Figure 4.4. MIH molar No.4. Intact MIH enamel was used in the MIH-intact sample. (a) buccal, (b) mesial, (c) palatal, (d) distal, (e) occlusal surfaces

For ease of handling, each tooth was embedded in Castapress transparent acrylic resin (Vertex-Dental B.V., Zeist, the Netherlands). The resin was cured for 25 min in a cold water bath under 2 bar pressure. The combined effect of positive pressure and heat dissipation during the resin’s exothermic setting reaction minimised air bubble generation.

Three sagittal sections were cut under water irrigation from each tooth through the affected area using a diamond grit-impregnated saw fitted to an automated precision sectioning machine (Accutom-50, Struers A/S, Ballerup, Denmark) (Figure 5.6). A sample holder moves towards the cutting wheel at a predetermined feed-speed; the lower the feed-speed, the more precise the cutting. The settings used were based on previous work (Farah et al., 2010c). The sectioning was done with medium force, 2000 rpm, and low feed-speed of 0.05 mm/second to avoid deviation of the cutting wheel. The cut sections were kept hydrated by storage in phosphate buffered saline (PBS).
The thickness of each cut section was 1 mm. With such thin sections, the dentine-enamel junction (DEJ) was easily identifiable and deep grooves were made in the enamel close to and parallel to the DEJ. Enamel pieces were then fractured off the tooth section. The adherent smear layer was removed by sonication. The samples contained in Eppendorf tubes with double distilled water were treated using an ultrasonic bath.

The mineral content of each enamel piece was dissolved in 1.5 ml of 20% TCA (with 10 µl proteinase inhibitor) for 1-2 days. The protease inhibitor was prepared by dissolving one pill of Roche proteinase inhibitor cocktail without EDTA in 20 ml of deionised distilled water. For samples that were incompletely dissolved after 24 h, the pellet recovered by centrifugation (13,200 rpm for 10 min at 4°C) was treated again with fresh 20% TCA.

After full dissolution of the mineral phase, the samples were centrifuged (13,200 rpm, 10 min at 4°C) and the pellets collected. The remaining mineral was removed by washing the pellets three times by re-suspension in 20% TCA for 5 minutes and centrifugation. Any remaining TCA was removed by adding to each pellet 300 µl of 80% acetone (stored at -20°C), followed by centrifugation at 13,200 rpm for 10 min at 4°C. The pellet was dissolved in 100 µl of deionised distilled water.

The samples were analysed by Dr Farah and Dr Klefmann at Centre for Protein Research, Department of Biochemistry, University of Otago, using SDS-PAGE and MS/MS. Running the SDS-PAGE for a short period separated the proteins from the high mobility minerals. 60 V was applied during the 30 minutes required for protein stacking and 200 V during the 15 min used for protein resolution in a 12% acrylamide gel. Only Coomassie stained protein in the gel close to the loading wells was used for the MS/MS.
Results

Over seventy proteins were identified in the three experiments, including the two pilot studies. Table 4.1 summarises the MS/MS results from the three studies. The numbers presented in the table indicate the absolute numbers of peptide queries matched using the Mascot search engine.

Pilot study 2 showed that removal of residual mineral content using TCA washes dramatically increased the numbers of matches detected in normal enamel but not MIH enamel. However, the attempts to minimise contamination by collagens and keratin were unsuccessful.

Twenty seven proteins were detected in MIH enamel but not sound enamel (light green cells in Table 4.1). Most of these gave only a single query match. Many of these proteins have previously been found in normal enamel. We note that haemoglobin (Hb) was only detected in MIH enamel with post-eruptive breakdown (PEB) and with a single query match.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Sound enamel</th>
<th>Sound enamel-No TCA Wash</th>
<th>Sound enamel-TCA Wash</th>
<th>MIH enamel-No TCA Wash</th>
<th>MIH enamel-TCA Wash</th>
<th>Sound enamel</th>
<th>MIH enamel-Intact</th>
<th>MIH enamel-fractured</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Weight (g)</td>
<td>0.006</td>
<td>0.011</td>
<td>0.008</td>
<td>0.009</td>
<td>0.007</td>
<td>0.044</td>
<td>0.009</td>
<td>0.008</td>
</tr>
<tr>
<td>collagen alpha-1(I) chain preproprotein</td>
<td>97</td>
<td>196</td>
<td>82</td>
<td>51</td>
<td>27</td>
<td>201</td>
<td>113</td>
<td>80</td>
</tr>
<tr>
<td>collagen alpha-2(I) chain precursor</td>
<td>80</td>
<td>199</td>
<td>81</td>
<td>37</td>
<td>52</td>
<td>137</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>collagen, type XI, alpha 2 isoform 3 preproprotein</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>keratin, type I cytoskeletal 10</td>
<td>19</td>
<td>18</td>
<td>41</td>
<td>27</td>
<td>58</td>
<td>10</td>
<td>38</td>
<td>42</td>
</tr>
<tr>
<td>keratin, type II cytoskeletal 1</td>
<td>39</td>
<td>18</td>
<td>48</td>
<td>31</td>
<td>62</td>
<td>20</td>
<td>33</td>
<td>63</td>
</tr>
<tr>
<td>keratin 2</td>
<td>15</td>
<td>14</td>
<td>25</td>
<td>18</td>
<td>47</td>
<td>9</td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>keratin, type I cytoskeletal 9</td>
<td>36</td>
<td>11</td>
<td>27</td>
<td>19</td>
<td>49</td>
<td>12</td>
<td>26</td>
<td>32</td>
</tr>
<tr>
<td>keratin, type II cytoskeletal 6B</td>
<td>4</td>
<td>6</td>
<td>18</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>keratin, type II cytoskeletal 6A</td>
<td></td>
<td>11</td>
<td></td>
<td></td>
<td>9</td>
<td>24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>keratin, type II cytoskeletal 6C</td>
<td></td>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>keratin, type II cytoskeletal 5</td>
<td>8</td>
<td>3</td>
<td>4</td>
<td>18</td>
<td>7</td>
<td>8</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>keratin, type I cytoskeletal 16</td>
<td></td>
<td>7</td>
<td>14</td>
<td>7</td>
<td>10</td>
<td>16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>keratin, type I cytoskeletal 14</td>
<td>6</td>
<td>3</td>
<td>6</td>
<td>8</td>
<td>17</td>
<td>8</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>keratin, type I cytoskeletal 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>keratin, type II cytoskeletal 1b</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>keratin, type II cytoskeletal 78</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>78</td>
<td>1</td>
</tr>
<tr>
<td>albumin preproprotein</td>
<td>29</td>
<td>95</td>
<td>97</td>
<td>95</td>
<td>18</td>
<td>28</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>pigment epithelium-derived factor precursor</td>
<td>3</td>
<td>17</td>
<td>1</td>
<td>1</td>
<td>12</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>biglycan preproprotein</td>
<td>1</td>
<td>16</td>
<td>1</td>
<td></td>
<td>11</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>alpha-2-HS-glycoprotein</td>
<td>10</td>
<td></td>
<td>8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serine proteinase inhibitor, clade A, member 1 precursor</td>
<td>9</td>
<td>17</td>
<td>12</td>
<td>12</td>
<td>10</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Sound enamel</td>
<td>Sound enamel- No TCA Wash</td>
<td>Sound enamel-TCA Wash</td>
<td>MIH enamel-No TCA Wash</td>
<td>MIH enamel-TCA Wash</td>
<td>Sound enamel</td>
<td>MIH enamel-Intact</td>
<td>MIH enamel-fractured</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>--------------</td>
<td>--------------------------</td>
<td>-----------------------</td>
<td>------------------------</td>
<td>---------------------</td>
<td>--------------</td>
<td>-------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Sample Weight (g)</td>
<td>0.006</td>
<td>0.011</td>
<td>0.008</td>
<td>0.009</td>
<td>0.007</td>
<td>0.044</td>
<td>0.009</td>
<td>0.008</td>
</tr>
<tr>
<td>vitronectin precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prothrombin preprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>matrix metalloproteinase-20 preprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kininogen-I isoform 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>antithrombin-III precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ameloblastin precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>osteomodulin precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lumican precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alpha-1-antichymotrypsin preprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPARC precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>complement component C9 precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>transforming growth factor-beta-induced protein ig-h3 precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>apolipoprotein A-I preproprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coagulation factor IX preproprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thrombospondin-1 precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Sound enamel</td>
<td>Sound enamel-No TCA Wash</td>
<td>Sound enamel-TCA Wash</td>
<td>MIH enamel-No TCA Wash</td>
<td>MIH enamel-TCA Wash</td>
<td>Sound enamel</td>
<td>MIH enamel-Intact</td>
<td>MIH enamel-fractured</td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
<td>--------------------------</td>
<td>-----------------------</td>
<td>------------------------</td>
<td>---------------------</td>
<td>--------------</td>
<td>-----------------</td>
<td>------------------</td>
</tr>
<tr>
<td></td>
<td>Pilot Study 1</td>
<td>Pilot Study 2</td>
<td>Pilot Study 2</td>
<td>Pilot Study 2</td>
<td>Pilot Study 2</td>
<td>Final Study</td>
<td>Final Study</td>
<td>Final Study</td>
</tr>
<tr>
<td>Sample Weight (g)</td>
<td>0.006</td>
<td>0.011</td>
<td>0.008</td>
<td>0.009</td>
<td>0.007</td>
<td>0.044</td>
<td>0.009</td>
<td>0.008</td>
</tr>
<tr>
<td>amelogenin, X isoform isoform 3 precursor</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>metalloproteinase inhibitor 1 precursor</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>clusterin isoform 1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>desmoglein-1 preproprotein</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>procollagen C-endopeptidase enhancer 1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>enamelin precursor</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>olfactomedin-like protein 1 precursor</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>histidine-rich glycoprotein precursor</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>filaggrin family member 2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>prolactin-induced protein precursor</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>complement C3 precursor</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>kallikrein-4 preprotein</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lysozyme precursor</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>hornerin</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>elongation factor 1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>actin, alpha skeletal muscle</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>protein S100-A9</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>haemoglobin subunit beta/gamma</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>zinc-alpha-2-glycoprotein precursor</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2F transcription factor 6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Sound enamel</td>
<td>Sound enamel- No TCA Wash</td>
<td>Sound enamel-TCA Wash</td>
<td>MIH enamel-No TCA Wash</td>
<td>MIH enamel-TCA Wash</td>
<td>Sound enamel</td>
<td>MIH enamel-Intact</td>
<td>MIH enamel-fractured</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>--------------</td>
<td>---------------------------</td>
<td>-----------------------</td>
<td>------------------------</td>
<td>---------------------</td>
<td>--------------</td>
<td>------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Sample Weight (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dermcidin preprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>arginase-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stabilin-1 precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>neuroserpin precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>transthyretin precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serotransferrin precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serine/threonine-protein kinase 13 isoform 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>proteoglycan 3 precursor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PREDICTED: similar to immunoglobulin lambda-like polypeptide 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trypsin-1 preproprotein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>beta-catenin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bleomycin hydrolase</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>keratinocyte proline-rich protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.1.** Protein identified as present in sound and/or MIH enamel from the 3 MS/MS studies. Grey columns denote MIH enamel, white ones sound enamel. Light blue cells indicate proteins indentified in MIH enamel samples only.
Discussion

Sound enamel proteins

Keratin and collagen are present in enamel in trace amounts (Acil et al., 2005; Eastoe, 1963; Fonseca et al., 2008; Lesot et al., 1988; Lin et al., 1993; Springer et al., 2005). Keratin may reflect remnants of the enamel forming cells, ameloblasts, trapped in the final tissue (Warshawsky and Josephsen, 1981). They are also commonly detected contaminants in MS/MS (Park et al., 2009) and their dominant abundance in the MS/MS results most likely reflects contamination from the skin. Considerable care was taken to avoid contamination of the cut enamel samples from dentine (i.e. thin teeth sections were prepared, with enamel cut away from the DEJ and ultrasonic cleaning of the samples), and from the skin (head cover, masks and gloves were used). It therefore seems likely that the keratin and collagen contamination occurred during SDS-PAGE process or the MS/MS procedure. In addition, the lower content of acidic amino acids in keratin makes it more easily detected with MS/MS.

The results of the MS/MS in this chapter may represent the most comprehensive list of enamel proteins to date. The protein compositions of sound and MIH appear similar. A few proteins detected in the MIH enamel were not detected in sound enamel, but most of those had peptide query matches at the threshold of detection (1 peptide match).

A recent advance of our understanding of the enamel matrix proteins resulted from analysis of the commercially available Emdogain® preparation that is used widely to promote regeneration of soft and hard periodontal tissues. The active ingredient of Emdogain® is Enamel Matrix Derivative (EMD) derived from porcine enamel organic matrix. MS/MS analysis of EMD identified a range of proteins in the preparation, most of which were also identified in the current investigation of human enamel. These proteins were: ameloblastin, immunoglobulin gamma chain, immunoglobulin heavy chain, cytoskeletal β-actin, ATP synthase, fetuin A (α2-HS glycoprotein in humans), α1-antichymotrypsin (Zilm and Bartold,
2011). The range of proteins identified in Emdogain® should be considered with caution as the mass production of this material may result in the inclusions of proteins external to enamel.

A large number of the proteins discovered in this MS/MS work have not been reported previously in enamel preparations. On the other hand, several of these proteins have been described to be involved in several steps of enamel formation, tooth differentiation, cell adhesion and movement, mineralisation and other non-specified activities of the ameloblasts or the inner enamel epithelium. Some other proteins may have been the remnants of the ameloblastic cellular layer responsible for the formation of enamel (Bartlett et al., 2006). The following discussion briefly describes activities ascribed to some of the proteins we have identified in enamel that are not considered to be part of the main enamel matrix.

**Metalloproteinase, serine proteinase and their inhibitors:** The metalloproteinase appears to be restricted to the secretory stage with a specific role, while the serine proteinase is more active during the maturation stage and appears to have a nonspecific role to remove all the remaining protein allowing for crystal growth (Robinson et al., 1998a). These proteinases can be retained in the maturing enamel, most likely by binding to the HA crystals (Brookes et al., 1998). Bartlett and Simmer (1999) predicted the presence of proteinase inhibitors in enamel since proteinase inhibitors are commonly present within the same tissues with their proteinase partners. Serine proteinase inhibitor (Davaadorj et al., 2010) and metalloproteinase inhibitor (Hannas et al., 2007; Yoshiba et al., 2003) were subsequently detected in developing enamel. They are expressed by ameloblasts and appear to have roles in matrix regulation.

**Transforming growth factor β2 (TGF-β2), and kinase enzymes:** TGF-β2 was reported to be expressed by ameloblasts during enamel formation and is thought to play an important role in tooth initiation, epithelial morphogenesis and ameloblast differentiation (Li et al., 2008). TGF-β2 also induces angiogenesis in the enamel organ (Dickson et al., 2001). TGF-β2 activates kinase enzymes and has an important role in the induction of cell differentiation into ameloblasts, elongation of the epithelial sheath, and secretion of enamel matrix proteins (Moriguchi et al., 2011).
**Osteomodulin:** This protein is expressed in ameloblasts and plays a role in the mineralisation process (Buchaille et al., 2000; Couble et al., 2004).

**Actin:** Actin is expressed by ameloblasts and has an important role in facilitating ameloblasts movement during enamel secretion and mineralisation. It is thought to be involved in the development of dental fluorosis (Li et al., 2005; Li et al., 2011; Sehic et al., 2010).

**Transferrin:** A radioautographic study demonstrated that transferrin transports iron into maturation-stage ameloblasts (McKee et al., 1987).

**Catenin:** This protein is important in directing the movement of rows of ameloblasts during the secretory stage of enamel formation, by directing their attachment and detachment (Bartlett et al., 2010). Catenin is also important in enamelin gene expression (Tian et al., 2010) and in shaping the final morphology of the individual tooth (Liu et al., 2008).

**Biglycan:** This member of the small leucine-rich proteoglycans family, appears to control the expression of amelogenin during enamel formation (Goldberg et al., 2002; Goldberg et al., 2005). Biglycan also plays an important, but not fully-understood, role in the development of other mineralised tissues like dentine and bone. An increase in the biglycan level in mice results in delayed tooth phenotype development (Ye et al., 2004) and inhibits the growth and proliferation of hydroxy apatite crystals in mineralised tissues (Boskey, 1997).

**Serum proteins:** Several serum proteins have been also found in developing enamel. These include serum albumin (Fincham et al., 1999; Limeback et al., 1989; Strawich and Glimcher, 1990), α2-HS glycoprotein and gamma globulins (Robinson et al., 1998a; Smith et al., 1995). Serum proteins are endocytotically engulfed by ameloblasts. Nanci et al (1996) used intravenous injection of immuno-labelled albumin and α2-HS glycoprotein into rats to show that serum proteins appeared inside the ameloblasts and in the enamel organ. The authors concluded that the uptake of serum proteins may occur in a non-selective manner and may be
related to the way ameloblasts remove unwanted and degraded proteins from the matrix. The finding, in the current investigation, of several serum proteins supports this conclusion.

The proteins discovered in mature enamel using MS/MS appear to be relics of enamel development that are trapped in the mature enamel. Although most of the proteins we have identified by MS/MS of mature enamel have been reported previously, most were only described in enamel tissue or cells isolated from developing enamel of animal origin and not from fully mature human enamel. The detected proteins may also represent remnants of the ameloblasts and other cells involved in enamel formation. This adds a new perspective to the fact that enamel makes an excellent marker for systemic insults that affected the body during the period of enamel formation.

Park et al (2009) performed MS/MS on extracted dentine proteins from human teeth. They identified in dentine several proteins identified in the current study. These include serum proteins, serine proteinases, immunoglobulins, and those proteins involved in the formation of the extracellular matrix. These similarities may shed new light on our understanding of the relationship between enamel and dentine.

**MIH enamel proteins**

The high level of collagen and keratin contamination has complicated analysis of the MS/MS results since it can mask the presence of other proteins and because it does not allow for a semi-quantitative analysis of the results. This explains why a certain protein, for example serum albumin, showed different numbers of peptide query matches for the same weight of enamel from different samples. These differences in the peptide query matches might also be genuine, reflecting different abundance in different samples. Larger protein samples may be needed to minimise the keratin contamination problem.

In our previous investigation (Farah et al., 2010b), only proteins with apparent molecular weights of 50-70 kDa during SDS-PAGE gel were analysed by MS/MS. This
minimised the collagen/keratin contamination and allowed more precise determination of the albumin quantity in MIH and sound enamel. The numbers of database matches detected for serum albumin were 3-7 fold higher for MIH enamel than for sound enamel. Similar results were found for antitrypsin and antithrombin III.

The inability to quantify the relative abundance of the proteins detected poses another problem. Understanding posttranslational modifications of proteins (e.g. oxidation, phosphorylation) in MIH enamel may help shed light on its biochemical pathogenesis. Phosphorylation of extracellular proteins is a critical step in the mineralisation of hard tissues like enamel and dentine (George and Veis, 2008). These modifications are usually described in terms of percentages and locations of amino acids modified. Using mass spectrometry, Finch et al. (1993) identified certain sites of oxidation of albumin when treated with hydrogen peroxide (e.g. Cys34, Met123, Met298, Met446, Met548). The extent of oxidation varied with location in the protein sequence, suggesting a relationship between oxidation and the three-dimensional conformation of albumin. Because it was not possible to determine the amount of each protein in MIH and sound enamel samples (only their presence or absence was detected), the percentage of modifications was also not possible to be determined.

The observed similarity in proteins between MIH and sound enamel, and the inability to quantify the amount of each protein or the posttranslational modifications, has made the present study more useful in describing the repertoire of enamel proteins. However, this study also showed the strong similarity between intact MIH enamel and MIH enamel with PEB, indicating a similar origin for the proteins. Hb was only detected in MIH enamel with PEB, but at only 1 query match. The confidence in this result is, therefore, weak. In addition, Hb was detected in intact MIH enamel using immunofluorescence as demonstrated in Chapter V.

Proteins identified only in MIH enamel or that are clearly in greater abundance in this tissue are proteins involved in tissue injury and its repair, or in bleeding and coagulation. These proteins include: prothrombin, antithrombin, SPARC protein, complements and immunoglobulins, coagulation factors, thrombospondin, hornerin and Hb. As stated above,
however, it cannot be stated with confidence based on these results that these proteins definitely reflect tissue trauma and bleeding.

The study most closely related to the current investigation is that of Mangum et al (2010). MIH enamel proteins were extracted by dissolving the mineral phase and precipitating the proteins in trifluoroacetic acid. Analysis of the extracted proteins by MS/MS identified the range of proteins present in MIH enamel. Table 4.2 describes the proteins indentified in Mangum et al (2010) study. There is a strong agreement between the findings of the two studies. However, Mangum et al (2010) explained the results differently. They argued that enamel isolated from intact MIH enamel contained elevated amounts of serum proteins, while that isolated from fractured MIH enamel contained salivary proteins, including haemoglobin. Based on differences in the protein profiles for the intact MIH enamel and fractured MIH enamel, they concluded that the surface integrity decided the proteomic profile of the enamel, i.e. intact MIH enamel obtained its elevated albumin from serum while salivary proteins gained access into fractured MIH enamel. However, the following reservations/explanations are important when considering their argument:

- It is unclear how carious enamel was excluded when fractured enamel was collected. Inevitably, carious enamel, saturated with salivary proteins, or even non-carious fractured enamel may absorb some salivary proteins. In the present study, when MIH enamel with PEB was collected, fractured enamel at the surface was avoided and only the part cervical to the fracture (but still within the affected MIH enamel) was collected to avoid surface contamination.

- The enamel was collected by scraping with a scalpel. This will most likely retain any adherent proteins on the surface. In the current study, outer enamel was avoided in order to avoid incorporating external proteins.

- Careful inspection of the SDS-PAGE gel and MS/MS results of the intact and fractured MIH enamel do not show considerable differences as claimed by the authors. Figure 4.5 (a) shows that the Coomassie stained SDS-PAGE profiles of proteins from intact MIH enamel and (b) of fractured MIH enamel are very similar.

- Haemoglobin is more abundant in blood than in saliva
Robinson et al (1998b) have clearly demonstrated that salivary proteins can penetrate into porous (demineralised) enamel only during the dynamic process of demineralisation/remineralisation, and that if already demineralised enamel is incubated in saliva, no salivary proteins penetrate into the enamel. This, arguably, also applies to hypomineralised enamel.

In Chapter V, we detected haemoglobin but not α-amylase in intact MIH enamel.

Figure 4.5. SDS-PAGE of (a) MIH enamel proteins isolated from intact MIH enamel, and (b) from fractured MIH enamel. The lanes appear similar, contrary to Mangum et al (2010) argument. [Adapted from (Mangum et al., 2010)]
The detection of serum proteins, and in particularly albumin, may be the factor responsible for the low mineral density reported for MIH enamel (Farah et al., 2010c; Fearne et al., 2004). Albumin can bind to HA crystals and inhibit their growth (Garnett and Dieppe, 1990; Robinson et al., 1992). A related finding is that MIH enamel also contained the serine proteinase inhibitors antitrypsin and antithrombin III, which may interfere with the proteolytic activity of enamel serine proteinase kallikrein 4 (KLK4). Secreted mainly during the transition and maturation stages of amelogenesis, KLK4 non-specifically degrades the proteins of the enamel matrix (Lu et al., 2008). This facilitates continued mineral growth required for full enamel maturation. Interference with the activity of KLK4 results in hypomaturation of the enamel. Mutations in the KLK4 gene result in hypomaturation amelogenesis imperfecta with hypomineralised enamel and increased amount of protein (Hart...
et al., 2004). Takagi et al (1998) demonstrated the presence of albumin in hypocalcified amelogenesis imperfecta (AI) in addition to abundance of amelogenin, and explained that as due to disturbance in protein degradation in AI. Unlike hypocalcified AI, however, the amount of amelogenin in MIH enamel appears comparable with sound enamel (Mangum et al., 2010).

In conclusion, the current investigation has identified several proteins not previously found in sound human enamel. These proteins represent an archive that may help in explaining the process of normal amelogenesis and in identifying insults to the developing tissue. Considerable similarity was also found between the proteins identified in sound enamel, MIH enamel and MIH enamel with PEB. Contamination with keratin and collagens prevented conclusions about quantitative differences in the proteins recovered from sound and MIH enamel. Replication of this study and the inclusion of a larger number of teeth will help validate the findings of this study.
Chapter V

Spatial Distribution of Albumin, Haemoglobin and α-Amylase in Sound and Hypomineralised Enamel

Introduction

This thesis investigates the hypothesis that MIH is due to physical or pathological trauma affecting the enamel during its formation. If MIH is of traumatic origin, blood proteins may penetrate into MIH enamel during its formation. Blood components entering forming enamel are expected to distribute according to the morphology of the MIH defect, and should not to be limited to the outer surface of the enamel.

Blood proteins, in particular serum albumin, are also found in saliva and crevicular fluid and may theoretically diffuse into enamel defects after or during tooth eruption. To
support the hypothesis of traumatic insult, and exclude the possibility of saliva/crevicular fluid penetrating into MIH enamel, it is necessary to demonstrate that proteins specific to saliva (represented by α-amylase) penetrate MIH enamel poorly, and that serum and blood proteins (represented by albumin and haemoglobin, respectively) penetrate enamel at greater depths.

The aim of this study was to determine the presence, distribution and possible gradient in enamel of two blood/serum marker proteins (haemoglobin and albumin), and a salivary marker protein (α-amylase) in MIH and sound enamel using direct immunofluorescence (DIF) detected by confocal laser scanning microscopy (CLSM). Dot blot analysis was used to confirm the specificity of the antibodies used in the study and to confirm the DIF findings.

Materials and methods

Direct immunofluorescence detection of albumin, haemoglobin and amylase

Enamel Samples

Five permanent molars with differing severities of MIH collected from five different children (Figures 5.1-5.5), and two permanent teeth with sound enamel collected from different individuals were chosen for this study from the pool of collected molars. The teeth were stored at 4°C in distilled water with thymol crystals until the time of the investigation.
Figure 5.1. MIH molar No.1. (a) buccal, (b) mesial, (c) palatal, (d) distal, (e) occlusal surfaces

Figure 5.2. MIH molar No.2. The temporary filling was lost during sample preparation. (a) buccal, (b) mesial, (c) lingual, (d) distal, (e) occlusal surfaces.
Figure 5.3. MIH molar No.3. (a) buccal, (b) mesial, (c) lingual, (d) distal, (e) occlusal surfaces.

Figure 5.4. MIH molar No.4. (a) buccal, (b) mesial, (c) palatal, (d) distal, (e) occlusal surfaces.
Enamel sample sectioning

For optimal visualisation of the samples using CLSM, each sample was cut with flat surfaces and even thickness. This is because an irregular surface would have produced uneven brightness and difficulties in focusing.

For ease of handling, each tooth was embedded in Castapress transparent acrylic resin (Vertex-Dental B.V., Zeist, the Netherlands) according to the protocol described in Chapter IV. Sagittal sections were cut with water irrigation through the affected part of each tooth using a diamond grit-impregnated saw fitted to a precise sectioning machine (Accutom-50, Struers A/S, Ballerup, Denmark) (Figure 5.6). The sectioning protocol is described in Chapter IV. To enable sample visualisation using transmitted light, sections were cut to a thickness of 0.5 mm. Cut sections were kept hydrated by storage in phosphate buffer saline (PBS). The cut sections were kept hydrated by storage in phosphate buffered saline (PBS).
Conjugating quantum dots to primary antibodies

Three different quantum dots (QDs) were originally selected for the study (Molecular Probes Inc., Eugene, Oregon). They are coated with polyethylene glycol (PEG) linker which has been shown to reduce nonspecific binding in fluorescent labelling (Bentzen et al., 2005). Table 5.1 summarises their specifications, and figure 5.7 shows their similar excitation but different emission wavelengths. Three rabbit polyclonal antibodies were used: anti-human albumin, anti-human haemoglobin and anti-human α-amylase (Sigma-Aldrich, St. Louis, Missouri).

Pilot work showed that hypomineralised enamel autofluoresces at 525 nm wavelength (green) when excited with the 405 or 488 nm laser wavelengths used to excite QDs. The autofluorescence was difficult to distinguish from immunofluorescence of the green QD-conjugated antibody complexes (Fig 5.8). The green QD was therefore unsuitable and not
used in the study. The orange QDs were conjugated to the anti-haemoglobin antibody, and the red QDs was conjugated to either anti-human albumin antibody or anti-α-amylase antibody.

<table>
<thead>
<tr>
<th>Quantum dot</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Colour of the quantum dot</th>
</tr>
</thead>
<tbody>
<tr>
<td>QD525</td>
<td>405/488</td>
<td>525</td>
<td>Green</td>
</tr>
<tr>
<td>QD565</td>
<td>405/488</td>
<td>565</td>
<td>Orange</td>
</tr>
<tr>
<td>QD655</td>
<td>405/488</td>
<td>655</td>
<td>Far red</td>
</tr>
</tbody>
</table>

Table 5.1. Specifications of the QDs used in the study
Figure 5.7. Absorption and fluorescence emission spectra of the QDs in pH 7.2. All three QDs have similar excitation wavelengths (blue line). (a) Green QD with 525 nm maximum emission wavelength. (b) Orange QD with 565 nm maximum emission wavelength. (c) Red QD with 655 nm maximum emission wavelength.
Figure 5.8. Pilot study using a green QD. (a) Autofluorescence of untreated MIH enamel and dentine. (b) MIH enamel and dentine treated with a QD-anti-albumin antibody showing slightly stronger fluorescence of the QD on a background of autofluorescent enamel. Distinguishing between untreated (autofluorescence of MIH enamel and dentine) and treated (immunofluorescence of the QD) samples using green QDs was not practical.

The manufacturer’s instructions for conjugating the QDs to their respective antibodies were followed. The conjugation reaction is preceded by: (1) preparing the required concentration of the antibodies for the reaction, (2) reducing the antibodies with dithiothreitol (DTT) (creating thiols in the antibodies), and (2) activating the QDs with succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate (SMCC). SMCC allows covalent bonding of the QDs to the antibodies. The succinimidyl ester of the SMCC chemically reacts with the primary amines in the amine-derivatised, PEG-coated QDs, and the maleimide reacts with thiols of the reduced antibodies.

The original concentrations of the antibodies were: 2.4 mg/ml for anti-human serum albumin antibody, 93.6 mg/ml for anti-haemoglobin antibody, and 7.8 mg/ml for anti-α-amylase antibody. As required for the conjugation reaction, the concentrations of the antibodies were adjusted to 1 mg/ml. For antibody reduction, 300 µl of 1 mg/ml antibody in PBS was reacted with 6.1 µl of 1 M DTT for 30 minutes at room temperature. For QD activation, 125 µL of 4 µM QD nanocrystals was reacted with 14 µl of 10 mM SMCC in DMSO for 1 hour at room temperature. The reduced antibody and the activated QD were
desalted through two separate desalting columns, with the first 0.5 ml of each eluate collected in an Eppendorf tube. The QD and the antibody solutions were incubated together for 1 hour at room temperature and the coupling reaction quenched with 10 µl of 10 mM 2-mercaptoethanol for 30 minutes. The solution was concentrated by centrifugal ultrafiltration at 4000 × g for 15 minutes, and passed through a separation column. The first ten drops of the coloured conjugate solution were collected. The conjugate (typically 1-2 µM) was stored at 4°C with 0.01% sodium azide added as preservative.

**Preparation of soya flour for the blocking agent**

Organic soya flour (Lotus Organic Foods, Cheltnham, Vic, Australia) was used as a blocking agent as the conventional blocking agent bovine milk powder contains albumin and was therefore unsuitable. Soya flour (0.4% w/v) was dissolved in PBS (pH 7.2) by shaking overnight. The suspension was centrifuged at 5,000 rpm for 10 minutes and filtered through a 0.22 µm sterile filter membrane (Millex-GP, Millipore Corp. Cork, Ireland) to reduce impurities and eliminate bacterial contamination.

**Reacting the conjugated primary antibodies with the enamel samples**

The cut enamel sections from each tooth were divided into three groups according to the three different proteins to be mapped, plus a group of untreated control sections. A total of 28 enamel sections (4 sections from each tooth × 7 teeth) were prepared. Each section was etched with 35% phosphoric acid for 15 seconds to remove the smear layer generated during sectioning. The etched surfaces were then washed with PBS buffer (pH 7.4), and immersed overnight in 0.4% (w/v) soya flour in PBS buffer to block out non-specific binding sites (Robinson et al., 1998b).

On the following day, the QD-antibody conjugate was diluted 10 fold in PBS and washed in PBS. The sections were separately reacted overnight with the corresponding QD-
antibody conjugate in the dark at room temperature. A pilot study showed that washing for at least 90 minutes was required to remove from the sample surface the non-specifically bound QD-antibody conjugates. The sections were therefore washed 2 × 60 minute in PBS with gentle stirring. The resultant samples were stored in humid containers at 4º C.

**Visualisation of enamel sections using CLSM**

Fluorescent images of 0.5 mm enamel sections were obtained using a Zeiss LSM 710 Upright Spectral Confocal Microscope (Carl Zeiss Jena GmbH, Jena, Germany). It has an upright configuration on an Axio Imager Z2 frame with 405 diode laser, argon laser, green HeNe and red HeNe laser, phase objectives and mercury burner. It provides laser excitation lines of 405, 458, 488, 514, 543 and 633nm, and 10 separate channels for detection. The 405 diode laser was used in the current study.

MIH enamel, dentine and sound enamel (with higher gain) all showed autofluorescence at 525 nm. This allowed QD fluorescence images acquired in a separate channel to be localised relative to enamel and dentine morphology by overlay on the autofluorescence.

Several factors affected the parameters used in CLSM detection of QD fluorescence:

1. Magnification: lower magnification allowed use of a higher master gain setting.
2. Autofluorescence of untreated MIH enamel: untreated MIH enamel showed an autofluorescence maxima at about 525 nm but minimal fluorescence was also detected at the far red and orange emission wavelengths used in this study. The gain was adjusted at these wavelengths so untreated MIH enamel gave no detectable fluorescence.
3. Immunofluorescence of treated sound enamel: while this did not affect the acquisition parameter setting directly, it enabled determination of the time needed to wash nonspecifically-bound QD-antibody complexes from enamel surfaces. Sound enamel treated
with QD-anti-human albumin showed no immunofluorescence after a 90 min wash while treated MIH enamel showed strong immunofluorescence.

Table 5.2 describes the parameters used for the acquisition of fluorescence data for the three different QD-antibodies at the 5x magnification. At the 10x magnification, the master gain was reduced so that detail was not masked by fluorescence saturation.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>QD</th>
<th>Excitation (nm)</th>
<th>Attenuation (%)</th>
<th>Pinhole size (µm)</th>
<th>Master gain</th>
<th>Digital offset</th>
<th>Digital gain</th>
<th>Frame size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti albumin</td>
<td>Red</td>
<td>405</td>
<td>4</td>
<td>68.4</td>
<td>780</td>
<td>-100</td>
<td>0.3</td>
<td>1024 x 1024 to 4096 x 4096</td>
</tr>
<tr>
<td>Anti Hb</td>
<td>Orange</td>
<td>405</td>
<td>4</td>
<td>92.6</td>
<td>600</td>
<td>-5</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Anti α-amylase</td>
<td>Red</td>
<td>405</td>
<td>4</td>
<td>89.8</td>
<td>600</td>
<td>-100</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2. Image acquisition settings.

Dot blots

Dot blots were used to: (a) confirm the activity and specificity of the antibodies used in the study, and (b) validate the findings of the DIF.

Dot blots to confirm the activity and specificity of the antibodies

Antigens for the three antibodies were prepared as crude fractions, i.e. serum (serum albumin), red blood cell lysate (Hb), and saliva (α-amylase). The serum and RBC lysate were prepared from 1 ml of human blood. The non-clotted blood was centrifuged at 3,000 rpm for
10 minutes and the supernatant separated from the pellet. The supernatant was centrifuged three times at 3,000 rpm for 10 minutes to remove residual RBCs. The cell-free supernatant was collected and denoted as serum. The pellet of red blood cells was re-suspended in 1 ml of PBS with EDTA (to prevent clotting), incubated for 5 minutes on ice (to wash the RBCs) and centrifuged at 3000 rpm for 10 minutes. This process was repeated two more times and the serum-free pellet of RBCs re-suspended in 1 ml PBS. A 100 µl sample of the RBCs was diluted in 1 ml PBS containing 0.1% Triton X 100 (Sigma-Aldrich NZ Ltd., Auckland) to lyse the cells and release the haemoglobin. The sample was incubated for 5 minutes on ice and centrifuged at 13,000 rpm 10 minutes. The supernatant was centrifuged once more at 13,000 rpm (to remove residual red cell membranes) and denoted as the RBC lysate.

One ml of non-stimulated whole saliva was collected and centrifuged three times at 3,000 rpm for 10 minutes. The supernatant recovered was denoted as the saliva.

The antigen-containing samples were diluted 100 fold in PBS. A six-step series of 5-fold dilutions was then prepared in a microtitre plate for the diluted serum, saliva and RBC lysate.

Five pieces of nitrocellulose membrane were used for dot blot analysis. One µl samples from each dilution series was added to each membrane (Fig 5.9). The membranes were air-dried and then incubated overnight in blocking solution containing 0.4% soy flour and 0.1% Tween® 20 (Sigma-Aldrich NZ Ltd., Auckland).

The membranes were washed 2 x 10 min with blocking solution and then separately incubated with an antibody (diluted 1000 fold in PBS) or control solution (Fig 5.9) for 1 hour with continuous gentle shaking. The membranes were washed 5 times with blocking solution and then immersed for 1 hour in alkaline phosphatase-conjugated secondary anti-rabbit antibody (Sigma-Aldrich NZ Ltd., Auckland). The membranes were then washed 5 times with blocking solution, and one final time with high pH (9.5) Tris buffer (10 mM Tris, 100 mM NaCl, 5 mM MgCl₂). Akaline phosphatase was detected by immersing each membrane in 10 ml of the high pH Tris buffer in which 1 tablet of SIGMA FAST™ BCIP/NBT (5-
Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium) (Sigma-Aldrich NZ Ltd., Auckland) had been dissolved. The alkaline phosphatase activity was usually detected within 1-2 minutes as a purple colour generated by antibody decorated dots.

**Figure 5.9.** Diagram of dot blots. Five similar membranes were prepared. One µl samples from each of the 5-fold dilution series were added to each of the 5 nitrocellulose membranes at the locations indicated by the coloured dots. After blockage, anti-α-amylase antibody was incubated with membrane 1, anti-Hb antibody with membrane 2, anti-albumin antibody with membrane 3, human serum with membrane 4 (control for non-specific IgG binding) and PBS with membrane 5 PBS (no-antibody control). The anti-rabbit alkaline phosphatase conjugate was added to all the membranes to decorate antigen-rabbit antibody complexes.

**Dot blots to validate DIF findings**

Dot blots were used to confirm the presence/absence of albumin, Hb and α-amylase in protein extracts from sound and MIH enamel.

Enamel samples from three MIH teeth (0.01g) and from three sound teeth (0.02g) were cut and dissolved in TCA and recovered by precipitation in ice cold 80%v acetone as described in Chapter IV. The resultant protein pellets was dissolved in 70 µl of double distilled water. Eight-step series of 5-fold dilutions were prepared in a microtitre plate for the proteins from sound and MIH enamel. Antigen-containing control samples from saliva, serum and RBC lysate were diluted 100-fold and arrayed as five fold dilution series. The nitrocellulose membranes were incubated with primary and secondary antibodies as described above.
Results

Direct immunofluorescence

Detection of albumin

Normal teeth

Single sections from the seven teeth selected for the study were used for albumin detection. Figure 5.10 shows a representative section from a sound tooth in which albumin was mapped. No albumin antigen (red fluorescence) was detected in sound enamel. Small but significant amounts were detected in dentine.

MIH molars

When the gain setting was adjusted so that no red fluorescence was detected from treated enamel of normal teeth and untreated MIH teeth, the affected part of antibody-treated MIH enamel showed red fluorescence indicating the presence of albumin antigen (Figs 5.11 – 5.13). The strength of the fluorescent signal and its distribution differed between teeth and individual sections. In some cases the enamel closest to the DEJ was most fluorescent. This observation may reflect albumin within Hunter-Schreger (HS) bands in deep enamel or a gradient of albumin between the dentine-enamel junction (DEJ) and the outer enamel. Fluorescence at the cusp tip was weaker than the rest of the affected MIH enamel (Fig 5.11-e). In certain areas fluorescent tuft-like structures extended from the DEJ into the enamel (Fig 5.12). Similar structures appeared in some histological sections of MIH enamel (Chapter VI).

The fluorescence disappeared as it was traced across the junction of affected MIH enamel and sound cervical enamel (Figs 5.11 - 5.13). Like enamel from normal teeth, sound cervical enamel from MIH teeth showed no fluorescence.
Figure 5.10. Photographic and CLSM images of a sound tooth section treated with QD-anti albumin antibody. (a) Photograph of the cut section. The red box indicates where the CLSM images were taken. (b) Channel 1: Autofluorescence of enamel and dentine. The master gain was increased to show the outline of the enamel surface. (c) Channel 2: Immunofluorescence of the QD showing minimal red fluorescence in dentine. (d) Channel 1 and 2 combined. CLSM images are at 5x.
Figure 5.11. Photograph and CLSM image of an MIH tooth section treated with QD-anti albumin antibody.

(a) Photograph of the cut section. Red box indicates where the CLSM images (b-d) were taken. Blue box indicates where CLSM image (e) was taken. Green box indicates where CLSM image (f) was taken.

(b) Channel 1: Autofluorescence of enamel and dentine at the junction of MIH and sound cervical enamel. MIH enamel shows much higher autofluorescence than sound cervical enamel.
(c) Channel 2: Immunofluorescence of the QD showing red fluorescence particularly in enamel close to the DEJ, and in the dentine. Red QD fluorescence (reflecting albumin) is stronger in MIH enamel closer to DEJ.

(d) Channel 1 and 2 combined.

CLSM images are at 5x.
(e) Channel 1 and 2 combined at the cusp tip. The level of red fluorescence indicates lower levels of albumin. 5x.
(f) Composite image of dentine and full thickness of MIH enamel showing the distribution of red fluorescence. No gradient of fluorescence was detected. 10x.
(b) Channel 1: Autofluorescence of enamel and dentine at the junction of MIH and sound cervical enamel. MIH enamel shows significantly higher autofluorescence than sound cervical enamel. Tuft-like structures extend from DEJ towards the outer enamel surface (red arrows).

Figure 5.12. Photographic and CLSM images of an MIH tooth section treated with QD-anti albumin antibody.

(a) Photograph of the cut section. Red box indicates where the CLSM images were taken.
(c) Channel 2: Immunofluorescence of the QD showing red fluorescence particularly in enamel close to the DEJ, and in the dentine. Red fluorescence is present in enamel close to the DEJ in the region of sound cervical enamel and is associated with the tuft-like structures.

(d) Channel 1 and 2 combined.

CLSM images are at 5x.
Figure 5.13. Photographic and CLSM images of an MIH tooth section treated with QD-anti-albumin antibody.

(a) Photograph of the cut section. Red box indicates where the CLSM images were taken.

(b) Channel 1: Autofluorescence of enamel and dentine at the junction of MIH and sound cervical enamel. MIH enamel shows much higher autofluorescence than sound cervical enamel.
(c) Channel 2: Immunofluorescence of the QDs showing red fluorescence in MIH enamel and in the dentine, but not in sound cervical enamel.

(d) Channel 1 and 2 combined.

CLSM images are at 5x.
Detection of haemoglobin

Normal teeth

One section from each of the seven teeth selected for the study was used for Hb detection. Figure 5.14 shows a representative section from a sound tooth in which Hb was detected. Very weak orange fluorescence was found in dentine, but not enamel.
Figure 5.14. Photographic and CLSM images of a sound tooth section treated with QD-anti Hb antibody. (a) Photograph of the cut section. Red box indicates where the CLSM images were taken.
(b) Channel 1: Autofluorescence of enamel and dentine. Master gain was increased to show the outline of the enamel surface. (c) Channel 2: Immunofluorescence of the QD showing minimal orange autofluorescence in dentine. (d) Channel 1 and 2 combined. CLSM images are at 5x.
**MIH molars**

MIH enamel and dentine showed very weak autofluorescence at the wavelength used to detect orange QD (565 nm). At the gain used for Hb detection in normal teeth and untreated MIH teeth, the affected part of MIH enamel showed strong orange fluorescence that indicated the presence of Hb (Figs 5.15 - 5.20). Sections from different MIH molars showed orange fluorescence in the MIH affected part of the enamel, but there was some variation in the distribution of fluorescence between teeth. In general, the orange fluorescence for Hb was weaker than the red fluorescence for albumin, possibly indicating lower level of Hb than albumin. Some regions of enamel close to the DEJ showed higher fluorescence that may be related to HS bands in deep enamel or the presence of a gradient of Hb. Fluorescence at the cusp tip appeared significantly weaker than the rest of the affected MIH enamel.

The orange fluorescence declined across the junction between affected MIH enamel and sound cervical enamel. Like enamel from normal teeth, sound cervical enamel from MIH teeth was not fluorescent. Dentine under the affected MIH enamel was always more fluorescent than dentine under the normal sound cervical enamel, and the orange fluorescence (Hb) was detected inside the dentinal tubules (Fig 5.17). CLSM of carious dentine showed no Hb penetration from saliva/crevicular fluid (Fig 5.19 e-g).

**Figure 5.15.** Photographic and CLSM images of an MIH tooth section treated with QD-anti Hb antibody.

(a) Photograph of the cut section. Red box indicates the location of the CLSM images.
(b) Channel 1: Autofluorescence of enamel and dentine.

(c) Channel 2: Immunofluorescence of the QD showing orange fluorescence, particularly in enamel close to the DEJ and in the dentine.
(d) Channel 1 and 2 combined. Red box indicates where 10x image in Fig 5.16 was taken.

CLSM images are at 5x.
Figure 5.16. 10x magnification of the labelled area in Fig 5.15 (d). Red box indicates where 20x image in Fig 5.17 was taken.
Figure 5.17. 20x magnification of the labelled area in Fig 5.16. Orange fluorescence can be seen in the dentinal tubules (red arrows) indicating the presence of Hb in dentine.
Figure 5.18. Photographic and CLSM images of an MIH tooth section treated with QD-anti Hb antibody.

(a) Photograph of the cut section. Red box indicates the location of the CLSM images.

(b) Channel 1: Autofluorescence of enamel and dentine.
(c) Channel 2: Immunofluorescence of the QD showing orange fluorescence, particularly in enamel close to the DEJ and in the dentine.

(d) Channel 1 and 2 combined.
CLSM images are at 10x.
Figure 5.19. Photographic and CLSM images of an MIH tooth section treated with QD-anti Hb antibody.

(a) Photograph of the cut section. Red box indicates the location of the CLSM images (b-d). Blue box indicates where CLSM images (e-g) were taken.

(b) Channel 1: Autofluorescence of enamel and dentine at the junction of MIH and sound cervical enamel. MIH enamel is significantly more autofluorescent than sound cervical enamel.
(c) Channel 2: Immunofluorescence of the QD showing orange fluorescence in MIH enamel but not sound cervical enamel.

(d) Channel 1 and 2 combined.
CLSM images are at 5x.
(e) Channel 1: Autofluorescence of carious dentine.

(f) Channel 2: Immunofluorescence of the QD showing very weak orange autofluorescence in carious dentine.

(g) Channel 1 and 2 combined.

CLSM images are at 10x.
Figure 5.20. Photographic and CLSM images of an MIH tooth section treated with QD-anti Hb antibody.

(a) Photograph of the cut section. Red box indicates the location of the CLSM images (b-d.

(b) Channel 1: Weak autofluorescence of sound cervical enamel.
(c) Channel 2: No orange fluorescence detected in sound cervical enamel.

(d) Channel 1 and 2 combined. CLSM images are at 5x.
Detection of α-amylase

Red fluorescence indicating the presence of amylase) was not detected in normal enamel, MIH enamel or dentine from any samples used in this study (Fig 5.21 and 5.22).

Figure 5.21. Photographic and CLSM images of a sound tooth section treated with QD-anti amylase antibody. (a) Photograph of the cut section. Red box indicates the location of the CLSM images (b) Channel 1: Autofluorescence of enamel and dentine. Master gain was increased to show the outline of the enamel surface. (c) Channel 2: Immunofluorescence of the QD showing minimal no red fluorescence. (d) Channel 1 and 2 combined. CLSM images are at 5x.
Figure 5.22. Photograph and CLSM image of an MIH tooth section treated with QD-anti amylase antibody.

(a) Photograph of the cut section. Red box indicates where the CLSM images were taken.

(b) Channel 1: Autofluorescence of enamel and dentine at the junction of MIH and sound cervical enamel. MIH enamel shows much higher autofluorescence than sound cervical enamel.
(c) Channel 2: No red fluorescence detected from enamel or dentine.

(d) Channel 1 and 2 combined.

CLSM images are at 5x.
Dot blots

Antibodies reactivity and specificity

The dot blot analysis confirmed the reactivity of the three antibodies against their corresponding antigens (Fig 5.23). No unexpected cross reactivity was detected. For this analysis the original samples of serum, saliva and RBC lysate were diluted 100 fold. Under these conditions, the anti-α-amylase (Fig 5.23-c) showed a weak positive result against saliva. An undiluted saliva sample showed stronger reactivity (5.23-e).

Validating the DIF results

Albumin antigen was detected in dot blots of the MIH enamel sample, but not the sound enamel sample. The Hb and α-amylase antigens were not detected in dot blots of the MIH or sound enamel samples (Fig 5.24).
Figure 5.23. Dot blot test of antibody reactivity and specificity. (a) Diagram of a typical dot blot membrane. On each membrane (b-f), three rows of 6 dots present a 5-fold dilution series for each antigen set out as in (a) with 1 µl samples applied. The first row represents sequential dilution for serum, the second row for saliva, and the third line for RBC lysate. 

(b) Membrane 1 showed 4 positive dots in the first line indicating binding of the anti-albumin antibody to serum albumin, and 2 weakly positive dots in the second line due to the presence of albumin in saliva. 

(c) Membrane 2 shows 2 positive dots in the second line indicating binding to α-amylase. 

(d) Membrane 3 shows 3 positive dots in the third line indicating binding to Hb in the RBC lysate. 

(e) Membrane 4 provides a negative control. The blot was treated with human serum instead of a rabbit antibody and no binding to the antigens by the goat anti-rabbit-alkaline phosphatase conjugate was found. 

(f) Membrane 5 is an additional negative control treated with PBS instead of a rabbit antibody. 

(g) Membrane uses a dilution series starting with undiluted saliva sample. It shows strong specific binding to saliva by the anti-α-amylase antibody.
Figure 5.24. Dot blot test for antigens extracted from teeth. (a) Diagram of a typical dot blot membrane. On each membrane, two rows of 8 dots and 1 row of 6 dots present 5-fold dilution series (from left to right) for each antigen set out as in (a). In b-d, the bottom row is the control antigen for each blot (serum on Membrane 1, RBC lysate on Membrane 2 and saliva on Membrane 3). (b) The anti-albumin antibody shows strong reactivity with serum and gave one weak positive dot with the most concentrated sample (circled) in the top MIH enamel line. (c) The anti-haemoglobin antibody shows a strong reaction with RBC lysate but no detectable reaction with the tooth extracts. (d) The anti-α-amylase reacts with saliva but not with the tooth extracts. (e) Use of human serum instead of rabbit antibody gave no positive dots. (f) Use of PBS instead of rabbit antibody gave no positive dots. The controls indicate that the alkaline phosphatase conjugate of the anti-rabbit secondary antibody did not non especifically bind to the antigens presented.
Discussion

It is important to establish the reactivity and specificity of the antibodies used for immunohistological analysis. Dot blot analysis established these properties for the three antibodies used in this chapter, even though the antigen sources used were not the purified antigens. On the other hand, the use of dot blots to detect the presence of the three protein markers in MIH and sound enamel only detected the most abundant of the three proteins, namely albumin. The negative results for $\alpha$-amylase and Hb mean either that these proteins were not present in the MIH or sound enamel, or they were present in amounts lower than the detection threshold for the technique as applied. The detection of Hb in MIH enamel with DIF is consistent with the latter possibility. The inability to detect $\alpha$-amylase by either dot blot analysis or DIF suggests that it does not penetrate into MIH or sound enamel in significant quantities. This interpretation was supported by the inability to detect $\alpha$-amylase peptide query matches in our MS/MS study (Chapter IV) of sound and MIH enamel.

The autofluorescence of MIH enamel and of dentine was very clear in this study. Sound enamel showed no fluorescence when excited with a 405 nm laser light except at very high gains. This autofluorescence of MIH enamel was similar to that reported for carious dentine when excited with a 488 nm laser at an acquisition of 529 nm (van der Veen and Bosch, 1995). The acquisition for the green QD is around the 525 nm wavelength, at which MIH enamel showed high autofluorescence. This feature was used to an advantage in visualizing the outline of the enamel and dentine and superimposing the red/orange QD fluorescence over the outline of the sample to localize where the QD-antibody complexes were distributed. It is noteworthy here that autofluorescence of MIH enamel when excited by laser can also be used to indicate the degree of the defect using DIAGNOdent pen. DIAGNOdent is a device that excites the fluorophores in carious/hypomineralised tissues with laser and then collects the resultant fluorescence of the tissue and converts it into a digital reading. The higher the number on DIAGNOdent pen (more autofluorescence) is, the more the protein content it posses (Farah et al., 2008; Farah, 2009). In carious tissue, bacterial
metabolites are thought to be the fluorophores responsible for the increased readings. In MIH enamel, the nature of the fluorophores is still unknown.

Similar to the findings of Shore et al (2000), no albumin was traced in sound enamel, whether from normal sound teeth or from sound cervical enamel. An exception was albumin detected at the DEJ in some but not all sections (Fig 5.12). The high fluorescence (auto- and immuno-) of MIH enamel showed a clear cut at the junction with the sound cervical enamel. This distinctive junction between hypomineralised and sound enamel has been demonstrated by previous studies to be between 0.5 – 1 mm in width (Farah et al., 2010c; Farah, 2009; Mahoney et al., 2004). The cervical enamel devoid of albumin has been previously shown to also have normal mineral density (Fig 5.25).

Both the autofluorescence and the signal for albumin antigen detected at the cusp tip were weaker than for the rest of the MIH enamel, suggesting a lower protein content and albumin content (Fig 5.11-e). This is also in accordance with previous findings showing higher mineral density and higher mechanical properties in the cusp tip (Farah et al., 2010c; Farah, 2009) (Fig 5.25). There is an inverse relationship between the mineral density and the amount of organic content of enamel (Farah et al., 2008; Spears, 1997). The reason why the cusp tips commonly show less protein and more mineral is unknown.

In certain areas of deep enamel, tuft-like structures showing higher autofluorescence also showed the presence of albumin. These tuft-like structures were also observed in histological sections (Chapter VI), and will be discussed in more details in the next chapter.

The distribution of Hb antigen in MIH enamel was similar to that of albumin, albeit less in intensity, reflecting less amounts of Hb. This difference in the amounts was also demonstrated in the dot blotting study. In certain areas, the fluorescence albumin, Hb or even autofluorescence of the MIH enamel did not appear to follow the enamel rods or interrod areas, but appeared to cover them and mask them completely.
Hb from saliva/crevicular fluid did not penetrate exposed carious enamel (Fig 5.19 e-g). Salivary proteins can penetrate into carious dental tissues (Shore et al., 2000), but the Hb concentration in saliva appears insufficient to create a detectable diffusion gradient into the carious dentine. Dentinal tubules are larger than the porosities of MIH enamel (Xie et al., 2008a) but do not show penetration by salivary Hb. Mangum et al (2010) argued that Hb in MIH enamel results from saliva/crevicular fluid gaining access to fractured MIH enamel but not intact MIH enamel. The absence of detectable Hb penetration in carious dentine, the failure of α-amylase to penetrate MIH enamel, and the detection of albumin and Hb in both fractured and intact MIH enamel are inconsistent with this suggestion. In addition, the albumin from saliva that penetrated demineralised enamel remains intact (Robinson et al., 1998b) while albumin found in MIH enamel was both intact and degraded (Mangum et al., 2010), possibly pointing to proteinase activity during enamel maturation. Another argument...
against the albumin and Hb originating in saliva is the different distributions of albumin in MIH and demineralised enamel. Shore et al (2000) determined the spatial distribution of albumin and α-amylase in demineralised enamel in order to determine whether albumin in demineralised enamel was from saliva, or endogenous as a remnant of the enamel matrix. Because both α-amylase and albumin were found together in the demineralised enamel, and both proteins were undetected in sound enamel controls, the authors concluded saliva was the source of albumin. Albumin and α-amylase were not uniformly distributed in carious lesions and were mostly found in the subsurface enamel that had undergone 10-20% demineralisation. The binding of albumin and α-amylase to the crystals in the demineralised region may have prevented deeper penetration into the enamel. This distribution of salivary albumin in demineralised porous enamel is different from its distribution in porous hypomineralised enamel, where deeper enamel (closer to the DEJ) usually showed greater amounts of albumin than other parts of the enamel. This observation rules out saliva as the source of the albumin (and Hb). The demonstration by Robinson et al (1998b) that albumin only penetrates enamel during demineralisation/remineralisation and that experimentally demineralised enamel (equivalent to hypomineralised enamel in the present argument) does not absorb albumin from an outside source is consistent with an endogenous source of albumin in MIH enamel.

If albumin and Hb do not penetrate MIH enamel from the saliva, a possible source is blood during enamel formation. Most sections from MIH enamel showed higher albumin and Hb immunofluorescence in the deeper enamel close to the DEJ. This may reflect a gradient of albumin/Hb from DEJ to the outer surface. The presence of this gradient is supported by this and other studies:

- The high autofluorescence of enamel close to the DEJ indicates higher organic content.
- Decalcified histological sections of MIH enamel showed higher organic content closer to the DEJ (Chapter VI).
The mineral density and mechanical properties of MIH enamel (which are inversely related to the amount of protein in enamel) are lower in deep enamel close to the DEJ (Farah et al., 2010c; Farah, 2009).

To explain this gradient and its negative effects on the enamel, two points must be explained; the dynamics of blood/serum flow in the tissues surrounding the developing enamel, and the effect of abundance of albumin present in enamel at different stages of development and vulnerability.

\[ \text{a) The dynamics of blood/serum flow in the tissues surrounding developing enamel} \]

Limeback et al (1989) reported that serum albumin was deposited in developing porcine enamel from both the enamel organ side and the DEJ side. Okamura (1983) showed that serum albumin is present in secretory stage enamel but not in maturation stage enamel. Albumin was detected passing between and through the ameloblasts. It is thought that albumin is pinocytosed from serum by the ameloblasts and then secreted into the immediate vicinity of developing enamel at the secretory stage. Albumin is not expressed by ameloblasts (Couwenhoven et al., 1989; Yuan et al., 1996). These findings were also confirmed by Mckee et al (1986) who also demonstrated, using radioautography, the penetration of several other serum proteins into developing enamel. Another immunohistochemical study found that albumin in dentine cannot reach the DEJ during the secretory/transition stage. In the late transition and maturation stages, albumin reaches the outer dentine and into the DEJ, but it was not clear whether it penetrates into the enamel (Shore et al., 1995). It therefore appears that albumin can in fact reach developing enamel from both sides: from the ameloblasts side during the secretory stage and from the DEJ side during the transition/maturation stage. However, it most likely only penetrates enamel during the secretory stage from the ameloblasts side.
b) **The effect of abundance of albumin present in enamel at different stages of development**

Albumin enters enamel during the secretory stage of normal enamel development. Albumin is then degraded (presumably by serine proteases) during the transition stage and disappears in the maturation stage (Robinson et al., 1994). The regulated presence of albumin in developing enamel is important for normal enamel development. If albumin directly contacts HA crystals, it prevents their growth (Garnett and Dieppe, 1990; Robinson et al., 1992). In the secretory stage, albumin appears to down-regulate crystal growth. During the transition stage it is degraded and removed so exponential crystal growth can occur in the maturation stage.

Interestingly, if albumin was allowed to reach developing enamel in excess amounts, it does not do so in the secretory stage, but only in the maturation stage. This was proven by Robinson et al (1994) where they studied how much albumin can gain access into developing rat enamel in different developmental stages post-mortem. In the secretory stage, albumin concentration did not increase in enamel after extended periods. However, in maturation stage enamel, a time-dependent concentration increase occurred. This is arguably because secretory stage enamel is saturated with solid-state amelogenin which prevents the albumin from reaching the HA crystals. In the transition and maturation stages, the enamel may be more vulnerable; the vascularity is at its highest, the protective capacity of amelogenin is declining rapidly due to its degradation, and the enamel is very porous (Robinson et al., 1996). The appearance of the postmortem samples in the work of Robinson et al resembles the appearance of MIH molars (Fig 5.26) (Robinson et al., 1992).
Figure 5.26. The effect of postmortem storage on bovine teeth. (a) A molar that has been stored frozen at -20°C for 48 hours. Dark discoloration due to leakage of blood into the more occlusal maturation-stage enamel (MB or M) can be seen in the frozen teeth, but not in the freshly dissected tooth in (b). The cervical secretory-stage enamel (S) remained normal in colour in both samples [Adapted from (Robinson et al., 1992)].

If blood reaches developing human enamel when the more occlusal part was in the vulnerable maturation stage, the cervical part would still be in the more protected secretory stage. In addition, in the maturing occlusal part, the deeper enamel closer to the DEJ would be more vulnerable than the outer enamel since it is more mature (Fig 5.27). Therefore, regardless of where the albumin (serum/blood) is reaching enamel from (ameloblasts side or DEJ side), its most deleterious effect will be in the deeper occlusal enamel, less in the outer occlusal enamel which is more protected, and completely sparing the cervical secretory enamel.
Figure 5.27. Schematic diagram demonstrating the maturation process of enamel. The darker the enamel the less mineralised it is in this diagram, and the closer chronologically it is to the secretory stage. When the cervical enamel is still passing through the relatively protected secretory stage, the rest of the occlusal enamel is passing through the maturation stage. The inner enamel close to the DEJ is more mineralised and more vulnerable to insult. The outer enamel is the least mature. Note that even in the cervical part passing through the secretory stage, the inner enamel close to the DEJ would be passing through the vulnerable maturation stage ahead of the surface enamel.

Therefore, although animal studies in which purposeful trauma was delivered to developing enamel showed blood and albumin reaching developing enamel from the ameloblasts side (Andreasen, 1976; Tarjan et al., 2002), the albumin (and possibly the other blood proteins) will go through the outer enamel with minimal negative effect (early maturation stage) and reach into deeper, more mature, enamel causing more hypomineralisation.

This model, however does not explain the presence of Hb inside the dentinal tubules in MIH dentine but not in dentine from sound teeth. Whether this Hb has reached dentine
through the DEJ from the enamel, or from the pulp is still unknown. A hyperaemic pulp may be the result of fever or trauma and both may result in the gradient seen in enamel.

In yet another histological animal study, fever was induced in pregnant rats, which resulted in varying degrees of enamel hypomineralisation and hypoplasia (Kreshover and Clough, 1953). It was observed that the pulp was hyperaemic, and the connective tissue overlying the ameloblasts from the other side was also hyperaemic. Blood accumulated between the ameloblasts and the predentine before enamel matrix formation. It was not possible to determine the source of the blood in that area. However, in other more mature sections, the bleeding between the ameloblasts and the predentine was replaced by oedematous-like material between the ameloblasts and the secreted enamel, and not between the secreted enamel and the dentine. Interestingly, the changes in enamel formation showed no correlation with the duration of the induced fever or the stage of pregnancy, perhaps pointing to the nonspecific nature of enamel defects. In other words, different conditions may result in similar manifestations of enamel defects.

In summary, MIH enamel showed the presence of albumin and Hb, but not α-amylase, pointing to blood as the source of these proteins. Whether blood reaches into developing enamel from the ameloblasts side or the DEJ, eventually the histological appearance will be similar with the proteins being more abundant close to the DEJ. The presence of Hb in dentinal tubules underneath MIH enamel may point to dentino-pulpal complex as the possible source of the blood, rather than the enamel organ.
Chapter VI

Histopathological Study of Sound and Hypomineralised Enamel

Introduction

The autofluorescence of MIH enamel detected using confocal laser scanning microscopy (CLSM) is likely to reflect its high organic content. The distribution of serum albumin and haemoglobin antigens mapped in sound and MIH enamel did not appear to correlate with the location of the enamel rods or interrod areas. These antigens appeared instead to overlay the rods, covering them completely in some areas. Within these areas, especially in the enamel close to the dentine-enamel junction (DEJ), the rod architecture of normal enamel was not seen, even when detected using the autofluorescence of the MIH enamel.

The aim of the current study was to investigate the architecture of the organic matrix of MIH enamel in order to understand distribution of the proteins observed using the CLSM (Chapter V). This was achieved by examining decalcified histological sections of MIH and sound enamel using transmitted light microscopy.
Materials and Methods

One MIH molar and one sound molar were chosen for this study from the pool of the collected molars. The teeth were stored at 4º C in distilled water with thymol crystals until the time of investigation. The histopathological sections were prepared at the Oral Diagnostic and Surgical Sciences Medlab Dental laboratory.

The two molars were fixed by immersion in neutral buffered formalin for 3 days.

Tissue fixation was followed by mineral phase decalcification. This was done by immersing the teeth in 10% formic acid (100 ml formalin, 220 ml formic acid, 2 L distilled water). Each week, the decalcification end point was assessed chemically by sampling 1ml decalcifying fluid in 1 ml of 10% ammonium oxalate, 1 ml of 10% sodium hydroxide for 30 minutes. If a precipitate or a white cloudy solution was observed, the decalcification process was considered incomplete and was continued for another week. Decalcification of the teeth required 3-4 weeks. The decalcified tissue was neutralized by washing with saturated lithium carbonate for or 2-4 hours and then distilled water for 40 minutes.

The decalcified teeth were then processed overnight on an automated treatment processor through a series of alcohol, xylene, and paraffin wax (5860º C). They were then embedded in wax moulds and cooled on ice.

Thin sections (3 µm) cut using an electronic rotary microtome (Microm HM 340E, Thermo Fisher Scientific, New Zealand) stained with haematoxylin and eosin (H & E) as follows: The cut sections were dried for 1 hour at 60º C on adhesive slides, and dewaxed in xylene for 5 minutes followed by 4 x 2-3 minute treatments with absolute alcohol, washed in running tap water and stained with Gills Haematoxylin for 4 minutes. The sections were washed in running tap water, placed in Scott’s tap water for 1 minute, washed again with running tap water, and stained with 1% eosin for 4 minutes. The sections were washed,
dehydrated 4 x 2-3 min in alcohol, cleared in xylene for 5 minutes and coverslipped with DPX synthetic resin.

Results

Most of the organic matrix of enamel in the sound tooth was lost during formic acid decalcification (Fig 6.1). In contrast, a substantial MIH tooth enamel matrix was retained, particularly close to the dentine-enamel junction (DEJ) (Fig 6.2).

Figure 6.1. Haematoxylin and eosin (H&E) stained histopathology image of a representative section of a sound tooth

(a) Cusp tip at 5x. Only a very thin section of enamel matrix was retained after decalcification. The dark pink area at the cusp tip is an artefact where the tip of the decalcified dentine has turned over (red arrow). The red box indicates the location of the magnified images (b-d).
(b) Cusp tip at 10x.

(c) Cusp tip at 20x.
(d) Cusp tip at 40x. The wide spaces inside the enamel matrix indicate where the enamel rods were located before decalcification.
Figure 6.2. H & E stained histopathological images of an MIH tooth section

(a) Cusp incline at 5x. Substantial enamel matrix was retained after decalcification. The red box shows the location of the magnified images (b-d).
(b) 10x. (c) 20x.
(d) Cusp tip at 40x. Spaces inside the enamel matrix indicate the location of enamel rods before decalcification. The small sizes of the spaces (compared to those of sound enamel in Fig 6.1 – d) indicate that the enamel rods were constricted or even completely missing in some areas. Most of the affected MIH enamel appeared to be occupied with organic matrix rather than mineralised rods.

In some areas in the affected MIH enamel, tuft-like structures (indicating high organic content) extended from the DEJ at varying distances into the enamel (Fig 6.3). The cervical part of MIH enamel was lost during decalcification, indicating high mineral content and minimal organic content. However, in most of the sections, a thin enamel matrix layer was retained close to the DEJ (Fig 6.4).
Figure 6.3. H & E histopathological image of an MIH tooth section at the cusp tip.

(a) Cusp tip at 5x. Tuft-like structures extend from the DEJ into the enamel (red arrows). The red box indicates the location of the magnified image (b).

(b) 10x magnification
Figure 6.4. H & E stained histopathological images at the cervical areas of an MIH tooth and a sound tooth

(a) Cervical area of the MIH tooth. 5x. In the decalcified section a narrow area of enamel matrix (red arrow) is retained in the cervical area between the coronally affected MIH area and the periodontal ligament cervically.

(b) Cervical area of the sound tooth. 5x. No enamel matrix is retained in the cervical area after decalcification.
Discussion

MIH enamel has been studied extensively at high magnification using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Electron microscopy, however, does not allow for easy and clear differentiation between the organic and the mineral parts of the enamel, and the distribution of the organic matrix is always inferred from the observed images. Other than a polarised light microscopy study of relatively thick histological sections (Jalevik and Noren, 2000) there are no reports in the literature on histological studies specifically for the organic matrix.

As expected, the organic matrix of sound enamel was scarce and most of it was lost during the decalcification process. The spaces inside the matrix correspond to the appearance of the enamel rods, which are wide and not interrupted by areas of thick organic material.

The images of MIH enamel were quite different, with substantial retention of the organic matrix. Spaces in the matrix representing the rods are small and constricted, reflecting thinner enamel rods and wider interrod areas. TEM of MIH enamel showed well-organised, but less densely packed rods, with an average interrod width of approximately 100 nm. The size of the hydroxyapatite crystals (HA) was similar to that of sound enamel (Xie et al., 2008a). SEM investigation of MIH teeth showed that in MIH enamel, although the basic rod structure was observable, a degree of disintegration of the rods was present (Fagrell et al., 2010; Jalevik et al., 2005). The more severe the defects were clinically, the thinner the rods and the wider the interrod areas were.

In some areas of MIH enamel, the width of the organic matrix left no space for the enamel rods. SEM has revealed membrane-like layers that were commonly found between different prism layers (Fagrell et al., 2010). This may be the thick organic content seen in the current histological study.
Polarised light microscopy of undecalcified 120 µm histological sections from MIH teeth can be used to measure porosity (Jalevik and Noren, 2000). The affected regions of MIH enamel showed different porosities, sometimes reaching more than 5%. The more discoloured the enamel, the more porous it appeared. This was confirmed by Farah et al (2010a; 2009). The junction with the sound cervical enamel followed the lines of Retzius.

TEM images of the junction area between affected MIH and sound cervical areas showed wider interrod areas than the normal enamel, but it was bridged by many HA crystals resulting in less voided interrod areas (Chan et al., 2010). The inner enamel closer to the DEJ was usually more porous. Similarly, the area closer to the DEJ in the current study was denser with organic matrix than the more superficial enamel. Graver et al (1978) argued that full maturation does not occur in the layer of sound enamel adjacent to the DEJ, and it remains richer in organic materials.

Tuft-like structures were seen extending from the DEJ into the enamel only in MIH enamel sections. Tufts are a common feature in histological sections of sound enamel, but it was not seen in sections from the sound tooth in this study. In general, enamel tufts projecting from the DEJ into varying distance in the enamel are not fully understood, but they represent branched, ribbon-like structures (Nanci and Ten Cate, 2007; Sognnaes, 1949) with higher organic content than the rest of prismatic enamel. The organic content of the tufts was shown not to be amelogenin (Amizuka et al., 2005), and its relationship to other enamel proteins like tuftelin, ameloblastin, keratin or enamelin is not understood (Fincham et al., 1999; Robinson et al., 1989; Robinson et al., 1975). A particular similarity exists between tuft proteins and keratin (Robinson et al., 1989, Robinson et al., 1975). Interestingly, in Chapter V, serum albumin showed higher concentration at the tufts. Since the tufts themselves are not fully understood, it is hard to conclude what the albumin presence in the tufts means.

The cervical part of MIH enamel has been shown to be of normal appearance under SEM (Fagrell et al., 2010; Jalevik et al., 2005), normal mineral density (Farah et al., 2010c), and normal mechanical properties (Farah, 2009; Mahoney et al., 2004). However, in this study, a thin layer of organic matrix was retained in the cervical enamel close to the DEJ. This layer was not observed in sound enamel from the normal tooth. In the polarised light
microscopy of Jalevik and Noren (2000) a thin area in the sound cervical enamel, close to the DEJ, showed higher porosity than the rest of the cervical enamel. In addition, in our previous work using x-ray microtomography (XMT) (Farah et al., 2010c), the mineral density of the deeper part of the sound cervical enamel close to the DEJ was low and relatively similar to that of the MIH enamel part (Fig 6.5). This hypomineralised area is most likely because MIH defects follow the incremental lines of enamel formation, which if followed from the cusp towards the cervical part, curve from the enamel surface occlusally towards the DEJ cervically.

No differences could be detected between dentine under MIH enamel and under sound enamel from the normal tooth. Previous SEM and polarised light microscopy studies showed the dentine under affected MIH enamel to be morphologically normal except for the presence of interglobular dentine in some areas (Heijs et al., 2007).

In summary, this histological study has shown that MIH enamel has constricted enamel rod spaces and very dense organic matrix especially in the deeper part adjacent to the DEJ. This deeper part seems to retain a somewhat denser enamel matrix even in the sound cervical enamel.
Figure 6.5. XMT images of cross-sections at different levels in an MIH molar. At the top left corner of each figure, are a macro-photograph and a sagittal section to show the level at which each cross-section was taken. (a) The colour coding. (b) Cross-section at a very cervical level. (c) Cross-section at a more occlusal level, but still within the sound cervical enamel. The white box shows a hypomineralised area localised to the deep enamel close to the DEJ. (d) At a more occlusal level within the affected MIH enamel, the hypomineralisation is clear and spans the thickness of the enamel [adapted from (Farah et al., 2010c)].
Chapter VII

General Discussion, Conclusions and Future Research

A major challenge in understanding the aetiology of MIH lies in studying an affected tissue while the development of normal sound tissue is only partially understood. Until recently, most of the literature on enamel formation focused on the major enamel proteins: amelogenin, enamelin, ameloblastin and tuftelin. However, as new findings emerge, including those from the current investigation, it has become clear that enamel formation is not only a more complicated process, but also probably has similarities to dentinogenesis in aspects that involve common proteins. Many factors, including proteins expressed in the ameloblasts, proteins from the serum, and even proteins of unknown origin have significant roles to play in enamel formation that involve complex interplay. Even proteins that were previously implicated in amelogenesis now appear to have additional functional roles. For example, amelogenin has previously been described as enamel specific protein with structural and mineralisation-modulating roles. A recent investigation found that it helps regulate cell signalling in periodontal ligament cells and osteoblasts (Matsuzawa et al., 2009).

Three aspects of this thesis aimed to investigate the role of blood and serum proteins in the development of MIH. The hypothesis was that MIH is of traumatic origin, most likely
physical in nature that occurs around the time of birth. Several findings from this work and other studies appear consistent with this hypothesis. On the other hand, since the molecular basis of enamel formation and the chronology of the events involved are not well understood, a conclusion that obstetric trauma is the cause of MIH development may be premature.

Tandem mass spectrometry (MS/MS) of enamel proteins showed that few proteins were found in MIH enamel only and not in sound enamel, or were in significantly greater abundance in MIH enamel. Most of these proteins are involved in tissue injury and repair or in bleeding and coagulation. Because of significant keratin contamination, these results cannot be considered conclusive.

Evidence from direct immunofluorescence (DIF) mapping of the three protein markers was stronger due to the use of an appropriate negative control. Albumin and Hb clearly penetrated MIH enamel but not mature sound enamel. The presence of these blood proteins in MIH enamel may have been due to penetration during enamel formation, as proposed for the pathogenesis of MIH. Alternatively, these proteins may have penetrated MIH enamel after the completion of enamel formation, either during tooth eruption or due to gingival bleeding. Blood is unlikely to enter into enamel during eruption as haemorrhage does not normally occur during eruption (Bhaskar, 1991). It is also unlikely that blood from gingival bleeding will enter hypomineralised enamel. Serum proteins do not penetrate demineralised enamel (or hypomineralised enamel in the case of MIH), and can only gain access to enamel during the dynamic process of demineralisation/remineralisation (Robinson et al., 1998b).

A declining gradient of albumin and Hb was detected from the dentine-enamel junction (DEJ) to the outer enamel surface. It was difficult to determine whether this gradient was due to bleeding through the DEJ, or through the ameloblasts but eventually concentrating in the deeper enamel. The finding of Hb in the dentinal tubules under affected MIH enamel but not under sound enamel points to the former possibility. Several questions arise: Why would blood penetrate into MIH enamel from dentine? Could that blood have diffused from the MIH enamel, across the DEJ and into the dentine? Or could both enamel and dentine be
affected by the insult that caused the MIH enamel? Could the particular teeth studied have had bleeding from the hyperaemic pulp into the dentine? It has not been possible to answer these questions within the scope of this limited study. Since the DIF study was performed on a limited number of teeth it would also not be appropriate to generalise. Clearly, more teeth need to be studied to reach any more general conclusions. However, previous studies have shown that dentine under MIH enamel usually appeared normal (Heijs et al., 2007; Mahoney, 2005). Also, an indirect immunofluorescence study found that the pulpal tissue was inflamed in MIH teeth, but was hyperaemic only in the more severely affected teeth (Rodd et al., 2007).

The DIF study has provided some significant methodological advances. The autofluorescence of MIH enamel appeared problematic in pilot work because it masked the fluorescence of the green quantum dots (QDs). However, this property was later used to advantage to provide a clear map of the anatomy of the enamel and the dentine, on to which immune-reacted red and orange QDs fluorescence could be superimposed. In addition, the time required for the quantum dot conjugation reaction and the necessary washing steps was demonstrated. A third important point was the storage of the samples. An important advantage of QDs is their extended life compared with fluorescent antibodies. However, the fluorescence of samples stored in PBS faded with time. This may be due to wash out of the QD-antibody complexes from the sample or due to precipitants from the PBS on the sample. Although this problem could be avoided by keeping sections well hydrated in a humid chamber it resulted in another problem. Highly fluorescent “bodies” of unknown origin appeared to precipitate on the sample. Extended washing with PBS failed to wash away these fluorescent bodies. It therefore appears preferable to study cut sections immediately or after storage dry, i.e. between two tightly sealed glass slides. For the present study, however, all samples were assessed before storage. No fluorescence background or precipitated fluorescent bodies were detected in either sound enamel or untreated MIH enamel samples.

The histopathological assessment of the enamel organic matrix showed that the matrix of MIH enamel was very dense, constricting the growth in width of the enamel rods, and perhaps even intervening between layers of the rods. Inner enamel, close to the DEJ, at the
sound cervical part also seemed to be of higher organic content. This follows the incremental lines of enamel formation. Tuft-like structures were detected in the MIH enamel but not in the sound enamel.

Based on the findings of this study, and as explained briefly in previous chapters, the following model for MIH pathogenesis is suggested (Fig 7.1):

During normal enamel development, serum (predominantly albumin) starts “leaking” into enamel during the secretory stage. This leakage possibly occurs along with the enamel tissue fluids that make up the fluid content of the enamel (ending up with minute amounts of water in mature enamel). Albumin is necessary in amelogenesis since it has the function of binding and transporting calcium (Okamura, 1983). Albumin reaches its maximum concentration during the late secretory/early transition stage (Robinson et al., 1994) and is subsequently degraded and removed in the late secretory/early maturation stage. This corresponds with the mineral growth, which remains constant during the secretory and transitional stages. The loss of proteins in the transition stage is not accompanied by an increase in mineral growth, but rather the resultant porosity is filled up with tissue fluids (presumably with serum proteins resulting in the maximum concentration of albumin in the early transition stage) (Robinson et al., 1988). Albumin then disappears in the maturation stage (Robinson et al., 1994). This temporal distribution of serum (with albumin as its main component) parallels with amelogenin secretion and removal. During the secretory stage, nascent amelogenin is the main protein of the organic matrix, and is present in a solid state (Brookes et al., 1995), that prevents the albumin from directly contacting the hydroxyapatite (HA) crystals. This “protective” role of amelogenin is important since if albumin is allowed to reach the crystals, it will prevent their growth (Garnett and Dieppe, 1990; Robinson et al., 1992). Amelogenin itself also appears to prevent the crystal growth by unknown mechanisms (Robinson et al., 1996). As the albumin concentration reaches its peak in the late secretory/early transition stage, amelogenin is being degraded by the serine proteinase into fluid-soluble molecules and removed. In order not to interfere with the crystal growth, the albumin is also degraded in the late transition stage, possibly by the same proteinase, and is almost completely removed, with minute amounts remaining in the maturation stage in crystal bound form (Robinson et al., 1996).
Figure 7.1. Enamel formation stages with respect to albumin. (a) Anti-albumin probing of rat enamel proteins isolated from progressive secretory-stage enamel. Albumin concentration increases gradually and starts showing break-down products towards the end of the stage. (b) Diagrammatic representation of the secretory stage showing dense amelogenin matrix (dark blue) and thin HA crystals (red circles) preventing most of the albumin (black arrows) from entering into the enamel. (c) Transition stage probing showing the highest concentration of albumin in the early part. Degradation of the albumin is still proceeding. (d) Diagrammatic representation of the transition stage showing tissue fluids (light blue) entering the enamel (now mostly porous and with little matrix), and bringing along serum proteins including albumin. The width of the crystals remains constant during this stage. (e) Maturation stage probing showing gradual reduction in the concentration of the 66 kDa albumin. The degraded products most likely are removed or become mineral bound. (f) Diagrammatic representation of the maturation stage showing fully mature crystals with minimum amounts of retained matrix. [Gel electrophoresis images adapted from (Robinson et al., 1996)].

A hyperaemic insult resulting in increased blood/serum entering the enamel usually has its most dramatic effect in the transition/early maturation stages, with apparently no, or at least repairable effects during the secretory stage. Transition and early maturation stages are therefore most vulnerable to the unregulated effects of excess albumin (Lyaruu et al., 2008;
Robinson et al., 1994). Albumin that exceeds the protective capacity of the solidified amelogenin binds HA crystals and prevents them from maturing.

In addition, in a study on bovine enamel to determine the rate and duration of enamel development, it was found that the time required for enamel to mature varied from one part to another in the same tooth and the enamel in the occlusal half required longer maturation phase than that in the cervical half (Deutsch et al., 1979). This means that the occlusal part of enamel is always at more risk than the cervical part.

Although this model successfully accounts for the structural and morphological aspects of MIH enamel, the current investigation has not identified, with certainty, the nature of this hyperaemic insult. The hypothesis that this is the result of an event that occurs around birth time, possibly through certain obstetrical practices, is yet to be confirmed or disproved. One possible method is tracing specific foetal proteins in MIH enamel, such as \( \alpha \)-fetoprotein, fetuin and foetal albumin. Their presence and percentage may prove that these proteins, trapped in the organic matrix, reached developing enamel around birth time since they are most abundant around that time and decline rapidly afterwards. Another approach is to compare the birth records of a group of children with MIH, with those of an individually matched control group. Certain events may be specifically examined, such as length of second phase of labour, mode of delivery, and head circumference as these have been linked to facial bleeding in newborn infants. Both methods are being conducted presently at the Department of Oral Sciences, Faculty of Dentistry.
References


Appendix 1

Health and Disability Ethics Committees

2 March 2007

Rami Amin Farah
University of Otago
Department of Oral Sciences
School of Dentistry
Dunedin

Dear Rami

Investigation of dental enamel hypomineralisation and hypoplasia, relationships with general health, family history and methods to improve the quality of the enamel.

Lead Investigator: Rami Amin Farah
Co-Investigators: Dr Bernadette K Drummond, Professor Michael Swain
School of Dentistry, University of Otago

MEC/06/12/177

The above study has been given ethical approval by the Multi-region Ethics Committee.

Approved Documents
- Information sheet for participants and their legal guardians dated November 23, 2006
- Consent form
- Structured questionnaire on general health and family history, to be administered by telephone
- Invitation letter: "Why we need your help!"
- Invitation letter to dentists

Accreditation
The Committee involved in the approval of this study is accredited by the Health Research Council and is constituted and operates in accordance with the Operational Standard for Ethics Committees, April 2008.

Progress Reports
The study is approved until 1 March 2009. The Committee will review the approved application annually and notify the Lead Investigator if it withdraws approval. It is the Lead Investigator's responsibility to forward a progress report covering all sites prior to ethical review of the project in March 2008. The report form is available on http://www.health.govt.nz/ethics/committees. Please note that failure to provide a progress report may result in the withdrawal of ethical approval. A final report is also required at the conclusion of the study.

Amendments
It is also a condition of approval that the Committee is advised of any adverse events, if the study does not commence, or the study is altered in any way, including all documentation eg advertisements, letters to prospective participants.

Please quote the above ethics committee reference number in all correspondence.

It should be noted that Ethics Committee approval does not imply any resource commitment or administrative facilitation by any healthcare provider within whose facility the research is to be carried out. Where applicable, authority for this must be obtained separately from the appropriate manager within the organisation.
Yours sincerely

Sue Fish
Multi-region Ethics Committee Administrator
Email: sue_fish@moh.govt.nz
Appendix 2

Instructions Sheet for Dentists

1. Please take few minutes to read the attached document entitled “Information for participants and their legal guardians” which gives some background about the study.
2. Give the parent/guardian a copy of the attached information sheet about the study, “Information for participants and their legal guardians”.
3. Please also explain the study to the child. This can be done by you and/or the child’s parent. We have also provided an information sheet for the child entitled “Why we need your help!” that the child may wish to read.
   Please note that we prefer teeth extracted from children under the age of 16 for our study.
   If the family’s first language is not English and they ask for an interpreter and one cannot be provided, then the child should not be included in the study.
4. If the child and the parent agree to participating in the study (that they are willing to give up the extracted tooth/teeth to be sent to us and that the parent agrees to being contacted for a telephone interview), please ask the parent/legal guardian to sign the attached Consent Form. Please remember that only a parent or a legal guardian can give consent on the child’s behalf for participating in the study. If the child is not accompanied by either of these, the child should not be included in the study.
   In the case where a parent agrees, on his/her child’s behalf, to participate in the study, and the child still refuses to donate the extracted tooth, no pressure should be put on the child and the child should not be included in the study.
5. Once the tooth/teeth are extracted, please wash it thoroughly from blood using sterile water or saline.
6. Put the tooth/teeth in the enclosed plastic vial (which already contains thymol crystals for disinfection).
7. Top up the vial with saline or distilled water (if you have both, saline is preferred).
8. Tightly seal the vial and fill-in the attached form on the child’s biographical data.
9. Put the vial in the plastic container, and keep it tightly closed with an adhesive tape.
10. Together with the signed consent form and the biographical sheet, put the plastic container in the enclosed self-addressed padded envelope and send them back to us.
11. If there are any related dental radiographs, please include them in the package. We will make a copy and send them back to you.
# Appendix 3

## Study of dental enamel defects and possible treatment methods

### Information about the child participant from whom the teeth were obtained

<table>
<thead>
<tr>
<th>Hospital/Department</th>
<th>:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name of the Dentist</td>
<td>:</td>
</tr>
<tr>
<td>Contact Telephone Number</td>
<td>:</td>
</tr>
<tr>
<td>Number for the Dentist</td>
<td>:</td>
</tr>
<tr>
<td>Name of the Child</td>
<td>:</td>
</tr>
<tr>
<td>Date of Birth</td>
<td>:</td>
</tr>
<tr>
<td>Consent Obtained?</td>
<td>:</td>
</tr>
<tr>
<td>(form to be attached)</td>
<td>:</td>
</tr>
<tr>
<td>Contact Details</td>
<td>:</td>
</tr>
<tr>
<td>Address</td>
<td>:</td>
</tr>
<tr>
<td>Telephone Number</td>
<td>:</td>
</tr>
<tr>
<td>Mobile Phone Number</td>
<td>:</td>
</tr>
</tbody>
</table>

Which teeth were extracted?

Are there radiographs? ..................

If yes, may we please have these to copy or could you please send us a copy?

Are there any other teeth in the mouth affected with enamel hypomineralisation or hypoplasia? (if yes, what are they?)

Please include this form with the package that you will send back to us.

Thank you for your help.
Appendix 4

Information for participants and their legal guardians

Study of dental enamel defects and possible treatment methods
November 23rd 2006

Principal Investigator: Rami Farah
PhD student in Dentistry
Department of Oral Sciences
School of Dentistry, University of Otago
Dunedin
Tel: 03 479 7879

Supervisors: 1) Associate Professor Bernadette Drummond
Head of Discipline of Paediatric Dentistry
Department of Oral Sciences
School of Dentistry, University of Otago
Dunedin
2) Professor Michael Swain
Head of Discipline of Biomaterials
Department of Oral Rehabilitation
School of Dentistry, University of Otago
Dunedin

Introduction

Your child is invited to take part in a study of a condition called molar incisor
hypomineralisation (enamel defects) and possible ways to improve the tooth enamel to
improve the success of dental fillings. You can take as much time as you like to consider if
your child and you are happy to donate to the study the teeth he/she is
having extracted. This time frame does not affect the planned treatment for your child. Taking part in this study is voluntary (your choice) and you/your child can decline to give the teeth to the study, and this will in no way affect your child’s continuing dental care.

**About the study**

This project is studying a condition which is affecting some of your child’s teeth. This condition is called molar incisor hypomineralisation, and means that the enamel of the teeth has not formed properly. This condition may present as white chalky spots, or yellow-brown areas on teeth, or rough pitted areas on teeth. It may be caused by fevers or other illnesses during the first years of life. How this happens is not well understood. The teeth are often very sensitive because of the thin enamel, and they may get more decay. The teeth are usually very difficult to fill because filling materials do not last.

Our study is looking at the causes of this condition to understand the damage to the tooth enamel. We hope this will help us to find ways to improve the enamel so that dental fillings will be more successful.

For this study, we need to look at teeth which have been extracted because of this condition. Therefore, we have contacted dentists from all over New Zealand and asked them to help us. We asked them to tell any parents and patients with molar incisor hypomineralisation whose treatment will involve extraction of the affected teeth about this study. They will seek consent for the damaged teeth to be donated to the study. All children who donate their teeth will receive a $10 book voucher.

Your child has been asked to take part in this study because the dentist has diagnosed that your child’s tooth/teeth that are being extracted have molar incisor hypomineralisation.

If you and your child consent to participating in this study, the following steps will be taken:

1. The dentist will ask you to sign a consent form on behalf of your child for participating in this study. Your child may sign it as well if they wish.
2. Your dentist will send us the extracted tooth/teeth as well as any related dental radiographs and we shall write to your child with the voucher.

3. We will study the teeth under an electron microscope, and test different materials that may harden enamel.

4. We will contact you by telephone to ask some questions about your child’s health and about any other family member(s) who may have/had this problem. The interview will take about 20 minutes. This information is important to try and determine if there are any causes for this problem that the children in this study share.

5. The interview will not be recorded but the answers will be written down in a coded file which will only be available for the researchers. The interview notes and the teeth will be given linked numbers and your child’s name will then be kept separate from this. The information we collect from you as well as the extracted teeth will be kept for 5 years while results are collected and analysed. We shall also publish the results in scientific and dental journals. The teeth and the collected information will then be destroyed in a proper ceremonial manner, and disposed off in accordance with the dental school medical waste disposal policy.

6. We shall send you a summary of the results at the end of the study. We shall also contact you if we discover any specific information about your child’s teeth that may help the diagnosis or his/her treatment.

**Benefits, risks and safety**

There will be no risks to your child donating the teeth to this study. There may be benefits for other children if we can develop improved treatments.

Taking part in this study will not cost you anything and your child will receive a $10 book voucher.
Participation

Your child’s participation by donating their teeth is entirely voluntary (you and your child’s choice). You and your child do not have to take part in this study, and if you choose not to take part, your child will receive the planned dental care, and no future care or treatment will be affected.

If you do agree to your child taking part, you are both free to withdraw from the study at any time, without having to give a reason and this will in no way affect your child’s future dental health/care. You may ask for the teeth to be removed from the study by phoning or writing to Mr Rami Farah at:

Rami Farah
PhD student in Dentistry
Department of Oral Science
School of Dentistry, University of Otago
Dunedin
Tel: 03 479 7879

General

If you wish, you may sign the part of the consent form which allows us to give information for your child’s dentist about any significant clinical factors that are discovered in the interview which could be of help for the dentist in treating your child. We will confirm this with you again by a phone call.

If you wish, we will contact you at the end of the study (possibly in three years time) and inform you of the results of the study.

You can get more information about the study by contacting the principal investigator at the address or telephone number provided in the first page of this sheet.

If you need an interpreter, one can be provided. You may have a friend, family or whanau support to help you understand the inconveniences and/or benefits of this study and any other explanation you may require.
During the telephone interview, you do not have to answer all the questions, and you may stop the interview at any time. Also, you may contact us at any time if you wanted to change your answer to any question, or if you want to completely omit an answer.

If you/your child have any queries or concerns regarding your child’s rights as a participant in this study you may wish to contact a Health and Disability Advocate, telephone

- Northland to Franklin 0800 555 050
- Mid and lower North Island 0800 42 3638 (4 ADENT)
- South Island except Christchurch 0800 377 766
- Christchurch 03 377 7501

Confidentiality

No material which could personally identify your child or you will be used in any reports on this study.

Your child’s tooth/teeth as well as your interview file will be given a reference number that will identify your child. This reference number, the extracted teeth and the interview file will be kept with the principal investigator and supervisors and will not be disclosed to any one else. There will be no names or other way of identification attached to the interview file or the teeth.

Results

You can get the results of this study by contacting the principal investigator at the address or telephone provided on the first page of this information. The results will be published in internationally recognised dental journals and will be presented at international conferences. There will always be a delay between data collection and data publication.
Statement of approval

This study has received ethical approval from the Multi-region Ethics Committee which reviews National and Multi regional studies.

Please feel free to contact the researcher if you have any questions about this study.
Hi there, my name is Rami Farah. I am a researcher at the School of Dentistry in Dunedin. I would like to ask for your help in understanding why your tooth has a problem.

I am studying under a special microscope teeth with problems that have been pulled out. I am trying to find a way to stop these teeth being sensitive.

I hope you will agree to send me your tooth/teeth. I will send you a $10 book voucher. I will also send your parents information about what I find.

If you have any questions you can ask your dentist or your mom or dad. If you still want to keep your tooth, you can take it home with you and that is fine with everybody. It is your decision whether to give us your tooth or not.

Thank you for your help.

Rami Farah
Appendix 6

CONSENT FORM

Study of dental enamel defects and possible treatment methods

<table>
<thead>
<tr>
<th>Language</th>
<th>Translation</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>English</td>
<td>I wish to have an interpreter</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Maori</td>
<td>E hiahia ana ahau ki tetahi kaiwhakamaori/kaiwhaka pakeha korero</td>
<td>Ae</td>
<td>Kao</td>
</tr>
<tr>
<td>Cook Island</td>
<td>Ka inangaro au I tetai tangata uri reo</td>
<td>Ae</td>
<td>Kare</td>
</tr>
<tr>
<td>Fijian</td>
<td>Au gardreva me dua e vakadewa vosa vei au</td>
<td>Io</td>
<td>Sega</td>
</tr>
<tr>
<td>Niuean</td>
<td>Fia manako au ke fakaaoaga e taha tagata fakahokohoko kupu</td>
<td>E</td>
<td>Nakai</td>
</tr>
<tr>
<td>Samoan</td>
<td>Out e mana’o ia i ai se fa’ amatala upu</td>
<td>Io</td>
<td>Leai</td>
</tr>
<tr>
<td>Tokelaun</td>
<td>Ko au e fofou ki he tino ke fakalilii te gagana Peletania kin a gagana o na motu o te Pahefika</td>
<td>Ioe</td>
<td>Leai</td>
</tr>
<tr>
<td>Tongan</td>
<td>Oku ou fiema’u ha fakatonulea</td>
<td>Io</td>
<td>Ikai</td>
</tr>
</tbody>
</table>

I have read and understand the information sheet dated November 23rd, 2006 for participants (or legal guardians of participants) donating extracted teeth for the study designed to investigate the nature of molar incisor hypomineralisation. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given. I also have had the opportunity to use whanau support or a friend to help me ask questions and understand the study.
I have had this project explained to me by the dentist arranging dental extractions for my child.

My child was given full information about the research in a form that he or she could readily understand.

My child was given the opportunity to ask questions and to have those questions answered to my child’s satisfaction.

I believe that _______________________________ (participant’s name) would have chosen and consented to participate in this study if he/she had been able to understand the information that I have received and understood.

I understand that taking part in this study is voluntary (my and my child’s choice) and that I/my child can decline participation and that I may ask for the teeth and information to be withdrawn at any time and that this will in no way affect my child’s future dental health care.

I understand that my child’s participation in this study is confidential and that no material which could identify him/her will be used in any reports on this study.

I understand that my child’s extracted teeth, the radiographs and the data pertaining to my child’s participation in this project may be retained by the researchers for 5 years for continuous research. I also understand that my child has the right to withdraw consent to the continued use or retention of personally identifiable health research data once he or she attains the age of 16.

I also have had the time to consider whether to take part.

I know who to contact if I have any questions about the study.

This study has been given ethical approval by the Multi-region Ethics Committee. This means that the Committee may check at any time that the study is following appropriate ethical procedures.

- I consent to the researchers storing my child’s extracted teeth for its later use as a part of this study.................YES / NO
- I consent to the extracted teeth being disposed off at the end of this study........................................YES / NO
- I would like the principal researcher to discuss the outcomes of the study with me..................................YES / NO
- I agree and believe my child would agree to his/her dentist being informed of relevant clinical information .......YES/NO
I ……………………………………………… hereby consent my child ………………………………………………… to take part in this study.

Date:

Signature:

Relationship to Participant: __________________________________________

Address for results: __________________________________________

Names of Researchers:
- Mr Rami Farah (Tel: 03 479 7879)
- Associate Professor Bernadette Drummond (Tel: 034797128)
- Professor Michael Swain (Tel: 03 479 4196)

STATEMENT BY PRINCIPAL INVESTIGATOR

I Rami Farah, declare that this study is in the potential health interest of the group of patients of which ……………………………………..... (name of participant) is a member and that participation in this study is not adverse to …………………………………….. (name of participant)’s interests and welfare.

Signed: _____________________________  Date _______ ___________________
Principal Investigator

Project explained by: Treating Dentist:

Project role: Identification of children to participate in the study.

Signature:

Date: