

**Role of Matrix Metalloproteinases in Caries**

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Declaration

I, the author, confirm that the Thesis is my own work. I am aware of the University’s Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). The entirety of the laboratory work and clinical data analysis presented in this thesis, unless otherwise stated, is that of the author. The author has been responsible entirely for writing up and drafting. A thesis editor has not been used.

This work has not been previously accepted for any other award at this, or any other, university.

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Abstract

**Background:** Cariogenic bacterial acids dissolve the inorganic elements in dentine caries and expose the dentine matrix. Host-derived matrix metalloproteinases (MMPs) play an essential role in caries progression by degrading exposed collagen. MMP inhibitors such as silver diamine fluoride (SDF) are a non-invasive treatment to arrest caries, often in combination with potassium iodide (KI) to minimise tooth discolouration.

**Aim:** To investigate MMPs in the context of caries diagnosis and management by correlating MMPs to ICDAS scores and then investigating the long-term anti-MMP potential of SDF in relation to two important clinical variables: the presence of KI and application time.

**Methods:** Twenty-four extracted teeth diagnosed with ICDAS were sectioned and 24 sections were analysed immunocytochemically with anti-MMP2 and anti-MMP9 antibodies. Positive staining was visualised by immunofluorescence using a VectorFluorDuetDouble Labelling Kit. Images of triplicate samples for each ICDAS score were analysed using ImageJ software. A further 45 carious dentine blocks were subjected to one of four regimens: i) baseline; ii) SDF application; iii) SDF+KI application, iv) control group. Blocks were incubated in natural saliva for 1, 4 and 12 weeks. Total MMP activity was determined with a colorimetric MMP assay and collagen degradation was measured with a hydroxyproline assay. Eighteen additional blocks were analysed after 1- or 3-minute SDF applications.

**Results:** MMP2 and 9 were undetectable in caries with ICDAS 0-2, and an increase was found in ICDAS 3. A significant increase in MMPs was observed at ICDAS scores of 4-6. There was a statistically significant positive correlation between ICDAS score and MMPs. SDF significantly inhibited total MMP activity at 1, 4 and 12 weeks by 93%, 91% and 55%, respectively, while the use of KI reduced activity to 55.5%, 48% and 20%. There was no significant difference in MMP activity between 1- and 3-minute SDF application times.

**Conclusion:** The presence of MMPs and degree of collagen degradation correlates strongly with caries assessed by ICDAS, validating the modern approach to caries treatment with a minimally invasive approach. Lower overall efficacy of SDF could negate any potential benefit of KI in reducing discolouration. Nevertheless, even a 1-minute application of SDF could be clinically effective in stopping caries activity.

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**List of abbreviations**

|  |  |
| --- | --- |
|  |  |
| **ABC** | Avidin-biotin complex |
| **ANOVA** | Analysis of variance |
| **AOI** | Area of interest |
| **AP** | Alkaline phosphatase |
| **BASCD** | The British association for the study of community dentistry |
| **BSP** | Bone sialoprotein |
| **CaF2** | Calcium fluoride |
| **CHX** | Chlorhexidine |
| **DGP** | Dentine glycoprotein |
| **DMFT** | Decayed, Missing due to caries, and Filled Teeth index |
| **DMP1** | Dentine matrix protein |
| **DPP** | Dentine phosphoprotein |
| **DSPP** | Dentine sialo phosphoprotein |
| **ECM** | Extracellular matrix |
| **EDJ** | Enamel-dentine junction |
| **EDTA** | Ethylenediaminetetraacetic acid |
| **ELISA** | Enzyme-linked immunosorbent assay |
| **GA** | General anaesthesia |
| **GAGs** | Glycosaminoglycan |
| **GCF** | Gingiva-crevicular fluids |
| **GIC** | Glass ionomer cement |
| **H2O** | Water |
| **H2O2** | Hydrogen peroxide |
| **HAP** | Hydroxyl apatite |
| **HCI** | Hydrochloric acid |
| **HIAR** | Heat-induced antigen retrieval |
| **HNSCC** | Head and neck squamous cell carcinoma |
| **HRP** | Horseradish peroxidase |
| **HYP** | Hydroxy proline |
| **ICCMS**  **ICDAS** | International caries classification and management system  The International Caries Detection and Assessment system |
| **IHC** | Immunohistochemistry |
| **KI** | Potassium Iodide |
| **MEPE** | Matrix extracellular phosphorylated glycoprotein |
| **MMPs** | Matrix Metalloproteinases |
| **NaOH** | Sodium hydroxide |
| **NCPs** | Non-collagenous protéines |
| **OPN** | Osteopontin |
| **OSCC** | Oral squamous cell carcinoma |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **PMNs** | Poly-morphonuclear leukocytes |
| **PRPs** | Proline- rich proteins |
| **PVPA** | Polyvinylphosphoric acid |
| **SDF** | Silver diamine fluoride |
| **SIBLING** | Small Integrin Binding Ligand N-linked Glycoprotein |
| **SMART** | Silver-modified atraumatic restorative technique |
| **TIMP** | Tissue inhibitor metalloproteases |
| **VHN** | Vickers hardness number |

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# CHAPTER 1

LITERATURE REVIEW

# Chapter 1: Literature Review

## Anatomy of the tooth

Teeth are made of four major tissues: enamel, dentine, pulp and cementum. The crown of the tooth is covered by enamel; the root of the tooth is covered by cementum.

### Enamel

  Tooth enamel is a hard and mineralised structure (95% by weight), and only 5% of its weight consists of organic matrix and water (Ten Cate, 1994). As a result, enamel acts as an insulating barrier that protects the tooth from physical, chemical and thermal forces that would otherwise damage vital tissues in the underlying pulp. Hydroxyapatite crystals (HAP) are the main mineral in tooth enamel. These crystals are densely packed in an ordered pattern and form enamel rods [enamel prism]. The intercrystalline spaces between the hydroxyapatite crystals form a network of diffusion pathways in the enamel called "microspores" (Fejerskov and Kidd, 2009).

### Dentine

  Dentine is a calcified tissue that makes up most of the tooth. Unlike enamel, dentine is a vital and sensitive tissue that forms throughout life (Ten Cate, 1994). Dentine is softer than enamel, less brittle and less mineralised. It is made up of 70% minerals by weight, 20% collagen and 10% water and non-collagenous proteins (NCPs) (Nanci, 2008; Fejerskov et al., 2015). Of the 20% collagen, 90% is type I collagen. Type III and V collagen have also been found in smaller amounts (Goldberg and Smith, 2004). Collagen molecules contain an intertwined triple helix of 2 ἀ-1 polypeptide chains and one ἀ-polypeptide chain. These chains are twisted together to form a right-handed superhelix molecule joined together to form a fibre. The spaces between the collagen fibres are called the "gab zone" (Butler, 1987). The fibres are stabilised by cross-links that form within and between the helical units, giving the collagen its stiffness and strength. The collagen network provides the plastic, tensile properties of the dentine, while the mineral component provides the hardness of the dentine. These properties give the enamel the necessary support (Nanci, 2008; Fejerskov et al., 2015).

## Dentine matrix proteins

Pre-dentine, a non-mineralised dentine, is formed by the secretion of thin collagen fibrils at the apical pole of odontoblasts (Fejerskov and Kidd, 2009). These collagen fibrils form a template through fibrinogenesis and cross-linking, which is mineralised.

Non-collagenous proteins (NCPs) are distributed between the collagen fibrils and accumulate on the tubule walls, which have been associated with the regulation of dentine mineralisation, growth and nucleation of HAP (Nanci, 2008; Fejerskov et al., 2015). The importance of NCPs in dentine mineralisation and the growth and nucleation of HAP is associated with the mineralisation of members of the Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) family. This family includes dentine matrix protein 1 (DMP1), dentine sialo-phosphoprotein (DSPP), bone sialoprotein (BSP), osteopontin (OPN) and extracellular phosphorylated matrix glycoprotein (MEPE) (Qin et al., 2004; Opsahl Vital et al., 2012). Members of the SIBLING family have been identified in both the ECM of bone and dentine; however, DSPP has been identified as specific to the dentine (Fisher and Fedarko, 2003).

During tooth development, DSPP is cleaved into three proteins: Dentine sialoprotein (DSP), Dentine phosphoprotein (DPP) and Dentine glycoprotein (DGP) (Yamamoto et al., 2015), with DPP being most abundant in dentine and considered key to the formation of dentine hydroxyapatite (Lussi and Linde, 1993). Proteoglycans, one of the NCP components of dentine, are concentrated in the gap zone and play an essential role in the mineralisation of dentine as they contain the core protein glycosaminoglycan' (GAGs). They play a role in preventing premature mineralisation, regulating the mineralisation process, and controlling the growth of HAP (Waddington et al., 2003).

## Demineralisation of dentine

When the tooth is exposed to acidic conditions, demineralisation occurs. Demineralisation involves the dissolution of HAP, which destroys the tooth structure (Fejerskov and Kidd, 2009). This can happen through three different processes: Caries, tooth erosion or the formation of the hybrid layer, which is necessary for the bonding of restorative materials.

## Dental caries

Dental caries is the result of an imbalance in the biofilm. Certain bacteria in the oral cavity cause local destruction of mineralised dental tissue (Fusayama, 1979; Caufield and Griffen, 2000). This can occur at any time during life and plays a significant role in the development of pulp inflammation and infection (Pitts, 2004).

### Prevalence of dental caries

Dental caries is a widespread disease worldwide. Its severity and prevalence vary from region to region and country to country and has been shown to have a negative impact on health-related quality of life (Baghdadi, 2015; Gilchrist et al., 2015). The most recent 10-year survey of children's dental health in England, Wales, and Northern Ireland (Children’s Dental Health Survey, 2013) showed a decline in the prevalence of dental caries in children between 1983 and 2013, which was attributed to prevention programmes and the use of fluorides, particularly in toothpaste. However, changes in prevalence between 2003 and 2013 were found to be statistically insignificant. In 2019, data from the National Dental Epidemiology Programme for England showed that 32.4% of 5-year-old children in England had dental caries (NDEP, 2019) and in 2012, data from the World Health Organisation (WHO) suggested that 60-90% of children worldwide had dental caries (WHO, 2012.). This shows that dental caries is still a major health problem (Lagerweij and van Loveren, 2015).

### Aetiology and pathogenesis of dental caries

Dental caries has a multifactorial aetiology (Fejerskov and Manji, 1990; Kidd, 1996.) with four factors required for the development of caries: cariogenic bacteria, fermentable carbohydrates, host (susceptible tooth surface) and time (Southam and Soames, 1993). However, these factors do not always lead to the same outcome. Different people are affected by dental caries to different degrees. This variation depends on many other factors, such as the shape of the tooth, the degree of oral hygiene (fluoride exposure) and the buffering capacity of the saliva (Fejerskov et al., 2015).

Although the oral cavity contains a wide variety of bacteria, a few species are thought to be closely associated with the development of dental caries, particularly Streptococcus mutans (*S. mutans*) and Lactobacilli (Fejerskov and Kidd, 2009). These species make up the majority of bacteria in dental plaque. Dental plaque is a biofilm adhering to the tooth surface that, if left undisturbed, enables the process of caries formation (Marsh and Bradshaw, 1995). It is believed that a single species of microorganism is not sufficient to cause caries, highlighting the crucial role of dental plaque (biofilm) in the development of this disease (Fejerskov, 2004). For many years, the role of bacteria in plaque and their relationship to dental caries has been described by two hypotheses. The first, the specific plaque hypothesis, states that only certain types of bacteria are involved in the development of dental caries. The second hypothesis, the non-specific hypothesis, explains the disease as the result of the activity of the entire microflora in the dental plaque. A later alternative hypothesis, the ecological plaque hypothesis, reconciled the main points of the previous two hypotheses. This hypothesis states that "disease is the result of a shift in the balance of resident microflora caused by a change in local environmental conditions" (Marsh, 2003). In dental caries, frequent episodes of low pH in plaque due to repeated sugar ingestion and lower sugar excretion favour the growth of acid-forming and acidogenic species, which leads to demineralisation of the tooth structure (Marsh, 2003; Fejerskov et al., 2015).

Cariogenic bacteria produce significant levels of organic acids, mainly lactic acid, as by-products of the fermentation of dietary sugars. These acids cause the pH in the mouth to drop, leading to the dissolution of HAP and the removal of minerals (such as calcium) from the tooth tissue. At this point, demineralisation occurs, but can be reversed by remineralisation. Remineralisation promotes the incorporation of calcium and phosphate into the demineralised tooth structure. The presence of fluoride triggers remineralisation by acting as a catalyst for the diffusion of calcium and phosphate to form a more stable, acid-resistant structure, "fluorapatite-hydroxyapatite" (Featherstone, 2004).

In dental caries, demineralisation and remineralisation are considered dynamic processes. However, if demineralisation continues, more minerals are lost, leading to a cavitation (Featherstone, 2004). Disturbances in the physiological balance between the factors that determine plaque composition lead to dental caries. If these disturbances cannot be prevented but can be controlled, the progression to cavitation can be prevented (Fejerskov et al., 2015).

### Caries diagnosis

Dental caries diagnosis is defined as "the art or act of recognising a disease by its signs and symptoms", while the identification of these signs and symptoms is associated with caries detection. Diagnosis and detection of caries are essential for risk assessment, treatment planning, epidemiological research, and clinical trials (Nyvad, 2004). There are various methods for caries diagnosis, such as visual and visual-tactile examination, X-ray examination and laser fluorescence devices.

Scientists and researchers have developed various reproducible and reliable diagnostic systems for the visual examination of dental caries (Backer Dirks et al., 1961; Jackson, 1950; World Health Organisation, 1997). In 2002, a group of caries researchers, paediatric dentists, restorative dentists, and epidemiologists developed a new system, the International Caries Detection and Assessment System (ICDAS) (Ismail, 2004). ICDAS was developed to provide an international evidence-based system for caries diagnosis and assessment that can be used in dental education, clinical application, research, and epidemiological studies (Pitts, 2004; Ismail et al., 2007; Braga et al., 2009) and that also allows standardisation of data collection and comparison between studies (Topping and Pitts, 2009).

According to ICDAS, teeth should be examined with compressed air and after tooth cleaning to detect the early signs of a caries lesion. Any plaque remaining on the surface should be removed with a ball explorer to check whether the tooth surface is discontinuous and whether a dental restoration is present (Ismail et al., 2007). The ICDAS system detects caries in two steps and its code contains two digits. The first digit represents the restoration status and the second represents the severity of the caries lesion (Ismail et al., 2007) (Table 1.1).

Several studies have been conducted to validate the ICDAS system and have shown high validity and reproducibility. Shoaib et al., (2009) found ICDAS to be a suitable system for proximal and occlusal caries diagnosis in primary teeth. Martignon et al., (2007) demonstrated a significant correlation between ICDAS scores and histological caries stages in both primary and permanent teeth. Jablonski-Momeni et al., (2008) also found the ICDAS system to be an optimal method for caries diagnosis in primary and permanent teeth.

**Table ‎1.1** ICDAS score for restorative and caries severity (Ismail et al., 2007).

|  |  |  |  |
| --- | --- | --- | --- |
|  | Restorative status |  | Caries severity |
| Code | Description | Code | Description |
| 0 | Un-restored and unsealed | 0 | Sound tooth structure |
| 1 | Partial sealant | 1 | First visual change in enamel after air drying |
| 2 | Full sealant | 2 | Distinct visual change in enamel without air drying |
| 3 | Tooth coloured restoration | 3 | Localised enamel breakdown with no visible dentine |
| 4 | Amalgam restoration | 4 | Underlying dark shadow from dentin |
| 5 | Stainless steel crown | 5 | Cavity with visible dentine |
| 6 | Porcelain, gold or preformed meta crown or veneer | 6 | Extensive cavity with visible dentine |
| 7 | Broken or lost restoration |  |  |
| 8 | Temporary restoration |  |  |

### Process of dentine caries

Cariogenic bacteria produce organic acids when they metabolise fermentable carbohydrates from food. These acids cause the pH in the mouth to fall below 5.5, which dissolves the mineral component of dentine (Chaussain-Miller et al., 2006; Fejerskov et al., 2015). This leads to the development of dental caries. In dentine, the process can be divided into two distinct phases: (i) the acidic dissolution of the mineralised phase of the tooth and (ii) the breakdown of the collagen matrix. As the bacterial acid dissolves the dentine mineral, it gradually exposes the organic extracellular matrix. The proteases produced by the bacteria degrade the components of the ECM and break down the collagen network, allowing the cariogenic bacteria to advance further towards the pulp. The nature of the tubular structure of dentine favours this progression (Kawasaki and Featherstone, 1997). S. mutants produce two types of proteases that degrade type I collagen and thus play an important role in the development of dental caries (Harrington and Russell, 1994).

Collagen is the major insoluble fibrous protein of the ECM and connective tissue. Sixteen different types have been identified, but 80-90% of the collagen in our bodies is either type I, II or III. The basic structure of all collagens is a triple helix of polypeptide chains interspersed with non-helical regions. (Ricard-Blum, 2011). The unique properties of each type of collagen result from interruptions in the helical and non-helical regions that cause cross-linking and/or folding to form other 3D structures, such as sheets. Collagen initially forms with pro-peptides that support structural changes by aligning disulphide bonds that lead to the formation of mature collagen. The pro-peptides are usually removed after secretion, leaving the tele-peptides, which are involved in inter- and intramolecular cross-linking (Gordon and Hahn, 2010; Ricard-Blum, 2011) and play an important role in increasing the tensile strength of collagen. Bacterial proteases degrade collagen in the telopeptide region and compromise the integrity of collagen fibrils. Katz and Park, (1987) demonstrated that the degradation of collagen by bacterial acids may not be as significant as previously thought, while Kawasaki and Featherstone (1997) stated that bacterial collagenases are very sensitive and cannot withstand the low pH values (4.3) that prevail during the demineralisation phase. Further *in vitro* studies suggest that bacteria in the oral cavity lack the enzymatic capabilities required for the degradation of collagen (Van Strijp et al., 1994; Van Strijp 1997).

These studies thus suggest that the contribution of bacterial proteases to collagen matrix degradation is limited, and host enzymes, including matrix metalloproteinases (MMPs), may play a role in this process (Tjaderhane et al., 1998) (see section 1.5).

### Caries histology

#### **Enamel caries**

Enamel is a highly mineralised structure, and the cariogenic bacteria produce acids, causing demineralisation which affects the crystals in the enamel, which it follows the direction of the enamel rods (Fejerskov and Kidd, 2009).

The first signs of demineralisation of the enamel can be seen on microscopic examination as an increase in the microporosity of the enamel. No clinical changes are observed at this stage. Further dissolution of the perikymata of the enamel and an enlargement of the intercrystalline spaces show up clinically as a white spot lesion (Black, 1932). As the enamel loses more minerals, several distinct zones become microscopically visible. These zones form a cone shape or triangular pattern with the base pointing towards the tooth surface and the tip towards the enamel-dentine junction (EDJ) (Kidd and Joyston-Bechal, 1997). These zones: the outer zone, the middle zone, and the inner zone. The outer zone, also known as the demineralisation zone, is the outermost layer of the enamel and is characterized by the loss of minerals, particularly calcium and phosphate, from the enamel matrix. The middle zone, also known as the subsurface zone, is characterised by the presence of a network of cracks and voids within the enamel. The inner zone, also known as the intrasubstance zone, is the innermost layer of the enamel and is characterised by the presence of bacterial infection and acid dissolution. These zones typically represent around 20-30%, 30-40%, and 20-30% of the total thickness of the enamel, respectively (Robinson et al., 2000; Fejerskov et al., 2015).

#### **Dentine caries**

#### Over time, as the demineralization of enamel progresses, the bacteria physically invade the dentine. The enamel cannot react because it is neither a cellular nor a vascular tissue, whereas the dentine is a vital tissue and reacts to the progression of caries. This reaction is seen in the formation of the sclerotic dentine along the dentinal tubules. Histologically, dentine caries can be described in four zones (Soames and Southam, 2005):

(*a) Zone of destruction:*

  The dentine in this zone becomes necrotic and liquefies. Cracks called "transverse fissures" appear at right angles to the direction of the dentinal tubules. Clinically, this zone is yellow and soft when caries is active and in the rapid phase. When the progression slows, it becomes brown and hard.

*b) Zone of bacterial invasion*

  When bacteria invade the tubules, they multiply, and the proteolysis process takes place in the tubules. At this stage, the tubules become softer, and the bacterial acids dissolve the collagen.

c*) Zone of demineralization:*

  The progressive front of dentinal caries is the demineralized zone, caused by bacterial acids, although there are no bacteria present in this region. Clinically, it is difficult to distinguish between the demineralized zone and the bacterial invasion zone.

d) *Zone of sclerosis:*

When the acid begins to penetrate the dentinal tubules, a defensive reaction occurs in them. This reaction helps to slow down the progression of the lesion by depositing a calcified material that seals the tubules and protects the pulp from the penetration of the acid.

More clinically relevant, dentine caries was first described by Fusyama and Terachima (1972) as having two layers: the outer layer and the inner layer, which is consistent with the biological understanding of the carious process. This description of the lesion is based on bacterial infection and the degree of degradation of the organic matrix of the dentine.

**The outer layer:**

 Bacteria in this infected dentine layer cause total denaturation of the organic matrix with the decomposition of the dentine and complete loss of dentine characteristics. The irreversible denaturation of the organic matrix is the main feature of infected dentine due to the disappearance of collagen I cross-link in the dentine. In this layer, cariogenic bacteria are frequently observed within the dentinal tubules (Fusayama, 1979; Marshall et al., 2001).

**The inner layer:**

 The caries-affected dentine in this layer differs from the caries-infected dentine because the tissue can be remineralised, the dentine is partially demineralised and fewer bacteria are present. Compared to healthy dentine, the collagen in caries-affected dentine shows a slight change. The mineral content of the dentine is reduced and mineral intra-tubular deposits form, which show up as tubular occlusions (Fusayama, 1979; Marshall et al., 2001). In caries treatment, it is important to distinguish between caries-infected dentine and caries-affected dentine. Collagen cross-linking in the inner zone remains normal and has the capacity to self-repair if the pulp remains vital (Nakajima et al., 1995).

## Matrix Metalloproteinase (MMP)

Matrix metalloproteinases or matrixins are a family of zinc (Zn)-containing, calcium (Ca)-dependent endopeptidases that belong to a larger protease family known as the "metzincin superfamily" (Nagase and Woessner, 1999).

  They were first discovered in vertebrates in the 1960s by Jerome Gross and Charles Lapiere who noticed enzymatic activity when they placed a tadpole tail in a collagen matrix plate during amphibian metamorphosis: the degradation of a collagen triple helix (Gross and Lapiere, 1962). This first collagenolytic enzyme was named interstitial collagenase (MMP1) and was later isolated from human skin (Eisen et al., 1968).

  The degradation of ECM plays an important role in many physiological and pathological processes. Under normal physiological conditions, MMPs play a key role in cell proliferation, differentiation, migration, remodelling and tissue repair (Mott and Werb, 2004; Nagase et al., 2006). However, upregulation of MMP enzyme expression and activity has also been found in several pathological conditions such as cardiovascular disease (Affara et al., 2009), arthritis (Tetlow et al., 2001), diabetes, and tumour growth and metastasis (Winberg et al., 2009).

### Structure of Matrix metalloproteinase

All matrixins are synthesised and secreted into the extracellular space as pro-enzymes (inactive form) called "zymogens" or "pro-MMPs". The MMP family shows considerable sequence homology but differs in substrate specificity (Murphy and Knäuper, 1997).

  For an enzyme to be recognised as an MMP, it must contain two domains: the pro-domain and the catalytic domain. The pro-domain normally consists of approximately 80 amino acids. It contains a cysteine switch motif that keeps the MMPs in their inactive form and is located at the N-terminus of the catalytic domain. The catalytic domain contains the zinc-binding motif that identifies the MMP family (Murphy and Knäuper, 1997; Visse and Nagase, 2003).

Members of the mammalian MMP family also share a basic structure consisting of common functional domains: signal peptide, pro-domain, and catalytic domain. The catalytic domain is connected to the C-terminal domain (hemopexin-like) via a linker region or flexible hinge consisting of about 75 amino acids. The C-terminal domain has a four-bladed β-propeller structure that provides a site for protein interactions. This is the site of interaction with MMP tissue inhibitors, tissue inhibitors of Metalloproteinases (TIMPs). This domain is absent in some types of MMPs such as MMP7, MMP23 and MMP26 (Murphy and Knäuper, 1997; Visse and Nagase, 2003).

### Classification of MMPs

The MMPs are a large multigene family with 24 members in vertebrates, 23 of which have been identified in humans (Parks et al., 2004).

Based on substrate specificity and domain organisation, the MMPs are classified into six groups: collagenases, gelatinases, stromelysins, transmembrane MMPs, matrilysin and other MMPs (Chaussain-Miller et al., 2006). Each group is described below:

1. **Collagenases:** This group includes MMP1, MMP8, MMP13 and MMP18

***a) MMP 1:*** also called collagenase 1. The main feature of this enzyme is its ability to degrade interstitial collagen types I, II and III (Visse and Nagase, 2003).

***b) MMP 8:*** also called collagenase 2, is synthesised in the bone marrow during the maturation of poly-morphonuclear leukocytes (PMNs) (Visse and Nagase, 2003). The function of MMP8 overlaps with that of other collagenases in the degradation of collagen types I, II and III, however, this enzyme prefers collagen type I as a substrate (Hasty et al., 1987). MMP8 is expressed by pulp tissue and odontoblasts of developed human teeth (Palosaari et al., 2000) and is considered the major collagenase in human dentine (Sulkala et al., 2007).

***c) MMP 13:*** Also called collagenase 3, is expressed in the skeleton during bone development and plays a role in restructuring the collagen matrix required for bone remineralisation. It is also expressed in the gums and during wound healing (Lindy et al., 1997). In pathology, MMP13 is found in destructive conditions such as periodontal disease and in many human carcinomas (Johansson and Ahonen, 2000). MMP13 is able to degrade type I collagen ten times more effectively than type II or III collagen (Lindy et al., 1997).

**2) Gelatinases:**

***(a) MMP2:*** also called gelatinase A, is expressed by odontoblasts, and is concentrated in dentine near the enamel-dentine junction (EDJ) (Goldberg et al., 2003). MMP2 digests collagen types I, II and III (Aimes and Quigley, 1995) and plays a key role in osteogenesis. Mutations in this enzyme lead to inappropriate destruction and resorption of bone (Martignetti et al., 2001).

***b) MMP 9:*** also called gelatinase B, and is expressed by neutrophils, macrophages, and osteoclasts (Visse and Nagase, 2003a). MMP9 mutations can play a role in numerous inflammatory diseases such as pericoronitis, periodontitis and various malignancies (Murphy and Knäuper, 1997; Visse and Nagase, 2003a). MMP9 is the major gelatinase found in saliva and gingival-crevicular fluid (GCF) (Van Strijp et al., 2003).

**3) Stromelysins:**

This group includes MMP3 (stromelysin 1) and MMP10 (stromelysin 2).

***MMP 3:*** has higher proteolytic activity than MMP10, although they have the same substrate specificity (Suzuki, 1990). MMP3 has been found in demineralised dentine and has been shown to play an important role in the organisation of the dentin matrix during odontogenesis (Boukpessi et al., 2008a). Another function of MMP3 is the activation of pro-MMPs, in particular the activation of pro-MMP 1 to the fully active MMP1 (Suzuki, 1990).

***MMP10:*** this MMP has the ability to digest collagen III, IV and V (Suzuki, 1990).

**4) Membrane-type MMPs:**

There are two groups of membrane-type MMPs (MT-MMP) that bind to cell surfaces (Kojima et al., 2000), which can be further subdivided into six subgroups: Group 1 (Type I) includes MT1-MMP (MMP 14), MT2-MMP (MMP15), MT3-MMP (MMP 16) and MT4-MMP (MMP24), while Group 2 (Type 2) includes MT5-MMP (MMP17) and MT6-MMP (MMP25).

**5) Matrilysins:**

This group includes MMP7 (matrilysin 1) and MMP26 (matrilysin 2). They are involved in the digestion of ECM components particularly collagen IV and X (Uría and López-Otín, 2000).

### Regulation of MMPs

For MMPs to fulfil their physiological or pathological functions, they must be present in a suitable cell type and location in an appropriate quantity and at the right time (Sternlicht and Werb, 2001). MMPs are specifically regulated by the activation and inhibition of the pro-enzyme form (Sternlicht and Werb, 2001). Most MMPs are precisely regulated by integrin-derived signals, phorbol esters, cell stress, changes in cell shape and extracellular matrix proteins (Kheradmand et al., 1998). In addition, the expression of MMPs is regulated by various cytokines and growth factors such as interleukin, interferon and TNF-α (Visse and Nagase, 2003).

  The regulation of MMPs may differ between types. For example, TGF-β induces the expression of MMP13 but suppresses the expression of MMP1 and MMP3 (Uría and López-Otín, 2000). Some MMPs are only specifically and uniquely expressed in a limited number of cell types. For example, MMP9 expression appears to be limited to odontoblasts and macrophages and MMP20 expression is restricted to the enamel organ of developing teeth (Sternlicht and Werb, 2001).

### Activation of MMPs

All MMPs are synthesised and expressed in the latent form, the zymogen (inactive pro-MMPs). When secreted, they must be activated to participate in the degradation of the ECM (Van Wart and Birkedal-Hansen, 1990). The process of pro-MMP activation is dynamic and complex, as several mechanisms have been described. To keep MMPs in the inactive form requires the formation of a complex resulting from the interaction between the Zn2+ of the active catalytic site and the conserved cysteine residue with the thiol group in the pro-peptide domain (Parks et al., 2004). This complex blocks the active site of the MMP. Disruption of this interaction converts the pro-MMP into an active proteinase in a process known as the "cysteine switch" (Van Wart and Birkedal-Hansen, 1990). (Figure. 1.1)

|  |
| --- |
| A screenshot of a cell phone  Description automatically generated |

**Figure 1.1** Schematic diagram demonstrating cysteine switch in MMP.

Pro-MMP2 is uniquely activated on the cell surface and requires the mediation of MT-MMPs (MT1-MT6). This activation requires the complex formation of MT1-MMP and TIMP2 via the C-terminal domain, which allows the inhibitory domain of TIMP2 at the N-terminal domain to bind to MT1-MMP. The accumulation of MT1-MMP at the cell surface facilitates the process of MMP2 activation. In contrast to MT1-MMP, the activation of MMPs by MT2-MMP is independent of TIMP2 (Itoh et al., 2001). MT1-MMP is also able to activate pro-MMP3 on the cell surface, which is mediated by the presence of active MMP2. This activation occurs directly and does not depend on TIMPs (Knäuper et al., 1996).

   Murphy (1999) described the role of acid etching in the activation of pro-MMPs, suggesting that when dentine is treated with citric and lactic acids and the pH is lowered as a result, the activity of pro-MMPs is triggered and the activity is maintained while the pH is neutralised. Apolonio et al. (2017) investigated the effect of a self-etching adhesive system on MMP2 and MMP9 in extracted permanent human teeth by measuring the activity before and after treatment with an enzymatic activity assay and in situ zymography. This study revealed a significant increase in the activity of MMP2 and MMP9 within the hybrid layer after etching, which could lead to collagen degradation in the hybrid layer and affect the longevity of the restoration. This finding is consistent with another study that investigated the activation of MMPs after the universal system and the self-etch system. They found that both etching systems have the potential to activate endogenous enzymes (Ahmet et al., 2020).

### Inhibition of MMPs

Two types of inhibitors can inhibit MMP activity: endogenous and exogenous.

  The major endogenous inhibitors, tissue inhibitors of metalloproteinase (TIMPs), inhibit the MMP family. These secretory proteins are divided into four types: TIMP1, TIMP2, TIMP3 and TIMP4 and form complexes with MMPs (Woessner Jr, 1991). The N-terminal domain of TIMPs is involved in biological activities and binds to MMPs, while the C-terminal domain is normally responsible for forming the complex with the inactive zymogen (Will et al., 1996). The balance between TIMPs and MMPs is thought to play a crucial role in normal and pathological tissue remodelling. In addition to their role in MMP activation, TIMPs have other biological functions, such as MMP transport and focusing on the cell surface by binding to MT-MMP, growth factor activity and promoting bone resorption activity (Sobue et al., 2001).

Exogenous MMP inhibitors are synthesised as therapeutic agents and several systemic inhibitors have been described (Sulkala et al., 2001). However, to combat caries lesions with MMP inhibitors, they should preferably be applied locally by incorporating them into mouth rinses or applying them directly to the lesion (Chaussain et al., 2013). Most of these inhibitors are synthetic and work by chelating calcium or zinc ions to the active site of the enzyme or coating the substrate and preventing MMP access (Mazzoni et al., 2012). The most widely tested MMP inhibitor is chlorhexidine (CHX), which has been shown to have an effective inhibitory effect on the endogenous enzymes MMP2, MMP8, MMP9 and cysteine cathepsins (Breschi et al., 2010; Scaffa et al., 2012). It has also been shown to positively influence the binding strength in the hybrid layer (Breschi et al., 2010). Although CHX has a broad-spectrum MMP inhibitory effect, its water solubility causes the agent to leach from the hybrid layer, compromising the long-term MMP inhibitory effect (Ricci et al., 2010) and failing to prevent the development of secondary caries (Maske et al., 2019). Polyvinylphosphonic acid (PVPA) has been introduced as an MMP inhibitor as an alternative to CHX, as its stability may prolong the longevity of the hybrid layer (Tezvergil-Mutluay et al., 2010). Quaternary Ammonium Methacrylate is another MMP inhibitor with a potent effect but requires a higher concentration than CHX (Tezvergil-Mutluay et al., 2011). Seventeen % of Ethylenediaminetetraacetic acid (EDTA) has been introduced as an endodontic irrigant and has been shown to inhibit MMPs when applied to dentine beams for 1 minute (Thompson et al., 2012).

Silver diamine fluoride is an agent that has been introduced as a desensitising agent to stop caries in primary and permanent teeth (Horst et al., 2016). A previous study has demonstrated its inhibitory effect on MMPs (Mei et al., 2012). This agent is discussed in more detail in section 1.7.

### Role of MMPs in the oral environment

As mentioned above, MMPs play an important role in physiological and pathological tissue remodelling. Physiologically, MMPs contribute to cell migration and remodelling during growth, enamel formation and wound healing (Hannas et al., 2007). In pathology, tumour cells produce MMPs that facilitate tumour metastasis to surrounding tissue (Ye, 2000).

  Biological processes in the oral cavity reflect the active role of MMPs in different areas. For example, MMP1 and MMP13 are able to degrade collagen types I, II and III and digest ECM molecules. The gelatinases (MMP2, MMP9) also digest denatured collagen and gelatine (Hannas et al., 2007). Several studies have shown the fundamental role of MMPs in oral tissue development.

#### **MMPs in enamel**

In amelogenesis, there are several phases in which enamel develops: the secretory phase, the transitional phase, and the maturation phase. During the secretory phase, the ameloblasts secrete a specialised enamel protein, amelogenin, into the enamel matrix. This makes up 90% of the enamel matrix (Nanci, 2008; Fejeriek., 2015). MMP20 (Enamelysin) plays a key role in enamel formation as it is secreted into the enamel matrix during amelogenesis, where it degrades amelogenin (Caterina et al., 2002).

#### **MMPs in dentine**

MMPs play an important role in matrix remodelling during dentinogenesis. Dayan et al., (1983) first noted collagenolytic activity in dentine, suggesting the presence of a complex of collagenase inhibitors bound to the collagen-dentine matrix during dentine development.

Several studies have subsequently identified and characterised the protease enzymes responsible for this collagenolytic activity (Palosaari et al., 2002; Goldberg et al., 2003; Sulkala et al., 2007; Mazzoni et al., 2009). The main MMPs identified in dentine, pre-dentine and odontoblasts are MMP2 (gelatinase A), MMP9 (gelatinase B), MMP8 (collagenase 2), MMP3 (stromelysin 1), MMP 13 and MMP20.

MMP2 plays a role in basement membrane degradation and remodelling during dentinogenesis (Sahlberg et al., 1992; Heikinheimo and Salo, 1995). Goldberg et al., (2003) demonstrated that MMP2 and MMP9 are located near the enamel-dentine junction (EDJ) in the later stages of incisor development in rats. They also demonstrated the key role of TIMP2 in dentine. High levels of a complex between MMP2 and TIMP2 were observed in odontoblasts in deep dentine and in the EDJ (Goldberg et al., 2003; Gobbi et al., 2021). The presence of MMP2 in dentine was also confirmed by Boushell et al., (2011), who highlighted the presence of MMP2 associated with odontoblasts, pre-dentine, dentine matrix of sound human dentine, and near demineralised pre-dentine.

  Another MMP identified in dentine is MMP9. Mazzoni et al., (2009) demonstrated that MMP9 is an intrinsic component of the fibrillar network of the organic matrix in human dentine. MMP9 was shown to be concentrated near the EDJ (Goldberg et al., 2003) in a complex with TIMP1 (Niu et al., 2011) and synthesised by odontoblasts after tooth formation was complete and It has been suggested that MMP9 is primarily related to pre-dentine formation. Once secreted by odontoblasts, they diffuse into the dentine matrix, where they become tightly bound to the collagen in the dentine matrix. The subsequent deposition of apatite crystals takes place during pre-dentine mineralisation (Boushell et al., 2011)

MMP3 has also been shown to be required for the formation of normal dentine by participating in the pre-dentine mineralisation process (Boukpessi et al., 2008). Mazzoni et al., (2011) further demonstrated by biochemical and histochemical methods that MMP3 is localised along collagen fibres and within intertubular dentine. Chaussain et al., (2013) found that MMP3 is present in its active form in demineralised dentine.

  In a 2007 study by Sulkala et al, collagenase enzymes in human dentine were characterised by western blotting with specific antibodies. They found that MMP8 was expressed by odontoblasts and mostly occurred as an unbound protein fraction, in contrast to gelatinases (MMP2-MMP9), whose activity was detected in the organic matrix (Sulkala et al., 2007).

### Role of MMPs in dental caries

As discussed earlier, demineralisation takes place when the tooth is exposed to acidic conditions. In dental caries, cariogenic bacteria (e.g., *S. mutans*) ferment the carbohydrates from the food and release lactic acid, which begins to dissolve the hydroxyapatite of the tooth. Once the dentine is reached, caries progresses rapidly due to the lower mineral content in the dentine and the smaller size of the crystals compared to the enamel. Acids produced by bacterial proteases dissolve the organic element and expose the dentine matrix. However, these proteases lack the ability to degrade intact collagen. MMPs are able to degrade most molecules of the extracellular matrix (Visse and Nagase, 2003). MMPs are secreted into the dentine matrix in an inactive form and need to be activated to degrade the ECM. When bacterial proteases produce acids, the oral pH drops below 5.5, which may favour the activation of endogenous host-derived enzymes (MMPs) (Tjäderhane et al., 1998). MMPs can withstand low pH for a short period of time but remain functional only if the oral environment is neutralised by the salivary buffer system (pH 7) (Tjäderhane et al., 1998).

Charadram et al., (2012) demonstrated differential activity of MMPs in the different layers of carious teeth, with higher activity in soft, infected dentine than in healthy dentine. This suggests that MMPs, especially MMP2, are activated through the caries process. This study also showed that the expression of MMP2 and TIMP2 was upregulated in odontoblasts near the carious lesion, suggesting that the acidic pH environment that occurs in caries induces the production and activation of MMPs.

  To summarise the role of MMPs in the denaturation of collagen in dental caries, the process is listed in four main steps:

(i)         Acids from bacterial proteases dissolve hydroxyapatite and expose the organic network.

(ii)        Pro-MMPs are activated by a decrease in pH.

(iii)      Active MMPs degrade the collagen network during the neutralisation period.

(iv)      Collagen degradation is enhanced by other MMPs.

Many researchers have identified different MMPs in the carious lesion, including MMP8, MMP2, MMP9 and MMP20. Among the different members of the MMP family, MMP8 has been shown to be the predominant collagenase in both healthy dentine and carious lesions (Sulkala et al., 2007). MMP8 is an unbound protein in the dentinal tubules, suggesting that this enzyme is responsible for initial degradation in collagen fibrils (Sulkala et al., 2007). The enzymes MMP2 and MMP9 are responsible for further degradation in the intertubular dentine. These collagenase enzymes have been identified in concentrated form near the EDJ, which may explain the early expansion and widening of dental caries along this junction (Sulkala et al., 2002). Boukpessi et al., (2008) demonstrated the pre-existence of MMP3 in human carious dentine using Western blotting analysis. Mazzoni et al. (2011) assessed MMP3 activity using zymographic analysis of dentine powder and indicated the apparent activity of MMP3 in demineralised dentine, which was, however, scarcely present compared to mineralised dentine. MMP3 cleaves glycoproteins and proteoglycans instead of attacking collagen. The degradation of MMP proteoglycans releases TGF-β from the extracellular matrix, which is involved in the activation of MMP9.

In vitro studies have demonstrated the role of MMPs in dental caries through the use of TIMPs, leading to a reduction in caries progression in rat dentine (Tjäderhane et al., 1998). Another in vitro study showed that the addition of active MMP8 to saliva prevents the remineralisation of carious dentine (Nordbo et al., 2003). MMPs have the ability to generate bioactive peptides from the degradation of the dentine matrix and release bioactive fragments and cytokines (Mott and Werb, 2004; Chaussain-Miller et al., 2006b). These bioactive peptides retain the activity of the parent protein and are able to influence the biological process of angiogenesis, proliferation and differentiation that constitutes the process of pulp healing (Dean and Overall, 2007). When released in the carious lesion, these peptides cause the activation of other MMPs and impair pulp healing (Smith et al., 2012).

The relationship between gene variants and dental caries has also been studied. Karayasheva et al., (2016) analysed single nucleotide polymorphisms of MMP2 and MMP3 and their association with susceptibility to dental caries in adults aged 20-30 years. Participants were divided into three groups according to their DMFT score: Group 1 (DMFT = 0), Group 2 (DMFT < 5) and Group 3 (DMFT > 5). Epithelial cells were collected from the buccal mucosa, DNA extracted and analysed for the two SNPs using PCR restriction fragment length polymorphism. This study found that gene expression of MMP2 and MMP3 was significantly higher in participants with caries lesions compared to the caries-free group. They concluded that these MMP genes most likely play a role in susceptibility to caries in adults (Karayasheva et al., 2016).

Antunes et al., (2016) investigated the association of MMP2, MMP3, MMP9, MMP20, TIMP1 and TIMP2 polymorphisms with early childhood caries and white spot caries in 786 children (2-6 years). Participants were divided into groups with and without caries and white spot lesions. Genomic DNA was extracted from buccal cells, and the polymorphism of the relevant enzymes analysed using TaqMan real-time PCR. An association between MMP9 and MMP20 and the development of caries and white spots in children was noted. A further study was conducted to investigate the association between dental caries and polymorphisms of MMP2, MMP3, MMP9, MMP13 and MMP20 genes in 782 school and preschool children with and without caries lesions. The MMPs gene variants were analysed using real-time PCR but no association was found between the selected MMPs genes and susceptibility to caries or the severity of caries lesions (Borilova Linhartova et al., 2020).

### MMPs in carious affected dentine

As previously mentioned, the carious dentine lesion consists of two layers that differ in their chemical and morphological structure: the inner layer (affected dentine) and the outer layer (infected dentine) (Fusayama and Terachima, 1972). Caries-affected dentine is close to normal dentine and is partially demineralised, with most of the collagen matrix intact. The clinical significance of this layer is that it can be remineralised and could serve as a suitable substrate for the adhesion of dentine restorations. However, the presence of endogenous MMPs in this layer has been confirmed in several studies (Van Strijp et al., 2003; Boushell et al., 2008; Shimada et al., 2009; Toledano et al., 2010; Charadram et al., 2012), which could compromise this as the organic matrix of caries-affected dentine is itself degraded.

Shimada et al (2009) demonstrated the localisation of MMPs through different layers of carious dentine, i.e., healthy, affected, and infected dentine, using immunogold labelling. The presence of MMPs was confirmed in all layers but did not show significant differences. However, MMP8 and MMP9 were identified in a lower concentration in the affected dentine layer compared to infected dentine. In contrast, MMP20 showed a significant reduction in the outer infected layer (Shimada et al., 2009). On the other hand, Toledano et al. (2010) found a different localisation of MMPs in the caries layers using immunofluorescence microscopy. They found that MMP2 had a lower intensity in the affected dentine than in the infected layer. The study suggests that the lower the intensity of MMP2 in the caries-affected dentine, the less the hybrid layer is degraded and the better the bond of the restorative material (Toledano et al., 2010).

**Table ‎1.2** Summary of the presence of increased expression of MMPs in dental tissue, caries lesions and saliva from individuals with caries.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sound dentine** | **Infected dentine** | **Affected dentine** | **Saliva of individuals with caries** |
| **MMP8**  (Sulkala et al., 2007) | **MMP8**  (Tjäderhane et al., 1998)  (Sulkala et al., 2007) | **MMP20**  (Shimada et al., 2009) | **MMP8**  (Nordbo et al., 2003) |
| **MMP9**  (Goldberg et al., 2003)  (Mazzoni et al., 2009)  (Gobbi et al., 2021) | **MMP9**  (Tjäderhane et al., 1998)  (Goldberg et al., 2003)  (Sulkala et al., 2007) |  | **MMP9**  (Tjäderhane et al., 1998) |
| **MMP2**  (Tjaderhane et al, 1998)  (Martin-De Las Heras et al., 2000)  (Goldberg et al., 2003b)  (Boushell et al., 2008)  (Gobbi et al., 2021) | **MMP2**  (Tjäderhane et al., 1998)  (Charadram et al., 2012) |  |  |
| **MMP3**  (Hall et al, 1999)  (Mazzoni et al., 2011)  (Chaussain et al, 2013) |  |  |  |
| **MMP13**  (Loreto et al, 2014) |  |  |  |
| **MMP20**  (Sulkala et al., 2002)  (Shimada et al., 2009) |  |  |  |

## Management of caries lesion

Dental caries, particularly in children, is a prevalent disease (Chen et al., 2019) that places a significant burden on patients and their families (Thomson, 2016) and an economic burden worldwide (Kassebaum et al., 2015). The concept of caries management has evolved as caries is no longer considered an infectious disease (Kidd, 2011). Nevertheless, caries management is one of the most important issues in healthcare (Laske et al., 2019).

  Dental decisions about treating caries lesions in children are complex and depend not only on the diagnosis of a particular tooth but also on patient management. Age, pain perception, cognitive development and cooperation of the child are some challenges in caring for children (Santamaría et al., 2020). Traditionally, the treatment of dental caries was based on the complete removal of the lesion under local anaesthesia and filling the cavity with a dental restoration. Young patients have cited this approach as a cause of anxiety and discomfort (Ladewig et al., 2018). Furthermore, the complete removal of the caries lesion increases the risk of pulp exposure (Ricketts et al., 2013).

  The understanding of the caries process and the fact that it is a slowly progressive disease has initiated the non-surgical treatment approach (Pitts et al., 2017). Minimally invasive dentistry is the preservation of healthy dental structure and minimally invasive methods should be used as much as possible, as this treatment option is no worse than conventional treatment and gives better results (Ericson et al., 2003; Banerjee et al., 2020; Schwendicke et al., 2021). Minimally invasive procedures support the use of a cariostatic agent to control and stop caries lesions. One such agent is silver diamine fluoride (SDF).

## Silver diamine fluoride (SDF)

Silver diamine fluoride is alkaline, and a colourless solution containing fluoride and silver with ammonia, which acts as a stabilising agent (Chu and Lo, 2008). The silver ion combines with the ammonia ion to form a diamine silver ion, which forms a more stable ion complex (Greenwall-Cohen et al., 2020). SDF contains 44,800 ppm fluoride, releasing three times more fluoride than stannous fluoride and sodium fluoride gels and varnishes (Rosenblatt et al., 2009).

  In the 1840s, the use of silver in the form of silver nitrate was introduced in dentistry to arrest caries lesions (Ea, 1891). In 1917, silver nitrate was used in conjunction with an ammoniacal solution called Howe's solution to control caries. This solution was believed to have an antibacterial effect and penetrated the affected dentine. In 1950, concerns were raised about the effectiveness of this solution, and its use was discontinued (Peng et al., 2012). SDF solution was developed in Japan in 1969 and was first used commercially as a cariostatic for children's primary teeth. It has also been used to prevent root caries in permanent dentition and sensitive teeth (Castillo et al., 2011; Li et al., 2016). In 2014, SDF (Advantage Arrest) was approved by the Food and Drug Administration (FDA) in the United States for the treatment of tooth sensitivity (Horst et al., 2016). SDF is used in several countries with various commercial products, e.g., Saforide in Japan, Cariestop in Brazil, FAgamin in Argentina and ammonia-free CSDA in Australia. In the UK, SDF is available for clinical use and is marketed by SDI as Riva Star for tooth sensitivity. It has been used 'off-label' for caries control, meaning a licenced dentist can prescribe it if it’s in the patient's best interest (Seifo et al., 2020).

### Clinical use of SDF

Studies and clinical trials have found that SDF can be used for a range of clinical applications like caries management, treating hypersensitivity and part of restorative treatments.

#### **Caries control and prevention**

The most common use of SDF is as a cariostatic agent. The efficacy of SDF in arresting caries lesions has been investigated in several clinical trials and systematic reviews. Chu et al., (2002) conducted a 30-month clinical trial in 3-5-year-old children to compare the effect of a 38% SDF application on caries arrest in upper primary anterior teeth with a 5% sodium fluoride varnish. They found that an annual application of a 38% SDF solution was more effective in arresting caries than the application of sodium fluoride at three-month intervals or the controls. Llodra et al., (2005) investigated the efficacy of SDF in controlling carious lesions on primary and permanent teeth and found that SDF was effective in controlling caries on primary teeth (56%) and first permanent molars (100%) in school-aged children. Another study compared the efficacy of 10% SDF with GIC fissure sealant in controlling caries lesions on the occlusal surfaces of the first permanent molars with a follow-up period of 30 months. In this study, SDF was found to have a higher capacity to arrest caries than other non-invasive treatments (Braga et al., 2009). Other studies compared 30% SDF with fluoride varnish and GIC restoration in arresting caries lesions and found that SDF was significantly more effective than other treatments (Dos Santos et al., 2012; Duangthip et al., 2016).

A systematic review and meta-analysis on the efficacy of 38% SDF as a cariostatic agent included eight studies on primary teeth comparing SDF with other non-invasive and restorative treatments (5% sodium fluoride and glass ionomer cement restoration) and found that this solution effectively arrests caries in 81% of lesions in primary carious dentine (Gao et al., 2016). More recently, a more general systematic review of intervention in the caries process in the primary dentition concluded that SDF is a highly effective cariostatic treatment for cavitated dentine lesions, provided there is no pulpal involvement (Schmoeckel et al., 2020). From all these above clinical trials and systematic reviews with high-quality evidence, it seems that SDF is superior to other non-invasive and restorative treatments or placebos in caries control.

SDF contains a high concentration of fluoride. When applied to healthy tooth surfaces, fluoride replaces the hydroxyl ions in the hydroxyapatite crystals and forms a less acid-soluble fluorohydroxyapatite, which consequently prevents caries lesions (Rosenblatt et al., 2009). The use of SDF to prevent the development of new caries lesions has also been studied. SDF showed a high prevention rate of 70% to 79% in primary teeth (Chu et al., 2002; Llodra et al., 2005) and 64% in permanent teeth (Rosenblatt et al., 2009). Oliveira et al., (2019) investigated the effectiveness of SDF in preventing caries lesions in the primary dentition in a systematic review. They included six randomised clinical trials and found that after a follow-up period of two years, the application of SDF to healthy teeth significantly reduced the development of new lesions compared with fluoride varnish and placebos. In an *in vitro* study, the caries preventive efficacy of SDF on 105 healthy enamel surfaces of bovine teeth was evaluated and compared to fluoride varnish by measuring the change in enamel surface density using Micro-CT after the samples were subjected to a demineralisation test. The results showed that enamel density was increased in the samples treated with SDF compared to the fluoride varnish treatment and the control. This study showed that SDF is an effective treatment for the prevention of dental caries (Ahn et al., 2020).

SDF can also detect caries lesions as it stains only demineralised tooth tissue. It has been found that when treated with SDF, a lower concentration of silver was detected on healthy enamel surfaces (Li et al., 2019). This explains why SDF does not stain healthy enamel surfaces. However, it should be noted that the staining of the lesion is permanent (Seifo et al., 2020). SDF staining is discussed in detail in section 1.7.5.4.

#### **SDF as part of caries restorative-based treatment**

There is a new paradigm in caries treatment that uses SDF as part of restorative treatment. This procedure is called the silver-modified atraumatic restorative technique (SMART), in which SDF is applied to a carious lesion, followed by a well-sealed GIC restoration. The advantage of this technique is that pulp involvement and tooth infection as a result of caries progression under the restoration is avoided (Seifo et al., 2020; Natarajan, 2022). However, no studies have yet been conducted to investigate the effectiveness of this technique compared to other treatment options.

#### **Sensitivity**

Managing dentine hypersensitivity was the first indication for the use of SDF solutions (Horst et al., 2016). The mechanism behind this is that silver ions penetrate and occlude the dentinal tubules of exposed dentine and reduce fluid shift through them. This reduces pain and reduces hypersensitivity (Horst et al., 2016; Seifo et al., 2020). Randomised control trials have demonstrated the efficacy of SDF as a desensitising agent (Castillo et al., 2011; Craig et al., 2012). However, further studies with longer follow-ups and comparisons with other sensitisation treatments are needed to fully understand its efficacy.

#### **Endodontic inter-appointment medication**

In endodontic treatment, the elimination of the microbiome and the use of an antimicrobial dressing in the root canals are essential steps to achieve successful results. A study by Hiraishi et al., (2010) investigated the use of 3.8% SDF as an antibacterial agent and for intracanal medication. Biofilms were created and treated with 3.8% silver, saturated calcium hydroxide solution, 5.25% sodium hypochlorite and 0.9% sodium chloride for 15 minutes and 60 minutes, respectively. Cleaned and shaped radicular dentine was treated with SDF for 3 days. The presence of silver salts on the dentine surface was examined by scanning electron microscopy. The authors demonstrated that sodium hypochlorite and SDF were effective against E. faecalis biofilms. Approximately 67% of the radicular dentine surfaces had silver deposits when SDF was used as an interappointment dressing, with the silver deposits penetrating the dentine tubules to a depth of 40 mm. The researchers concluded that SDF has the potential to be used as an interappointment dressing or as an antimicrobial root canal irrigation, especially if staining of the dentine is not a major problem.

### SDF mechanism of action

The caries inhibitory effect of SDF is attributed to three main modes of action: antibacterial effect, remineralisation effect, and inhibitory effect on endogenous enzymes (Figure 1.2). Table 1.3 summarises studies that have investigated the effect of SDF on cariogenic bacteria and the remineralisation effect in enamel and dentine. Table 1.4 summarises studies that have investigated the effect of SDF on the inhibition of endogenous enzymes.

#### **Antibacterial effect**

There are a number of ways in which silver ions exert an antimicrobial effect. They block the bacterial electron transport system by interacting with its life-sustaining enzymes (Marx and Barillo, 2014). Silver ions deactivate enzymes by interacting with their thiol group and bacterial DNA, leading to mutation and death of the bacterial cell (Russell and Hugo, 1994). Silver can also penetrate and destroy bacterial cell wall structures by accumulating silver ions in the cell and inactivating bacterial RNA and DNA (Lansdown, 2002). In addition, silver ions combine with the hydroxyapatite crystal to form silver-containing hydroxyapatite, which has been shown to have the ability to limit bacterial adhesion and reduce cytotoxicity (Chen et al., 2006).

|  |
| --- |
| **Diagram  Description automatically generated** |

**Figure 1.2** Diagrammatic representation summarising the mechanism of action of SDF.

SDF also contains fluoride, which appears to have a bactericidal effect by inhibiting biofilm formation due to its ability to bind to bacterial cellular components. In addition, fluoride can affect the enzymes responsible for the metabolism of carbohydrates and sugar absorption (Mei et al., 2013a).

  Many studies have investigated the antibacterial effect of SDF. Suzuki et al., (1976) found that SDF inhibited the adhesion of *S. mutans* to tooth surfaces with a lower minimum inhibitory concentration compared to sodium fluoride and silver ammonium nitrate. Knight et al., (2005) showed that SDF significantly reduced the growth of S. mutans on tooth surfaces compared to controls. Different concentrations of SDF (12%, 30% and 38%) were tested for their bactericidal activity. Although all showed a significant inhibitory effect, the higher concentration of 38% SDF was able to prevent biofilm formation for 48 hours, suggesting a key role in the development of a new biofilm (de Almeida et al., 2011; Savas et al., 2015). Furthermore, SDF showed a high inhibitory effect on a two-species biofilm containing *Lactobacillus acidophilus* and *S. mutans* (Mei et al., 2013a) and a multi-species biofilm containing *L. acidophilus, S. mutans, S. sobrinus* and *Lactobacillus rhamnosus* (Mei et al., 2013b).

The complementary use of fluoride varnish with SDF was investigated in a laboratory study by Yu et al., (2018). They investigated the inhibition of bacterial growth by 38% SDF alone and compared it with SDF and fluoride varnish together. They found that the addition of fluoride varnish reduced the antibacterial effect of SDF and therefore recommended not to use the varnish as an additional application to SDF.

#### **Remineralisation effect**

The cariostatic effect of SDF is attributed to its ability to remineralise enamel and dentine. Suzuki et al (1976) demonstrated that when enamel powder was treated with SDF, silver phosphate was formed, which was thought to play a role in the hardness of enamel lesions. They also found that silver and fluoride were involved within 10 to 20 μm of the enamel surface. In another study, it was found that enamel blocks treated with SDF had reduced lesion depth and caries progression compared to controls (Klein et al., 1999). SDF has also been shown to increase the surface microhardness of carious dentine lesions at a depth of 150-200μm (Chu and Lo, 2008).

Mei et al. (2013a) investigated the inhibitory effect of SDF on demineralised dentine samples by examining the dentine's lesion depth, surface morphology and crystal characterisation using a scanning electron microscope, X-ray diffraction and spectrometry. They found that the SDF-treated samples had reduced lesion depth compared to the controls. In addition, they observed dense granular grains on the dentine surface, indicating the formation of extrafibrillar minerals leading to a more potent inhibitory effect of SDF on demineralised dentine. In another study, they compared mineral density, surface morphology and crystal properties between SDF-treated primary teeth and active lesions without SDF treatment. They found a highly remineralised zone with high phosphate and calcium content on the SDF-treated teeth, suggesting that the clinical use of SDF positively contributes to the remineralisation of dentine (Mei et al., 2014). This is in line with another study by Knight et al. (2005), who treated demineralised dentine discs with SDF and exposed them to cariogenic challenge for two weeks. They found that SDF-treated discs had significantly higher fluoride uptake and lower phosphate and calcium loss than water-treated disc.

#### **Inhibitory effect on endogenous enzymes**

 As mentioned earlier, MMPs and cathepsins are endogenous enzymes that play an essential role in the caries lesion development. These enzymes mediate the breakdown of collagen after the initial mineral loss that exposes the collagen matrix. Inhibition of these enzymes therefore leads to arrest of caries lesions and inhibits collagen degradation (Chaussain-Miller et al., 2006).

The first study to investigate the inhibitory effect of SDF on MMPs was conducted by Mei and colleagues in 2012. They compared three different concentrations of SDF (12%, 30% and 38%) with silver nitrate solution and sodium fluoride. The activity of purified recombinant human MMP2, MMP8 and MMP9 was assessed by fluorometric assay and showed that SDF has a concentration-dependent effect on MMPs. The strongest inhibition was observed at 38% SDF and may explain the clinical success of 38% SDF in inhibiting dental caries and the limited effect of 12% SDF previously observed in *in vivo* studies (Chu and Lo, 2008). Furthermore, Mei et al. (2012) found that SDF was significantly more effective than sodium fluoride and silver nitrate in inhibiting MMP2, MMP8 and MMP9. However, it should be noted that recombinant MMPs were used in this study, which may not represent the matrix-bound MMPs in natural carious dentine and may not contain all the MMP activities typically found in dentine.

Cysteine cathepsins are a family of enzymes activated by low pH that are thought to be associated with the activities of MMPs in dentine and synergistically contribute to the degradation of the dentine matrix, leading to the progression of caries lesions (Tersariol et al., 2010). Previous studies have also demonstrated the inhibitory effect of 38% SDF on cathepsins B and cathepsins K (Mei et al., 2012; Mei et al., 2014).

The pathogenesis of caries differs between enamel and dentine. Dentine contains 20% collagen, therefore caries lesions in dentine are not only investigated on the basis of mineral loss as in enamel, but quantification of collagen degradation is also important (Nansi, 2008). Mei et al. (2013a) investigated the effect of SDF on the maintenance of the collagen matrix after treating dentine blocks with 38% SDF and subjecting them to pH cycling by incubating the samples in demineralising and remineralising solutions for eight days. They measured the degree of collagen degradation and found that SDF had a significant inhibitory effect on the demineralisation of dentine and thus on collagen degradation, compared to the control and the other experimental treatments. The researchers also found that dentine surfaces treated with SDF had a higher percentage of intact collagen than surfaces treated with water and had a protective effect on collagen degradation (Mei et al., 2013b). However, these studies examined in vitro demineralised tooth sections and not natural carious lesions, which may not represent the natural caries process.

**Table ‎1.3** Summary of studies studying the effect of SDF on cariogenic bacteria and remineralisation effect in enamel and dentine.

|  |  |  |
| --- | --- | --- |
| **Mechanism of action** | **Author, year** | **Main findings** |
| **Antibacterial effect** | Suzuki et al., 1976 | SDF inhibited the adhesion of S. mutans to tooth surfaces and had a lower minimum inhibitory concentration compared to sodium fluoride and silver ammonium nitrate |
| Knight et al., 2005 | SDF significantly reduced the growth of S. mutans on tooth surfaces compared to controls |
| De Almeida et al., 2011 | 38% SDF is able to prevent the formation of biofilm for 48 hours |
| Chu et al., 2012 | SDF-treated dentine had a lower count of bacteria compared to control |
| Mei et al., 2013a | SDF showed a high inhibitory effect on the two-species biofilm containing Lactobacillus acidophilus and S. mutans |
| Mei et al.,2013b | SDF showed a high inhibitory effect on the multi-species biofilm containing L. acidophilus, S. mutans, S. sobrinus and Lactobacillus rhamnosus |
| Savas et al., 2015 | SDF has the ability to prevent the development of new biofilm formation |
| Yu et al., 2018 | the addition of fluoride varnish reduced the antibacterial effect of SDF |
| **Remineralisation effect** | Suzuki et al., 1976 | SDF play a role in the hardness of enamel lesions |
| Klein et al., 1999 | reduced enamel lesion depth and reduced caries progression in SDF-treated samples compared to controls |
| Knight et al., 2005 | SDF-treated discs had significantly higher fluoride uptake and lower phosphate and calcium loss than water-treated discs |
| Mei et al., 2013 | SDF-treated dentine samples had reduced lesion depth compared to the controls with formation of dense granular grains on the dentine surface indicating the increase in microhardness of dentine lesions |
| Mei et al., 2014 | Formation of highly remineralised zone with high phosphate and calcium content on SDF-treated teeth |

**Table ‎1.4** Summary of studies investigated the SDF inhibitory effect on endogenous enzymes and collagen degradation.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Author**  **Year** | **Study type/method** | **sample** | **Targeted enzyme\ protein** | **Period of analysis** | **Main finding** | **Limitation of the study** |
| Mei et al., 2012 | Laboratory-based study  Fluorescent MMP kit | Recombinant enzymes | MMP2, MMP8 MMP9 | 60 min | SDF inhibited MMP2, MMP8 and MMP9 significantly more than sodium fluoride and silver nitrate | The use of recombinant proteins to represent matrix bound MMPs in dentine in the study of MMP activity has limitations including measuring a specific MMP at a specific time and not the combined action of MMPs; host MMPs can cause activation and deactivation effects on other MMPs.  Measuring the immediate effect and not considering the long term effect of SDF on MMPs |
| Mei et al., 2013a | Laboratory-based study  Immuno-labelling method | Human in vitro 30 demineralised dentine blocks obtained from permanent third molars | Intact collagen | 7 days | SDF had a high inhibitory effect on the demineralisation of dentine and on collagen degradation | The use of demineralised dentine beam and not natural caries.  Short period incubation time and does not reflect the long-term effect of SDF on collagen. |
| Mei et al., 2013b | Laboratory-based study  Hydroxy-proline assay | Human in vitro 72 demineralised dentine slices obtained from 18 permanent third molars | Hydroxy proline | 21 days | dentine surfaces treated with SDF had a higher percentage of intact collagen than surfaces treated with water | demineralised healthy dentine blocks were incubated with a high concentration of bacterial collagenase for a more extended incubation period. Bacterial collagenase leads to different effects on collagen molecules than mammalian. Therefore, prolonged use of bacterial collagenase is expected to result in a greater amount of degradation which does not reflect what normally occurs clinically. |
| Mei et al., 2014 | Laboratory-based study  Fluorescent cathepsin kit | Recombinant enzymes | Cathepsin B and K | 60 min | 38% SDF has an inhibitory effect of on cathepsins B and cathepsins K | The use of demineralised dentine beam and not natural caries. |

### The clinical technique of SDF

#### **SDF concentration**

Different SDF concentrations are available for clinical use. The difference in efficacy between 12% and 38% SDF in arresting caries has been investigated in several clinical trials, which found that 38% SDF was more effective than 12% (de Almeida et al., 2011; Savas et al., 2015; Fung et al., 2018). Laboratory-based studies have also found that 38% SDF is more effective than 12% in inhibiting the activity of MMPs and preventing collagen degradation (Mei et al., 2012, Mei et al., 2013). A systematic review by Tolba et al., (2019) evaluated and compared the efficacy of 12% and 38% SDF in arresting dentine caries lesions in primary teeth. They included 3 clinical trials and found that 38% SDF had a greater chance of arresting caries compared to 12% SDF. The main conclusion from all these studies was that a higher concentration of SDF has a higher efficacy and thus it is recommended that 38% SDF is used in caries treatment.

#### **Removal of carious tissue**

Minimal removal of dental tissue is a more conservative strategy that leads to more favourable outcomes and reduces the risk of iatrogenic pulp exposure. It is not always necessary to remove caries tissue to control lesion progression. This has been supported by strong evidence supporting the stepwise caries removal and the Hall technique as an alternative to complete caries removal (Innes et al., 2007; Ricketts et al., 2013; Schwendicke et al., 2013). Whether caries removal affects the efficacy of SDF was investigated in the study by Chu et al (2002). They found that caries excavation prior to the application of SDF showed no significant advantage in arresting the caries lesion.

#### **Re-application frequency**

Clinical trials have investigated the impact of the frequency of SDF use on its effectiveness. Randomised control trials comparing annual and biannual applications found that biannual SDF applications resulted in better caries control outcomes (Zhi et al., 2012; Fung et al., 2018). The same conclusion was reached in a systematic review, which found that annual and biannual SDF applications stopped carious dentin lesions with a success rate of 79 and 91 %, respectively (Duangthip et al., 2015). However, it is important to assess the success of SDF treatment clinically by checking for caries activity and progression. A clinically arrested lesion is darker and harder to probing.  If lesions prove to be active, reapplication of SDF is indicated (Seifo et al., 2020).

#### **Application time**

SDF should be applied to the carious lesion so that it can be absorbed into the carious tissue for optimal effectiveness. It is recommended that SDF be applied for three minutes (Horst et al., 2016). However, a one-minute application has been suggested, as it is sometimes challenging for young and uncooperative patients to allow SDF to take effect for longer(Greenwall-Cohen et al., 2020). The potential impact of application duration on SDF efficacy has been investigated in two recent studies (Table 1.5). In their *in vitro* study, Srisomboon et al., (2021) investigated the effect of three application times of SDF (30, 60 and 180 seconds) on mineral precipitation in demineralised dentine samples from permanent teeth. The samples were incubated in simulated body fluid for two weeks. The precipitation of silver chloride and calcium phosphate in the lesions was measured by infrared spectrometry and X-ray tomography microscopy. They found that the mineral precipitation in the dentinal tubules was comparable in all experimental groups.

   Punhagi and colleagues (2021) evaluated the effect of 1- and 3-minute application times of SDF on the remineralisation of primary enamel by measuring the microhardness of the samples after they had been subjected to pH cycling and comparing it with the microhardness after SDF treatment. The internal porosity of the samples was also assessed using micro-CT. The results of this study showed that there was no difference in the remineralisation of enamel between the two application times, However, these laboratory studies only focused on the effect of the application time of SDF on mineral density, which is only one aspect of the mechanism of action of SDF. The modifying influence of application duration on antibacterial activity and metalloproteinase inhibition has not yet been investigated.

**Table ‎1.5** Studies investigated the potential influence of SDF application time on mineral density.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Author, year** | **Study type/method** | **sample** | **Outcome measure** | **Main finding** | **Limitation of the study** |
| Srisomboon et al., 2021 | Laboratory-based study  Infrared spectrometer and X-ray tomography microscopy | Human in vitro 72 demineralised dentine slices obtained from 13 extracted permanent third molars | Mineral precipitation | mineral participation in the dentinal tubules was comparable among the three application times: 30, 60, and 180 seconds | Demineralised dentine lesion and not natural caries  Measured the immediate effect only. |
| (Punhagui et al., 2021) | Laboratory-based study  Microhardness test | Human/  90 enamel slices obtained from 45 exfoliated primary molars | Mineral precipitation  Change in microhardness | there was no difference in the remineralisation of enamel between 1 and 3 minutes of application time of SDF | Demineralised enamel lesion and not natural caries  Measured the immediate effect only. |

### Advantages and disadvantages of SDF

SDF is a simple, non-invasive, and easy-to-use treatment to arrest dental caries. It is also relatively inexpensive, especially compared to other restorative treatments and when general anaesthesia is required (Schmoeckel et al., 2020). The use of SDF does not require caries removal and local anaesthesia is not necessary. Therefore, it is a beneficial technique when dealing with an anxious, uncooperative, or medically compromised child who cannot tolerate dental treatment. It helps to improve cooperation by enabling effective minimally invasive treatment, followed by successful follow-up and coping strategies if needed (Horst et al., 2016; Seifo et al., 2020). SDF is a cost-effective and beneficia intervention at a population level for treating dental caries, particularly in low-resource settings and has the potential to reduce treatment costs and improve access to dental care for underserved populations (Zhi et al., 2019: Gao et al., 2021). Using SDF to arrest caries can help promote oral health in several ways. First, SDF is a non-invasive treatment that can halt the progression of caries without the need for a restorative procedure, such as a filling. This can be especially beneficial for children, as it can reduce the need for more invasive treatments and make their dental experiences more comfortable. Additionally, SDF has been shown to be effective in reducing the incidence of new caries and improving the effectiveness of child-friendly oral care (Rj et al., 2011). By helping to prevent the development of new caries and arresting the progression of existing caries, SDF can help to promote overall oral health and prevent more serious dental issues from developing in the future. Arresting caries lesions, especially in uncooperative young patients, can also help avoid tooth extractions (Seifo et al., 2020).

The success of SDF in arresting caries relies on maintaining good oral hygiene by the patient, which in some cases is difficult to achieve or predict. In addition, some parents may feel that the traditional restorative treatment is delayed or avoided for their children when the dentist treats their children's caries with SDF because SDF is a non-restorative treatment. In other words, SDF does not fully restore the tooth in the same way that a filling or other restorative treatment would. Instead, it is used to halt the progression of caries and prevent further deterioration of the tooth. This may be a concern for some parents because traditional restorative treatments, such as fillings, are often seen as the "standard" or "preferred" way to treat caries. As a result, parents may feel that their child is not receiving the most appropriate or effective treatment when SDF is used (Seifo et al., 2022). It is important for dentists to communicate with parents and explain the benefits and limitations of SDF treatment in order to help them understand why it may be used in certain situations.

### Furthermore, monitoring the caries lesion activity and the clinical treatment success after SDF treatment can be difficult for several reasons. One reason is that SDF causes a visible darkening of the treated tooth, which can make it difficult to accurately assess the progress of the caries lesion. Additionally, SDF does not fully restore the tooth, so it is not possible to determine the depth or extent of the caries lesion in the same way as with a restorative treatment such as a filling. As a result, it can be difficult to accurately monitor the caries lesion activity and determine the overall treatment success with SDF (Seifo et al., 2020). There are some ways that the treatment success of SDF can be monitored. For example, radiographic examination can be used to assess the progression of the caries lesion and determine the overall treatment success. Additionally, the use of clinical examination and patient feedback can provide some insight into the effectiveness of SDF treatment. Therefore, regular dental check-ups and monitoring are essential to ensure that any changes in the caries lesion are detected and treated promptly (Horst et al., 2016; Seifo et al., 2020).

### Side effects of SDF

#### **Adverse effects and toxicity**

No randomised control trial has reported severe adverse effects after the use of SDF. In very few cases, a very mild, transient, localised gingival reaction has been reported (Seifo et al., 2020). Major concerns have been raised due to the high fluoride concentration in the solution (Gotjamanos and Afonso, 1997). Although 38% SDF contains 44,800 ppm, one drop of the solution contains 2.3 mg of fluoride, compared to 11.3 mg from 5% fluoride varnish. Therefore, fluoride toxicity and the risk of fluorosis should not be a concern (Seifo et al., 2020).

  There is no study or data showing serum concentrations of silver and fluoride in children after the application of SDF. However, a study in adults showed that serum concentrations of silver and fluoride after treatment were very low and did not reach toxic levels (Vasquez et al., 2012). No study has reported an allergic reaction to SDF; however, this treatment is contraindicated in patients with a silver allergy (Horst et al., 2016).

#### **Adverse effect on the pulp**

It has been suggested that SDF should not be used for deep lesions near the pulp because silver ions can penetrate the pulp complex (Hu et al., 2018) However, an *ex vivo* study (Gotjamanos, 1996) investigated the effect of SDF on the dental pulp of carious teeth applied 3-55 months prior to tooth extraction as part of orthodontic treatment. The pulp responded favourably to SDF by forming abundant reparative dentine. More recently, a laboratory study by Rossi et al., (2017) examined the effects on the pulp complex histologically after SDF treatment. They found that the dentinal tubules to which SDF was applied were sealed with minimal penetration. They also observed the formation of tertiary dentine and that the pulp tissue showed an inflammatory response without silver precipitation. The study showed that SDF has a minimal negative impact on the pulp complex.

#### **Affecting bond strength**

The effects of SDF on adhesive strength are contradictory in the literature. Some studies suggest that SDF has a negative effect on bond strength by forming an interface at the tooth-restoration complex and closing the dentinal tubules, resulting in less penetration of the adhesive material. However, other studies have found that the bond strength was improved. in 2006, Knight et al. investigated the effect of SDF on the bond strength of GIC and found no effect provided that SDF was washed and dried before GIC was placed. The effect of SDF on composite bond strength was investigated in dentine (Quock et al., 2012) and enamel (Kj et al., 2018). Both studies showed that no adverse effects on bond strength were observed.

#### **Discolouration**

SDF can permanently discolour clothing and temporarily discolour the skin; it may take a week to disappear, however, it is not associated with pain or damage (Seifo et al., 2020). Discolouration of caries lesions is the most commonly reported disadvantage (Chu et al., 2002; Duangthip et al., 2016; Llodra et al., 2005). Caries tissue becomes permanently black within two minutes of the application of SDF and this increases in intensity for up to six hours (Patel et al., 2018). When a caries lesion is treated with SDF, silver phosphate is formed, which is photosensitive and turns black when exposed to light or reducing agents (Knight et al., 2006).

The black colouration of the caries lesion raises aesthetic concerns (Primus, 2017). In the USA, Crystal et al., (2017) investigated parents' views and attitudes towards the discolouration of their children's primary teeth after SDF treatment. They found that parental tolerance depended on the position of the treated tooth in the dental arch. They showed high acceptance (67.5%) when the treatment and staining occurred in the posterior region compared to the anterior region (29.7%). However, in the same study, parental acceptance was found to increase significantly to 60%, especially in the anterior region, when a decision had to be made between SDF treatment or treatment on GA. Parental socioeconomic status was also an influential factor in the acceptance of staining. However, in China, one study reported that staining was not a problem for parents and could even be considered an indicator of successful treatment (Chu, 2002). When SDF is applied to caries, silver precipitates in the lesion and the excess silver forms silver phosphate, which is the main cause of discolouration. To counteract the side effect of discolouration, Knight et al (2006) suggested that the application of salt after SDF application could eliminate the unwanted discolouration by preventing the formation of silver phosphate. An example of such a salt is potassium iodide (KI), which reacts with silver to form a white, creamy silver iodide that can be easily washed off after treatment.

Several studies have examined the effect of using KI after SDF on reducing staining. Miller et al., (2016) investigated whether there was a difference in restoration discolouration intensity between teeth treated with SDF and SDF+ KI. Twenty extracted permanent teeth with cavitated caries lesions were divided into two groups; in the first group, 38% SDF was applied and restored with GIC. The second group received the same treatment with the application of KI before restoration. The samples were incubated in artificial saliva at 37°C for one month. The intensity of discolouration was recorded using a six-point visual scale. No significant differences in discolouration were observed between the two groups. However, baseline discolouration was not recorded, and follow-up was limited to one month.

Nguyen et al., (2017) evaluated the effectiveness of KI in reducing discolouration after SDF treatment with different restorative materials (composite, GIC and resin-modified glass ionomer) in 45 extracted healthy and carious permanent teeth. The intensity of discolouration between the groups was assessed by visual examination and photo analysis immediately after treatment and after 28 days. All groups treated with KI had minimal to no discolouration after four weeks. However, the SDF-only treated samples were significantly darker than the KI treated groups. Discolouration varied between the groups and restorative materials; however, all teeth treated with KI had a better outcome in terms of colour than those treated with SDF only.

Another study by Zhao et al., (2017) investigated the effect of treatment with SDF and KI on tooth discolouration and prevention of secondary caries in GIC restorations in 30 premolars. They subjected the samples to a cariogenic biofilm test and evaluated the colour of the restoration with a dental spectrometer before and after treatment with a follow-up of 14 days. This study found that discolouration was observed in all groups, but it was lower in the SDF+KI group.

   The discolouration potential of SDF and the influence of KI on the degree of black discolouration over time were investigated by (Patel et al., 2018) by studying 35 extracted carious primary molars. The teeth were treated with either SDF+KI or SDF alone, and the discolouration was analysed over seven days using image processing software. The results showed that after the application of SDF, black staining started within two minutes and increased up to six hours after application. The use of KI immediately after SDF application did not result in any detectable discolouration of the carious dentine or surrounding enamel. These results are consistent with other studies that found KI to reduce discolouration in the short term (Garg et al., 2019; Zhao et al., 2019).

Recently, Detsomboonrat et al., (2022) conducted a study to determine the optimal concentration of KI that would effectively reduce discolouration after the application of SDF. Their study included 24 extracted carious teeth divided into six groups with different KI concentrations. To measure the colour, the teeth were photographed before and 1, 3, 7 and 14 days after the application of SDF and SDF+KI. They found that the application of KI could reduce the degree of discolouration in a dose-dependent manner, but the subsequent colour change over 14 days was minimal.

From the studies to date, it appears that SDF can visibly stain carious tissue and that the use of KI can reduce the staining potential. However, studies with longer follow-up periods are needed to evaluate the long-term efficacy of KI in minimising staining from SDF treatment.

### Effect of KI on the effectiveness of SDF

The use of KI following SDF may have implications beyond aesthetics, as concerns have been raised about the impact of KI on the efficacy of SDF (Koizumi et al., 2016; Zhao et al., 2017). Several studies have investigated the effect of KI on the antimicrobial action of the SDF (Knight et al., 2006; Hamama et al., 2015; Vinson et al., 2018; Abdullah et al., 2020; Haiat et al., 2021). Knight et al. (2006) investigated the permeability of *S. mutans* into demineralised dentine. This *in vitro* study included 40 dentine slices incubated with a continuous culture of *S. mutans* and assigned to 4 experimental groups comparing the effect of applying SDF alone, KI and SDF+KI on bacterial migration in dentine slices. Some migration of *S. mutans* was noted in all groups, however, significantly lower penetration of *S. mutans* was observed in the discs treated with SDF alone and SDF+KI compared to KI alone and the control.

Hamama et al (2015) evaluated the antibacterial effect of SDF+KI (Riva Star, SDI, Bayswater, Victoria) on 45 dentine discs obtained from healthy premolars and compared it with other SDF agents. After application, the discs were infected with *S. mutans* and analysed by confocal laser microscopy. This study showed that the discs treated with SDF+KI had significantly lower bacterial viability in intra-tubular dentine. However, this study did not compare the antimicrobial effect of SDF alone and SDF+KI.

Vinson et al. (2018) investigated the inhibitory effect of SDF with and without KI on *S. mutans* biofilm. Quantification of bacteria after treatment was calculated using an automated counting machine. This study found that SDF alone showed the highest bacterial inhibition compared to SDF+KI. However, the study used a biofilm with only one species (*S. mutans*), and it is known that the biofilm associated with caries lesions contains a more complicated microbial consortium (Samaranayake, 2018).

   Abdullah et al. (2020) investigated the effect of KI on the antibacterial efficacy of SDF on in situ biofilms in five volunteers. They compared SDF alone and SDF+KI on bacterial inhibition by quantification using PCR. This study found that SDF had a strong effect on bacteria, however, no significant difference was found between SDF and SDF+KI. The authors concluded that the antibacterial effect of SDF is not modulated by the application of KI. However, the biofilm in this study was collected from individuals with caries-free dentition. Biofilms of the carious lesion have been shown to have a different composition than caries-free sites, leading to a shift towards aciduric and acidophilic bacteria (Marsh and Bradshaw, 1995b). In a recent study, a systematic review of the possible influence of KI on the antimicrobial efficacy of SDF was conducted by Haiat et al. (2021) Seven studies were included, which generally showed that there was no significant difference between the antimicrobial effect of SDF alone and in combination with KI, suggesting that KI does not interfere with the antimicrobial effect of SDF.

The effect of KI on the caries inhibitory effect of SDF is unclear, as conflicting results have been reported. Knight et al. (2005) found in their research that KI not only reduced discolouration after SDF treatment but also had no adverse effect on the caries inhibitory effect of SDF. On the other hand, Li et al., (2016) reported in their study the comparison of the preventive potential of SDF and SDF+KI in preventing the development of new root caries lesions in 323 elderly randomly assigned to three intervention groups: Water treatment, SDF treatment, SDF+KI treatment and placebo. All subjects received oral hygiene instructions and fluoride toothpaste. The condition of the root surfaces was examined every six months for 30 months. This study found that although SDF and SDF+KI both inhibited the development of new root lesions, SDF alone showed higher efficacy than SDF+KI (95% credible interval)

.

  In agreement with Li et al (2016), a study by Zhao et al (2017) investigated the effect of treatment with SDF + KI on the prevention of secondary caries as part of the restorative treatment of caries lesions with GIC in 30 premolars. Treatment with SDF + KI had the potential to prevent the formation of secondary caries around GIC restorations, however, it was not as effective as SDF alone suggesting that KI influences the efficacy of SDF in preventing the development of secondary root caries (Zhao et al., 2017).

The influence of KI on the effectiveness of SDF in preventing enamel caries was investigated by Sorkhdini et al., (2021) when they studied the development of new caries lesions on human enamel samples after the application of SDF and SDF+KI. After treatment, the samples were subjected to a 7-day pH cycle test. They found that KI did not affect the efficacy of SDF and that both treatments were effective in primary coronal caries prevention. However, it should be consider that 7 day Ph cycling is not suitable to simulate enamel lesion development and therefore these results needs to be interpreted with caution.

## Summary

Dental caries is a complex and multifactorial disease and one of the most prevalent diseases worldwide. Its treatment is an ongoing public health issue. The decision to treat the lesion is based on accurate detection and diagnosis. However, there are differences in deciding at what stage of caries restorative intervention is required. Caries is a biofilm-mediated disease when cariogenic bacteria contribute to the process. Endogenous enzymes (MMPs) play a fundamental role in caries progression, and their expression and localisation in carious lesions have been studied. There is limited understanding and research in the literature on the role of MMPs in caries diagnosis and treatment. In terms of diagnosis, the relationship between MMPs in carious lesions and the surface appearance of caries as determined by clinicians has not been investigated, which is clinically important to obtain detailed information about the lesion at diagnosis and to decide on a personalised care plan. Various techniques have been used to study MMPs in dental tissues, such  
as zymography (Goldberg et al., 2009; Mazzoni et al., 2010; Nui et al., 2011;  
Mazzoni et al., 2018), ELISA (Nui et al., 2011), scanning electron  
microscopy (Mazzoni et al., 2007; Shimada et al., 2009 Mazzoni et al.,  
2018) and immunofluorescence (Toledano et al., 2010; Vidal et al., 2014).  
Zymography measures the activity of proteolytic enzymes (Leber and  
Balkwill, 1997), but requires that the dentine is in powder form to measure  
activity. Although this technique is effective, it does not allow localisation of  
the MMPs in the tooth section. ELISA can be used to investigate the  
concentration of the target enzyme, however, obtaining the protein extracts  
from the tissue is necessary, and localisation of the MMPs in the tooth  
section is again impossible. Immunostaining proved  
to be a suitable method for localising MMPs in tooth sections   
representing varying degrees of caries severity.

The treatment of caries in young patients is challenging and requires new or alternative approaches to control the lesion. SDF is one of these approaches. It is simple, easy to use and consistent with the concept of minimally invasive dentistry. SDF has been shown to inhibit MMPs; inhibition of these endogenous enzymes in dentine is one of the mechanisms of action of SDF in arresting caries. Previous studies have shown conflicting results regarding the influence of using KI after SDF to remove discolouration on the caries-inhibiting effect of SDF. Furthermore, no study has yet investigated the possible influence of KI on the anti-MMP potential of SDF. Therefore, further studies are needed to explore all the possible clinical effects of the combination of SDF and KI so that clinicians have the necessary evidence to make a clinical decision on the use of this combination.

# CHAPTER 2

HYPOTHESIS, AIMS & OBJECTIVES

# Chapter 2: Hypothesis, aims and objectives

## Null Hypothesis

Previous data in the literature showed the presence of MMPs in caries lesions, which play an important role in caries progression. We first hypothesised that MMPs do not correlate with the stage of caries and do not differ with the depth of caries. Secondly, we investigated SDF as an MMP inhibitor and hypothesised that the use of KI after SDF does not affect the anti-MMP potential of SDF.

## Aims and objectives:

The overall aim of this PhD programme is to investigate matrix metalloproteinase enzymes in the context of caries diagnosis and management by identifying, locating and linking MMPs to ICDAS scores and then investigating the long-term inhibitory effect of SDF on MMP activity in relation to two important clinical variables: the presence of KI and the duration of application. The specific aims of each chapter are summarised below.

**1)** **Chapter 5:** **Correlation between MMP presence and caries surface appearance**

* **Aim:** to investigate the localisation of MMP2 and MMP9 and their correlation with caries lesions assessed by the ICDAS score. The specific objectives were as follows:
* To perform immunocytochemical analysis of primary human teeth with carious dentine.
* To identify and characterise the location and distribution of MMP2 and MMP9 in different depths of human primary carious dentine.
* Search for a correlation between MMPs and carious lesions assessed by ICDAS scores.
* Quantify the amount of denatured collagen in dentine with varying degrees of caries severity as assessed by ICDAS.
* Relate the MMPs to the degree of collagen degradation in the caries lesion assessed by the ICDAS score.
* To investigate whether the MMPs in caries lesions correlate with diagnostic tests: laser fluorescence pen, histological score, and radiological scores.

**2)** **Chapter 6: Caries -inhibitory effects of Silver Diamine Fluoride: influence of time and potassium iodide**

* **Aim:**   to investigate the long-term inhibitory effect of SDF on MMP activity in relation to two important clinical variables: the presence of KI and application time. The specific objectives were as follows:
* To investigate the long-term effect of SDF on total MMP activity in caries lesions.
* To evaluate if Potassium Iodide (KI) application following SDF affects the anti-MMP activity potential of SDF in caries lesions.
* To assess and quantify the effect of SDF and SDF with KI on collagen degradation.
* To investigate different application times of SDF and their effect on MMP activity.
* To evaluate the effect of SDF application on the surface hardness of non-cavitated caries lesions.

# CHAPTER 3

MATERIALS & METHODS

# Chapter 3: Materials and Methods

## Overview

The study was conducted at the School of Clinical Dentistry, Sheffield. The project involved in-vitro laboratory studies investigating matrix metalloproteinases in extracted human primary carious teeth in relation to caries diagnosis and management. The sample comprised 65 primary teeth from 16 child patients obtained from a previous study. The teeth were supplied anonymously with no reference to the donor and no means of identifying the individual.

For caries diagnosis, an investigation of MMP expression in dental caries in 31 extracted teeth and the correlation of their expression with the clinical diagnosis and surface appearance of caries recorded by ICDAS was carried out using immunocytochemical techniques.

Investigations relating to caries management comprised two experiments conducted on 24 extracted teeth to investigate the MMP-inhibitory effects of silver diamine fluoride (SDF) and the influence of application time and potassium iodide (KI) on MMP activity using a Generic MMP activity assay and a Hydroxyproline assay.

Of the 65 available teeth, 55 were included in the experiments, as mentioned above. The remaining 10 teeth were recorded with an ICDAS score of 0-2 and were, therefore, not suitable for the above-mentioned investigations. However, they were used for a further line of enquiry to investigate the effect of SDF+/-KI on the surface microhardness of non-cavitated lesions.

## Experimental material

### Collection of teeth for the preliminary experiments

To establish “proof of principle”, 20 human primary molars were used to test immunostaining methods and assay procedures. These primary teeth were taken from a historical bank established in the School of Clinical Dentistry prior to the Human Tissue Act (Reid and Brooks, 1982) (Human Tissue Act, 2004).

### Collection of teeth for the main study

#### **Ethical approval**

This research project follows on from the work conducted by Subka et al., (2019). Subka and her colleagues undertook a clinical diagnosis of 1030 proximal surfaces in human carious primary molars from 82 children (54% boys, 46% girls) aged 5-10 years (mean age = 6.4; SD = 1.3 years), with 80% aged between 5- and 7-years. The clinical examination and assessment were performed at the paediatric dentistry clinic of the Charles Clifford Dental Hospital, Sheffield. Participants who required dental extractions under general anaesthesia (GA) as part of their normal treatment plan were included. Extracted teeth were then subject to histological validation of the pre-extraction ICDAS score for caries extent.

Ethical approval for their work was obtained from the National Health Services Research Ethics Committee (NHS REC; Reference 12/YH/0214) and from Sheffield Teaching Hospitals Research Governance Department (protocol number STH16301) (Appendix 1).

Ethical approval was granted to retain the teeth/histological sections obtained from paediatric patients whose legal guardians specifically consented to their retention and further use; this sample comprised 65 teeth from 16 patients (Appendix 2). The original approval, obtained on the 21st of August 2016, permitted these teeth to be retained in a Biobank for five years (Appendix 3). Approval from the University Research Ethics Committee for this project to use existing research, clinical or other data that has been robustly anonymised was received on the 1st of October 2020. (Appendix 4)

#### **Sample retrieval from biobank**

Samples (65 teeth) were retrieved from the Biorepository, located within the University of Sheffield Medical School. Each tooth was sectioned and stored in a labelled vial. The mesial surface was marked with a black permanent marker (STAEDTLER permanent Lumocolor) to aid the identification of tooth surfaces in each section. Samples were stored in distilled water at -80℃ in the biobank. Following retrieval, samples were stored at -80℃ in the dental school laboratory facilities until required.

#### **Sample characteristics**

After retrieving tooth samples (65 teeth from 16 patients) from the biobank, they were organised and sorted according to their original ICDAS score (as previously allocated by Subka et al., 2019). Table 3.1 shows the number of teeth per patient and available samples according to each ICDAS subgroup. Each tooth was previously sectioned longitudinally using a water-cooled band saw 0.2 mm thick (EXAKT-Apparatebau GmGH, Norderstedt, Germany) to provide multiple sections of 500μm thickness and stored in a labelled vial prior to freezing at -80℃ degrees in the biobank. A flow chart summarising the distribution of samples according to the purpose of the experiment is illustrated in Figure 3.1.

**Table ‎3.1** Table showing the total number of teeth included in the study and the distribution of tooth samples (surfaces) according to ICDAS scores.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Patient’s Serial No** | **Number of teeth per patient** | **Number of surfaces available according to each ICDAS score** | | | | | | | **Total number of surfaces with a recorded ICDAS** |
| **ICDAS 0** | **ICDAS 1** | **ICDAS 2** | **ICDAS 3** | **ICDAS 4** | **ICDAS 5** | **ICDAS 6** |
| **1** | 5 | 3 | 0 | 3 | 0 | 0 | 2 | 2 | 10 |
| **2** | 6 | 0 | 0 | 5 | 2 | 0 | 3 | 0 | 10 |
| **3** | 2 | 2 | 2 | 0 | 0 | 0 | 0 | 0 | 4 |
| **4** | 3 | 1 | 1 | 1 | 0 | 0 | 2 | 1 | 6 |
| **5** | 4 | 1 | 1 | 3 | 0 | 1 | 1 | 1 | 8 |
| **6** | 5 | 2 | 0 | 5 | 0 | 0 | 0 | 3 | 10 |
| **7** | 2 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 4 |
| **8** | 4 | 2 | 0 | 0 | 0 | 0 | 3 | 2 | 7 |
| **9** | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 1 |
| **10** | 2 | 0 | 0 | 1 | 1 | 0 | 2 | 0 | 4 |
| **11** | 5 | 3 | 0 | 5 | 0 | 0 | 0 | 2 | 10 |
| **12** | 4 | 0 | 0 | 4 | 0 | 0 | 2 | 2 | 8 |
| **13** | 6 | 3 | 1 | 3 | 0 | 1 | 1 | 3 | 12 |
| **14** | 3 | 2 | 0 | 1 | 0 | 0 | 1 | 2 | 6 |
| **15** | 8 | 0 | 0 | 8 | 0 | 0 | 1 | 1 | 10 |
| **16** | 5 | 2 | 0 | 3 | 1 | 0 | 3 | 1 | 10 |
| **Total** | **65** | **22** | **6** | **43** | **4** | **3** | **21** | **21** | **121** |

***(a) Sample size and distribution for immunostaining procedures***

One section per tooth was used for immunostaining studies; this was the middle section from each ICDAS-scored tooth. Each section had two surfaces which had been clinically examined to allocate an ICDAS coding (Figure 3.2); therefore, the number of samples will subsequently be referred to as the number of surfaces (n). The total number of surfaces used was 48, and the distribution of these samples according to immunostaining procedures is illustrated in Figure 3.3.

Diagram

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**Figure 3.1** Flow chart summarising the sample distribution according to the purpose of experiments.

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**Figure 3.2** Photograph showing the mesiodistal section of a lower primary second molar with ICDAS score recorded for each surface (1) demonstrates ICDAS 2 recorded for the mesial surface and (2) demonstrates ICDAS 5 recorded for the distal surface.

|  |
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**Figure 3.3** Sample size and distribution according to immunostaining technique. (n=number of surfaces, IHC = immunohistochemistry, IF = immunofluorescence).

***(b) Sample size and distribution for assays***

A total of 87 dentine blocks (15 used for hydroxyproline assay and 72 for Generic MMP assay) were obtained from extracted primary molar teeth from the biobank. For the hydroxyproline assay, three sections from different teeth for each ICDAS score were included, making a total number 15 block samples in this assay.

For the MMP activity assay, three sections per tooth were used (Figure 3.4). Therefore, from 24 teeth, 72 carious dentine blocks were obtained (Figure 3.5).

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**Figure 3.4** Schematic diagram to illustrate the three serial sections that were used from each tooth.

**Diagram

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**Figure 3.5** The distribution of dentine carious blocks according to the purpose of the experiment. (SDF= sodium diamine fluoride, KI= potassium iodide, MMP=matrix metalloproteinase).

## Preparation of teeth samples/sections

### Sample used in preliminary experiments

#### **Tooth sectioning**

An experienced technician (Hayley Stanhope), working in the histology laboratory, School of Clinical Dentistry, University of Sheffield, carried out the sectioning of the teeth. Each tooth was mounted in wax and then serially sectioned longitudinally in a buccolingual direction using a water-cooled bandsaw 0.2 mm thick (EXAKT-Apparatebau GmGH, Norderstedt, Germany) to achieve several cuts. Each section was approximately 150 microns (μm) thick. All sections were stored in containers containing 10% formalin until required.

### Samples used in Immunostaining procedures

#### **Preparation of tooth sections**

The archived tooth samples obtained from the previous work of Subka et al. (2019) were sectioned longitudinally in a mesiodistal direction. Each section was approximately 500μm thick. Following preliminary immunohistochemical study results relating to the present study, it was determined that 150μm was the maximum section thickness that would allow viable staining; therefore, the 500μm sections needed to be further ground to 150μm.

#### **Grinding of teeth sections**

Several attempts were made to cut the 500μm sections into the required reduced thickness of 150μm. However, this did not prove possible even with the variety of equipment available within the host laboratories; thus, an alternative approach was sought to grind the sections down to the required thickness.

Dr Marina Malinowski, Department of paediatric dentistry at the University of Leeds, facilitated the investigator’s access to laboratories at the University of Leeds where additional grinding instrumentation was available. Access permission was obtained on the 25th of February 2021 to use the facilities in the Lab, including sectioning of teeth using a precision diamond wire saw well 3242 and grinding teeth sections using a custom-made grinding device. The process of sectioning teeth and training to use the grinding device was undertaken under the supervision of Dr Malinowski at the University of Leeds laboratories. As grinding the teeth sections was considered time-consuming and to decrease the burden of travelling to Leeds, Dr Malinowski agreed to the grinding device being brought to the dental school laboratories at the University of Sheffield for the author to undertake the grinding of the main samples.

Whole primary molar teeth from the historical bank were used to test and perfect the grinding process before starting with the main samples. Firstly, teeth were cut longitudinally to provide 500μm thick sections to resemble the main sample sections; a precision diamond wire saw well 3242 (Delaware Diamond Knives. Inc) was used for this purpose. Teeth were embedded in wax and fixed in a sample holder. The tooth sample was positioned on a slotted table while the sample holder rotated 360 degrees for the correct direction of the appropriate cutting axis. (Figure 3.6)

Following the sectioning of teeth to 500μm (Figure 3.7), the grinding device (Figure 3.8) was used to grind the 500μm thick sections to 150μm. The device consists of two grinding plates (Figure 3.9). Samples were attached to a metal plate using nail varnish on one of the grinding plates, and a weight holder was placed on them and left to dry for at least one hour to achieve the maximum attachment (Figure 3.10). The grinding process commenced once the metal plate with the sample attached had been securely screwed to the grinding plate.

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**Figure 3.6** A Photograph demonstrating the Precision Diamond Wire Saw Well 3242 (Delaware Diamond Knives. Inc).

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**Figure 3.7** A Photograph illustrating a primary molar tooth which had been sectioned into four 500μm thick sections.

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| A picture containing metalware, catch, dirty  Description automatically generated |

**Figure 3.8** A Photograph to show a close-up view of the grinding device.

|  |
| --- |
|  |

**Figure 3.9** A photograph to show the parts of the grinding device.

|  |  |
| --- | --- |
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**Figure 3.10** (A) Tooth samples attached to a metal plate using red nail varnish. (B) A weight holder was placed on tooth samples to ensure maximum retention and allowed to dry for one hour.

This revised approach still proved challenging as the tooth samples repeatedly fell off the metal plate despite multiple attempts to secure them. In order to overcome this problem, the grinding of samples was undertaken using only one grinding plate. Deionised distilled water (Invitrogen, UK) was used as a lubricant while grinding sections on the device (Figure 3.11). The thickness of the teeth sections was monitored using a micrometre (Sealey, UK). Grinding was stopped when the desired thickness (150μm) was achieved uniformly across the sample (Figure 3.12).

|  |  |
| --- | --- |
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**Figure 3.11** Grinding of samples commenced using one grinding plate by applying circular movement to the tooth by hand.

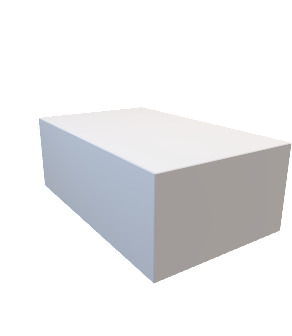
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**Figure 3.12** A micrometre was used to measure the thickness of samples following grinding.

### Samples used in assay procedures

#### **Preparation of carious dentine blocks**

Carious dentine blocks (n=78) were obtained from extracted human primary molars with proximal coronal carious lesions extending into dentine. All teeth sections had been previously stored in freezers at -800 C and were retrieved from the tissue bank as described previously in section 3.1.2.2. The teeth were left to defrost at room temperature for three hours. A diamond tapered fissure bur (Henry Schein, UK) with a high-speed dental handpiece (W&H, UK) under water cooling was used to obtain dentine samples (blocks) with dimensions of 3mm (width) x 3mm (length) x 500μm (thickness). (Figure 3.13)



3mm

3mm

500μm

|  |
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**Figure 3.13** A diagram to show the preparation and dimensions of carious dentine blocks.

Each dentine block was placed in a labelled vial with a unique ID number and allocated to one of the three experimental subgroups depending on the purpose of the experiment.

#### **Saliva (incubation media) collection**

Ethical approval for the collection of normal human saliva had previously been granted by the University of Sheffield Ethics Committee (Reference Number 040965; 02/08/2021).

Carious dentine blocks were incubated in natural saliva to mimic the oral environment for experiments involving the long-term application of SDF and to allow measurement of collagen degradation following each incubation period.

Saliva was collected at a one-time point from one donor (a female aged 39 years with a clear medical history and no medication history). Saliva was obtained according to a standard protocol. As stimulated saliva predominantly contains parotid gland products, notably amylase, it can potentially mask subtle changes in less prevalent salivary proteins. Thus, this experiment involved the collection of the whole saliva. The saliva sample was collected at 9 am. The donor had refrained from eating or drinking for one hour prior to collection and had rinsed her mouth with water immediately before sampling. A glass vial was used to prevent the loss of “sticky” salivary proteins, which could potentially adhere to a plastic surface during storage.

The saliva sample was centrifuged for 10 mins at 2000 rpm to remove food debris and high molecular weight proteins (GenFuge, Proyen, UK).  Following centrifugation, saliva was sterilised using a syringe filter (0.22μm) (StarLab, DE) to remove potentially contaminating bacteria from the oral cavity. Subsequently 400μL aliquots of saliva were stored in glass vials (Figure 3.14).

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**Figure 3.14** Diagram to outline the stages of saliva collection and preparation.

## Immunohistochemistry

### Overview

All immunohistochemical experiments, to localise MMPs in carious dentine, were carried out under the supervision of Dr Lynne Bingle, School of Clinical Dentistry, University of Sheffield. Polyclonal antibodies against MMP9 (ab74277, Abcam) and MMP2 (ab235167, Abcam) produced by Abcam were used; details are shown in Table 3.2. Table 3.3 provides details of all immunohistochemical reagents used.

**Table ‎3.2** Primary antibodies used in the immunohistochemistry experiments.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Antigen** | **Type** | **Host species** | **Supplier** | **Dilution** |
| MMP9 | Polyclonal (human) | Rabbit | Ab74227, Abcam | 1:750 |
| MMP2 | Polyclonal (human) | Rabbit | Ab235167, Abcam | 1:750 |

### Training

Prior to staining the teeth, the investigator received immunohistochemical training from Dr Bingle. Normal, human salivary gland tissue sections, taken from the Oral pathology tissue bank in the School of Clinical Dentistry, were used to practice the technique and achieve optimum staining results.

### Immunohistochemical procedures

Each immunohistochemistry process was conducted over two consecutive days, in two stages with the time for each stage being fixed to prevent any time variations and experimental errors. All stage one experiments were started at 2:00 pm, and stage two was started the following day at 9:00 am.

|  |  |
| --- | --- |
| **Material** | **Supplier** |
| Avidin-Biotin Complex (ABC) Reagent | Vector Laboratories. UK |
| Diaminobenzidine Tetrahydrochloride substrate (DAB) | Vector Laboratories. UK |
| DPX | Distrene, Plasticiser and Xylene |
| Hydrogen peroxide (H2O2) | Sigma Aldrich, Poole, UK |
| ImmPRESS® Duet Double Staining Polymer Kit Peroxidase/Alkaline Phosphatase (HRP Anti-Mouse IgG/AP Anti-Rabbit IgG) | Vector Laboratories. UK |
| Methanol | Fisher chemical, Loughborough, UK |
| Mounting medium | Fisher Scientific, Loughborough, UK |
| Multi-well plastic plate | Falcon, UK |
| Normal Goat Serum 100% | Vector Laboratories. UK |
| Phosphate buffer solution (PBS) | Sigma-Aldrich, Poole, UK |
| Secondary antibody | Vector Laboratories. UK |

**Table ‎3.3** Immunohistochemical reagents and kits used in this study.

#### **First stage (day one)**

***1. Preparation of Phosphate Buffered Saline (PBS)***

The *PBS* was made up of the following constituents: *5L distilled water + 42.5g NaCl (0.15M) + 5.8g K2HPO4 (0.015M) + 1.25g KH2PO4 (0.018M).*

Teeth sections were washed three times in PBS (Sigma-Aldrich®) for 5 minutes to remove any residuals from samples.

***2. Quench enzymatic activity***

Many normal tissues retain endogenous peroxidase activity. As the final step in the process relies on peroxidase activity giving a colour reaction, it is necessary to quench endogenous activity prior to any immunostaining. This was achieved by incubating the sections in 3% hydrogen peroxide (H2O2) in methanol (Sigma Aldrich, Poole, UK) for 20 minutes. H2O2 is available as a 30% solution; therefore, a 1-in-10 dilution was prepared as follows:

*30ml H2O2 + 270ml Methanol*

The solution should not be used after a storage period of more than seven days; thus, fresh preparations were made up as necessary. Multi-well (12-well) plastic plates (Falcon®) were used for ease of handling of samples. Each tooth section was placed in a separate well using metal forceps, and then H2O2 was applied (Figure 3.15). The sections were then further rinsed with PBS.

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**Figure ‎3.15** A photograph to demonstrate Quenching of enzymatic activity with H2O2 using a multi-well plastic plate.

***3. Antigen retrieval***

As samples were stored in formalin, the potential availability of antigens can be obscured. Thus, various antigen retrieval-processing methods are available to unmask the antigenic site, and in this case, heat treatment with sodium citrate buffer, pH6, was used to amplify the presence of antigens in the tooth sections. This buffer solution was prepared as follows:

Citrate buffer (pH 6) = 1.18g Sodium citrate (tribasic, 0.01M) + 400ml distilled H2O

The citrate buffer solution was placed in a glass flask, and the samples were carefully placed at the bottom of the flask using metal forceps to ensure the separation of samples. The citrate buffer was heated in a microwave (Igenix, China) for 8 minutes before cooling in PBS.

***4.* *Blocking with serum***

Although antibodies show preferential binding to specific antigens, they may partially bind to nonspecific proteins and cause “background staining”. To reduce this, 100% normal goat serum (Vector Laboratories, UK) was used for blocking non-specific sites. The serum of the animal from which a secondary antibody has been raised should be used. Samples were placed on glass slides in a humidified chamber, and normal goat serum was added to cover the section entirely. The serum was left on the sections for 30 minutes at room temperature (20℃).

***5. Incubation with primary antibody***

MMP9 and MMP2 antibodies were used separately as primary antibodies and were prepared by diluting in 100% normal serum. Dilutions of antibodies from 1:100 to 1:1000 in normal goat serum were prepared and explored to determine the optimum concentration as follows:

* 1:100
* 1:150
* 1:200
* 1:300
* 1:500
* 1:750
* 1:1000

The samples were placed on slides, and the primary antibody was applied and incubated at 4℃ overnight in a humidified chamber (Figure 3.15).

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**Figure 3.15** Schematic diagram to illustrate the application of normal serum and primary antibody.

#### **Second stage (day two)**

All samples were washed in PBS twice for 5 minutes.

***1. Preparation of secondary antibody and ABC reagent***

The secondary antibody (Vector Laboratories, UK), from the same species as the normal serum used for blocking, was prepared according to the manufacturer’s instructions as follows:

*Secondary antibody = 10ml PBS + 3 drops normal serum + 1 drop biotinylated secondary antibody*

At this point, the ABC reagent (Vector Laboratories, UK) was prepared, as it needs to stand for 30 minutes before application. This complex acts as an enhancement solution., it was prepared, according to the manufacturer’s instructions, as follows:

*ABC reagent = 5ml PBS + 2 drops reagent A (Avidin) + 2 drops reagent B (Biotin)*

Reporter intensity is a role of the localised enzyme activity, and improved sensitivity is reached by increasing the number of enzyme molecules bound to the target antigen. Avidin consists of four structural subunits (tetramer). Each unit has the ability to bind to Biotin. Initial mixing of Biotin and Avidin from partially biotinylated Avidin allows further Biotin binding on the secondary antibody. The multiple biotin-binding sites in each Avidin molecule led to this amplification. The result is a greater concentration of enzyme at the antigenic site and, therefore, an increase in signal intensity and sensitivity upon the addition of substrate.

The staining method of the Avidin-Biotin complex is illustrated in Figure 3.19.

Diagram

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**Figure 3.16** Staining method of Avidin-Biotin Complex (ABC).

***2. Application of secondary antibody and ABC Reagent:***

After rinsing with PBS, the samples were placed on slides in a humidified chamber, and the secondary antibody (Vector Laboratories, UK) was added to cover the sections and left for 30 minutes at room temperature. Following further washing with PBS for 2 X 5 minutes, ABC reagent was applied to the samples and left for 30 minutes at room temperature. After completion, the samples were washed with PBS (2X5 minutes). (Figure 3.21)

***3. Preparation and application of diaminobenzidine tetrahydrochloride substrate (DAB)***

According to the manufacturer's instructions, DAB (Brown stain) (Vector Laboratories, UK) was prepared as follows:

*DAB = 5ml distilled H2O + 2 drops of buffer stock solution + 4 drops of DAB + 2 drops of H2O2.*

DAB solution was applied to all samples (Vector Laboratories, UK), left for 5 minutes, and then rinsed with distilled H2O to stop further colour development. (Figure 3.20)

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**Figure 3.17** Schematic diagram demonstrating stage two procedure steps (secondary antibody applied to the samples followed by BPS wash. ABC applied to the samples and followed by BPS wash, and then DAB was applied followed by rinsing with distilled water).

***4. Mounting steps***

All samples were placed on glass slides and mounted in DPX (Distrene, Plasticiser and Xylene) mounting medium (Fisher Scientific, Loughborough, UK). The slides were left for at least two days before being checked under the light microscope and imaged using an Olympus BX51 microscope and Colorview IIIu camera with associated Cell^D software (Olympus soft imaging solutions, GmbH, Münster, Germany).

## Double staining immunohistochemistry

### Overview

Immunohistochemistry depends on the ability of antibodies to bind antigens with high affinity and specificity and the potential to conjugate antibodies to tracer molecules such as enzymes and fluorophores. These tracers allow for visualising the bound antigen by colorimetric reactions or fluorescence. IHC is usually performed with a single antibody, but a double staining technique with more than one primary antibody enables the simultaneous detection of more than one protein (Reid and Brooks, 1982).

A double immunohistochemistry procedure was performed with ImmPRESS® Duet Double Staining Polymer Kit Peroxidase/Alkaline Phosphatase (HRP Anti-Mouse IgG/AP Anti-Rabbit IgG) (Vector Laboratories). This is an enzymatic, non-biotin amplification system that produces highly specific and sensitive staining, with low background, of two different target antigens on the same tissue section. Anti-rabbit IgG and Anti-mouse purified secondary antibodies are independently conjugated with alkaline phosphatase (AP) enzyme and horseradish peroxidase (HRP), then harmonised at optimised dilutions to provide a stable pre-diluted, ready-to-use HRP/AP ImmPRESS polymer formulation. Table 3.4 shows the components of ImmPRESS® Duet Double Staining Polymer Kit.

**Table ‎3.4** Components of ImmPRESS® Duet Double Staining Polymer Kit.

|  |  |
| --- | --- |
| **Product name** | **Volume** |
| BLOXALL Blocking solution | 15mL |
| ImmPRESS Duet Reagent | 15mL |
| ImmPACT DAB EqV Reagent 1(chromogen) | 7.5mL |
| ImmPACT DAB EqV Reagent 2 (diluent | 7.5mL |
| ImmPACT Vector Red Diluent | 15mL |
| ImmPACT Vector Red Reagent 1 | 240μL |
| ImmPACT Vector Red Reagent 2 | 180μL |
| Normal horse serum (2.5%) | 15mL |

### Procedure

The double staining procedure was performed according to the manufacturing instruction with some modifications. Samples were defrosted for 3 hours and washed in PBS for 5 minutes in 12-well multi-well plastic plates. Endogenous peroxidase was quenched by incubating the samples in 3% hydrogen peroxide (H2O2) for 20 minutes. Antigen retrieval was carried out using heat-induced antigen retrieval (HIAR). Citrate buffer solution (pH 6.0) was placed in a glass flask. The samples were handled carefully and placed at the bottom of the flask separately using metal forceps.

The citrate buffer was heated in a microwave for 8 minutes. The samples were washed and cooled in PBS for 5 minutes after microwaving. 100% normal horse serum was used to block non-specific protein binding. Samples were placed on glass slides in a humidified chamber, and serum was added to cover the section entirely. The serum was left on the sections for 30 minutes at room temperature (20℃). Excess serum was tipped off the slides, and the primary antibodies, polyclonal rabbit MMP9 antibody (ab74277, Abcam) and polyclonal mouse MMP2 antibody (ab86607, Abcam) were diluted (1:750) in 100% normal horse serum and applied to the samples and incubated at 4℃ overnight in a humidified chamber.

Samples were washed in PBS for 5 minutes in multi-well plates and placed on slides where they were incubated with ImmPRESS Duet Reagent (HRP Anti-Mouse IgG/AP Anti-Rabbit IgG) for 10 minutes. The samples were then washed twice for 5 minutes. ImmPACT DAB EqV Substrate was prepared following the manufacturer’s instruction by mixing equal volumes of the provided Reagent 1 and Reagent 2. Samples were incubated with the substrate for approximately two minutes until the desired stain intensity was developed. The samples were washed twice before being incubated for 20 minutes with ImmPACT Vector Red Substrate, which was prepared and used immediately by mixing 2.5ml of ImmPACT Vector Red Diluent with 40ul of ImmPACT Vector Red Reagent 1 and 30μl of reagent 2.

The samples were washed twice with PBS and placed in a vial with distilled water; they were placed on slides and mounted in DPX (Distrene, Plasticiser and Xylene) mounting medium. The slides were left for at least two days before being checked under the light microscope and imaged using an Olympus BX51 microscope and a Colorview IIIu camera with associated Cell^D software (Olympus soft imaging solutions, GmbH, Münster, Germany).

## Immunofluorescence

### Overview

Double immunofluorescence staining is a technique that allows the detection of two biomarkers in the same sample. Reliable and reproducible results can therefore be obtained for tissue sections where the two target antigens overlap (co-localise) in the same structure. They also allow for co-localization studies to determine potential relationships between analytes.  VectaFluor™ Duet Double Labelling Kit (DyLight® 488 Anti-Rabbit IgG, DyLight® 594 Anti-Mouse IgG) (Vector Laboratories) was used to achieve double-label immunofluorescence. Details of the primary antibodies used are shown in Table 3.5. Table 3.6 provides details of all the immunofluorescence reagents and kits used, and Table 3.7 shows the components of the VectaFluor™ Duet Double Labelling Kit.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antigen | Type | Host species | Supplier | Dilution |
| MMP9 | polyclonal (human) | rabbit | ab74227, Abcam | 1:750 |
| MMP2 | monoclonal (human) | mouse | Ab86607, Abcam | 1:750 |

**Table ‎3.5** Primary antibodies used in the immunofluorescence experiments.

**Table ‎3.6** Immunofluorescence reagents and kit used in this study.

|  |  |
| --- | --- |
| **Material** | **Supplier** |
| Hydrogen peroxide (H2O2) | Sigma Aldrich, Poole, UK |
| Methanol | Fisher chemical, Loughborough, UK |
| Multi-well plastic plate | Falcon, UK |
| Normal horse Serum (100%) | Vector Laboratories. UK |
| Phosphate buffer solution (PBS) | Sigma-Aldrich, Poole, UK |
| VectaFluor™ Duet Double Labelling Kit (DyLight® 488 Anti-Rabbit IgG, DyLight® 594 Anti-Mouse IgG) | Vector Laboratories. UK |
| VectaShield Antifade Mounting Media | Cat#: H-1200; Vector Laboratories, Peterborough, UK |

**Table ‎3.7** Component of VectaFluor™ Duet Double Labelling Kit.

|  |  |
| --- | --- |
| **Product name** | **Volume** |
| Normal horse serum (2.5%) | 15mL |
| VectaFluor Duet Reagent | 15mL |

### Sample characterisation

For the immunofluorescence study, 24 proximal surfaces were analysed. There were a similar number of surfaces within each caries subgroup, as shown in Tables 3.8 and 3.9.

  The main dental variables were the position of the teeth in the dental arch (upper or lower arch) and whether they were first or second primary molars. Of the total sample, 6 (25%) were from maxillary teeth and 18 (75%) from mandibular teeth. Table 3.8 shows the dental arch variable within each caries subgroup. Seven (29%) were first primary molars, and 17 (71%) were second primary molars (Table 3.9).

### Specificity controls

***Endogenous tissue background control***

Tooth sections were inspected using a fluorescent microscope with red and green excitation filters to ensure no signal was inherent to the dental tissue itself.

***Negative control/ No primary antibody control***

This control was prepared by incubating tooth sections in serum without primary antibodies. This was followed by incubation with secondary antibodies and detection reagents.

**Table ‎3.8** Number of samples in each ICDAS subgroup according to dental arch variable.

|  |  |  |  |
| --- | --- | --- | --- |
| **Caries lesion assessed by ICDAS** | Number of surfaces (n) | | |
| Maxillary teeth  (n) | Mandibular teeth (n) | Total  (n) |
| ICDAS 0 | 0 | 3 | 3 |
| ICDAS 1 | 0 | 3 | 3 |
| ICDAS 2 | 1 | 2 | 3 |
| ICDAS 3 | 3 | 0 | 3 |
| ICDAS 4 | 0 | 3 | 3 |
| ICDAS 5 | 1 | 2 | 3 |
| ICDAS 6 | 0 | 3 | 3 |
| Control (negative) | 1 | 2 | 3 |
| Total | 6 | 18 | 24 |

|  |  |  |  |
| --- | --- | --- | --- |
| **Caries lesion assessed by ICDAS** | Number of surfaces (n) | | |
| Primary first molar  (n) | Primary second molar (n) | Total  (n) |
| ICDAS 0 | 2 | 1 | 3 |
| ICDAS 1 | 1 | 2 | 3 |
| ICDAS 2 | 2 | 1 | 3 |
| ICDAS 3 | 3 | 0 | 3 |
| ICDAS 4 | 2 | 1 | 3 |
| ICDAS 5 | 2 | 1 | 3 |
| ICDAS 6 | 2 | 1 | 3 |
| Control (negative) | 3 | 0 | 3 |
| Total | 17 | 7 | 24 |

**Table ‎3.9** Number of samples in each ICDAS subgroup according to teeth variable.

### Procedure

The general outline of this experiment is illustrated in Figure 3.18. VectaFluor Double Labelling Kit reagents were equilibrated to room temperature. Samples were defrosted and washed in PBS for 5 minutes in multi-well (12-well) plastic plates. Antigen retrieval was carried out by heating citrate buffer in a microwave for 8 minutes. Samples were then washed and cooled in PBS for 5 minutes. Samples were placed on slides and incubated for 30 minutes with 100% normal horse serum to block non-specific protein binding. Excess serum was tipped off, and samples were incubated with the mouse MMP2 primary antibody (ab86607, Abcam) diluted in appropriate diluent (1:750) at 4℃ overnight in a humidified chamber. The following day, samples were washed with PBS twice before they were incubated with polyclonal rabbit MMP9 primary antibody (1:750) (ab74277, Abcam) at 4℃ overnight in a humidified chamber. From this point, all procedures were performed in the dark. Following further washes with PBS 2x5 minutes, samples were incubated with VectaFluor Duet Reagent for 30 minutes and washed twice with PBS for 5 minutes.

Samples were mounted in a media suitable for fluorescence, VectaShield Antifade Mounting Media (Cat#: H-1200; Vector Laboratories, Peterborough, UK), then left to air dry at room temperature for 24 hours in the dark and sealed with nail polish to immobilise the coverslip. The slides were stored in a light-tight box at 4℃ and analysed as soon as possible to reduce the chance of photo-bleaching. Images were taken using a fluorescence microscope (Carl Zeiss, Vistec Inc., Germany) and analysed with ImageJ software (Maryland, USA).

**Diagram

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**Figure 3.18** Schematic flow diagram to outline the main stages in the double staining immunofluorescence technique.

### Image analysis

#### **Image analysis software**

ImageJ is a public, open-source image processing programme developed at the National Institutes of Health (NIH) (Schneider et al., 2012). ImageJ allows the viewing, submission, and editing of biological images. It is widely used in scientific research because it provides a platform that supports many different types of microscopes and offers easy ways to measure and analyse images. ImageJ can calculate the area and pixel value statistics of user-defined selections and intensity-reduced objects. In this experiment, ImageJ was used to quantify the mean intensity of areas with positive expression.

#### **Field selection**

Specific areas of the image could be isolated for later analysis using the tool MACRO, which defined the area of interest (AOI) within the ImageJ software. Once created, an AOI can be saved and re-applied to subsequently captured images to ensure consistency of results (blind analysis).

#### **Field of interest**

Figure 3.19 demonstrates the five different fields employed for quantitative analysis, which are:

* Field 1: dentine-pulpal junction area
* Field 2: inner third of dentine
* Field 3: middle third of dentine
* Field 4: outer third of dentine
* Field 5: enamel-dentine junction area

Three readings were taken from each field of interest and the mean intensity for each field was calculated.

Diagram

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**Pulp**

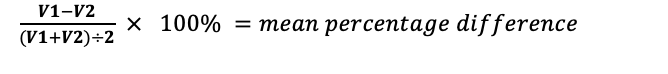
**Dentine**

**Enamel**

**Figure 3.19** Schematic diagram of the coronal dentine showing the five fields employed for the quantitative analysis. (A) field 1: dentine-pulpal junction area; (B) field 2: the inner third of dentine; (C) field 3: the middle third of dentine; (D) field 4: the outer third of dentine; (E)field 4: enamel-dentine junction.

#### **Reproducibility assessment**

The reproducibility of field selection and image analysis for the quantification of MMP9 in different fields of interest was measured 4 weeks after the first measurement by the same investigator on 7 captured images of tooth sections chosen randomly to represent different depths. In total, there were 32 different sets of 3 repeat measurements. Pearson correlation coefficients were calculated to determine the strength of agreement between the two measurements. In addition, the mean percentage difference between the initial measurement (v1) and the repeat measurement (v2) was determined as follows:



## Hydroxyproline assay

### Overview

The hydroxyproline (HYP) assay quantifies hydroxyproline amino acids in tissue lysates and biological fluids. The assay measures the percentage of solubilised collagen fragments in the tissue, reflecting the amount of collagen degradation based on the assumption that 90% of the dry mass of demineralised dentine contains type I collagen (Linde et al., 1980). In addition, hydroxyproline constitutes 9.6 mass% of type I collagen fibrils (Goldberg et al., 2011). The assay was performed using a Hydroxyproline Assay Kit (Colorimetric) (ab222941, Abcam) to evaluate the collagen degradation in caries lesions and incubation medium (saliva) and is based on the oxidation of HYP to a pyrrole intermediate and the reaction with Ehrlich’s reagent to form a brightly coloured chromophore to be detected at OD 560 nm. Table 3.10 provides details of all the reagents used in this assay. Table 3.11 shows the components of the HYP assay Kit.

**Table ‎3.10** Kit and Reagents used in hydroxyproline assay.

|  |  |
| --- | --- |
| **Material** | **Supplier** |
| 96 well plate | Greiner BIO-One, Stonehouse, UK |
| Concentrated hydrochloric acid (HCl),  10 N | Sigma Aldrich, Poole, UK |
| Hydroxyproline Assay Kit (Colorimetric) | ab222941, Abcam |
| Sodium hydroxide (NaOH) ,10 N | Sigma Aldrich, Poole, UK |

**Table ‎3.11** Component of hydroxyproline (HYP) assay Kit.

|  |  |
| --- | --- |
| **Product name** | **Volume** |
| Chloramine T concentrate | 600μL |
| Developer solution | 5mL |
| DMAB Concentrate | 5mL |
| Hydroxyproline standard | 100μL |
| Microplate sealing film | 1 unit |
| Oxidation buffer | 10 mL |

### Procedure

#### **Standard preparation**

Hydroxyproline standards were prepared by diluting 20μL of the 1 mg/mL hydroxyproline standard solution with 180μL of deionised water (Invitrogen, UK) to prepare 200μL of 0.1μg/μL standard. Using the standard solution, a standard curve was prepared at 0, 6, 12, 18, 24, 30μL to generate a corresponding standard of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 μg/well (0= blank) in duplicate.

#### **Sample preparation**

In this project, collagen degradation was measured in the following samples:

1. Tissue lysates of carious lesions with different ICDAS scores to correlate MMPs for each ICDAS score with collagen degradation.
2. Carious dentine blocks in incubation media (saliva) for different incubation times following the application of SDF+/- KI.

***(a) Tissue lysate of carious lesions***

Caries dentine blocks (n=15) were obtained from proximal coronal carious dentine in extracted human primary molars as described previously in section 3.3.3.1.

100μL of dH2O was added to each sample and homogenised using a pestle and mortar. 100μL of sample homogenate was transferred to a pressure-tight polypropylene vial and heated at 120℃ for 2 hours with 100μL of 10 N Sodium hydroxide (NaOH) (Sigma Aldrich, Poole, UK).

***(b)Saliva (incubation media)***

100μL of the incubation medium was collected from each incubation vial and transferred to a labelled polypropylene vial. 100μL of 10 N concentrated NaOH (Sigma Aldrich, Poole, UK) was added to each vial and hydrolysed at 120℃ for 1 hour.

From this point, both samples (carious lysate and saliva) followed the same procedure:

Following alkaline hydrolysis, vials were left to cool. Residual NaOH in each vial was neutralised by adding 100μL of 10 N concentrated hydrochloric acid (HCl) (Sigma Aldrich, Poole, UK). The vials were centrifuged (GenFuge, Progen, UK) at 10,000 x g for 5 minutes to pellet any insoluble debris following hydrolysis, and the supernatants were collected and transferred to newly labelled vials.

**3.7.3.3 Assay procedure**

An aliquot of 10μL from each vial and standard dilutions were transferred to a 96-well plate (Greiner BIO-One, Stonehouse, UK) and evaporated to dryness by heating the plate at 65℃ on a microplate incubator (JENCONS PLS, Bedforshire, UK) until a crystalline residue was formed. 100μL of oxidation solution was prepared by mixing 6μL of chloramine T concentrate and 94μL of oxidation buffer, and this was added to each well to incubate at room temperature for 20 minutes. To each well, 50μL of Developer was added and incubated for 5 minutes at 37℃, then 50μL of DMAB Concentrate was added and mixed thoroughly. The plate was sealed with a microplate sealer film and incubated at 65℃ for 45 minutes in the microplate incubator.

**3.7.3.4 Measurement**

The absorbance was measured at OD 560 nm using a spectrophotometer (Tecan, Switzerland). In order to obtain the corrected absorbance, the mean absorbance value of the blank (0) hydroxyproline standard was subtracted from all standards and sample readings. A pre-established calibration curve from a linear regression equation of the hydroxyproline absorbance against the hydroxyproline concentrations in the standards was obtained, and the hydroxyproline (μg/mL) was measured.

## Generic MMP activity assay

### Principle

The SensoLyte® Generic MMP Assay kit (Anaspec, San Jose, CA, USA) was used to detect the residual total MMP activity in carious dentine and measure the anti-MMP potential of SDF+/- KI. The assay involves incubating an MMP source with a chromogenic thiopeptide substrate cleaved by the MMPs to release a sulfhydryl group. This group reacts with 4,4’- dithiodipyridine or Ellman’s Reagent, a colour-developing thiol-reactive agent, and was detected at 412 nm using a microplate reader. Table 3.12 provides details of all the reagents and kits used in this assay. Table 3.13 shows the component of The SensoLyte® Generic MMP Assay Kit.

**Table ‎3.12** Kit and reagents used generic MMP assay.

|  |  |
| --- | --- |
| **Material** | **Supplier** |
| Deionised water | Invitrogen, UK |
| Human recombinant MMP9 (rh-MMP9) | (AS-55576-1, Anaspec, San Jose, CA, USA) |
| Riva Star | SDI, Bayswater, Victoria |
| SensoLyte® Generic MMP Assay kit | (Anaspec, San Jose, CA, USA) |

**Table ‎3.13** Component of The SensoLyte® Generic MMP Assay Kit.

|  |  |
| --- | --- |
| **Product name** | **volume** |
| Assay buffer | 20mL |
| MMP colorimetric substrate | 100μL |
| MMP inhibitor | 10μL |
| Reference standard | 10μL |
| Stop solution | 5mL |
| Trypsin | 100μL |
| Trypsin inhibitor | 100μL |

### Procedure

In this project, residual MMP activity in carious teeth using The SensoLyte® Generic MMP Assay kit was measured in the following:

1. Carious lesions before and after application of SDF +/- KI following three incubation periods in natural saliva: 1 week, 4 weeks and 12 weeks.
2. Carious lesions before and after different application times of SDF (1 minute or 3 minutes).

#### **Sample preparation**

Caries dentine blocks (n= 63) were obtained from proximal coronal carious dentine in extracted human primary molars with an ICDAS score of 5 and 6, as described in section 3.3.3.1. In this assay, carious dentine samples were used as the MMP source for the Generic Assay to detect the SDF effect, with and without the use of KI, on the activity of MMP and to detect the effect of different application times of SDF on MMP activity. The distribution of samples according to the purpose of the experiment is illustrated in Figure 3.5.

#### **Silver Diamine Fluoride (SDF) and Potassium Iodide (KI)**

The SDF and KI used in this project are available for clinical use and marketed as Riva Star (SDI, Bayswater, Victoria)**.** TheRiva Star system comprises two colour-coded capsules (Figure 3.20):

1. The silver capsule contains 38% SDF.
2. The green capsule contains a saturated solution of KI.

Application system (micro-brushes) is also supplied with the kit.

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**Figure 3.20** A photograph showing Riva Star product by SDI (SDI, Bayswater, Victoria).

#### **Preparation of positive control**

Human recombinant MMP9 (rh-MMP9) (AS-55576-1, Anaspec, San Jose, CA, USA) was used as the positive control to represent MMP found in human dentine. Rh-MMP was activated immediately before the experiment by adding 1μL of Trypsin (supplied in the kit) to 2μL of rh-MMP and incubated at 37℃ for 2 hours. Following incubation, 1μL of Trypsin inhibitor was added to the activated enzyme.

#### **Experimental treatment**

**Experiment 1**

The general outline of this experiment is illustrated in Figure 3.21. Residual MMP activity was measured in carious lesions before and after the application of SDF +/- KI. Three serial carious dentine blocks were used from each tooth in each comparison. A baseline reading of the enzyme activity was measured from the first dentine block. The 38% SDF from the silver capsule was applied to the second dentine block using the micro-brush available in the kit and left for 1 minute. The third block was treated with SDF for 1 minute, and then KI from the green capsule was applied using the micro-brush until the white precipitation stopped forming. The blocks were then dipped into wells that contained 250μL dH2O for two minutes and transferred to another well containing 250μL deionised water (Invitrogen, UK) for another two minutes to remove the absorbed SDF (Thompson et al., 2012).

The samples were incubated in natural saliva and divided into three groups according to the incubation period (4 teeth [n= 10 samples] per group). Each sample was placed in an ID-labelled vial. Each group contained 4 samples for a baseline reading, 4 samples treated with SDF, 4 samples treated with SDF + KI and 2 samples as a control.

1. Group 1: Incubation period for 1 week:
2. Group 2: incubation period for 4 weeks
3. Group 3: Incubation period for 12 weeks

Following each incubation period, the final reading of the residual total MMP activity was measured.

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**Figure 3.21** The general outline of the experiment to measure residual MMP activity in carious lesions before and after application of SDF +/- KI following three incubation periods in natural saliva: 1 week, 4 weeks and 12 weeks. (MMP= matrix metalloproteinases, SDF=Silver diamine fluoride, KI=Potassium Iodide).

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**Figure 3.22** Flow diagram to outline the main stages in experiment 1

**Experiment 2**

The general outline of this experiment is illustrated in Figure 3.26. Residual MMP activity was measured in carious lesions following different application times of SDF (1 minute and 3 minutes). Three serial carious dentine blocks were used from each tooth in each comparison. A baseline reading of the enzyme activity was measured from the first dentine block. The 38% SDF from the silver capsule was applied to the second dentine block using the micro-brush available in the kit and left for one minute. The third block was treated with 38% SDF for three minutes. The blocks were then dipped into wells containing 250μL dH2O for two minutes and transferred to another well containing 250μL deionized water for another two minutes to remove the absorbed SDF (Thompson et al., 2012).

The samples were divided into 3 groups according to the application time (5 teeth [n= 15 samples] per group). Each group contained 5 samples for a baseline reading, 5 samples treated with 38% SDF for 1 minute, 5 samples treated with 38% SDF for 3 minutes and 3 samples as a control. Following treatment with SDF, the final reading of the residual total MMP activity was measured.

**3.8.2.5. Assay procedure**

Chromogenic thiopeptide substrate solution was prepared by adding 100μL of MMP substrate and 4.9μL assay buffer (dilution of 1:50) supplied in the kit. Samples were placed in a 96-well plate and incubated in the substrate solution for 90 minutes at 37℃. They were then removed from the wells, and the substrate degraded by MMPs was measured at OD 412 nm using a spectrophotometer.

**Experiment 3:**

The aim of this experiment was to determine the agreement in MMP activity between serial blocks within each tooth. Three serial blocks from three teeth were assayed for basic activity using the Generic Assay so that the number of blocks was 9 (n=9).

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**Figure 3.23** Flow diagram to outline the main stages in experiment 2.

## Microhardness analysis

### Overview

The microhardness test is used to analyse the hardness of samples. In this study, the Vickers hardness tester (Foundrax, UK) was used (Figure 3.24). In this test, the surface of the sample is depressed by applying a certain force. This load is maintained for a certain time (in seconds). The resulting indentation is analysed with a calibrated optical microscope, and the hardness is calculated by measuring the dimensions of the two diagonals of the square indent.

|  |
| --- |
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**Figure 3.24** A photograph of Vickers Hardness Tester (Foundrax, UK).

### Procedure

In this project, surface micro-hardness was measured on the following samples:

1) Enamel from non-cavitated carious lesions (ICDAS 2) before and after the application of SDF +/- KI.

2) Enamel from non-cavitated carious lesions (ICDAS 2) and dentine adjacent to the lesion and compared to sound enamel (ICDAS 0) on the opposite proximal side of the tooth and dentine adjacent to the sound enamel. (Figure 3.28)

|  |
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**Figure 3.25** Schematic diagram demonstrating the area of microhardness analysis in experiment 2. (A) Sound enamel (ICDAS 0), (B) Enamel of non-cavitated carious lesions (ICDAS 2), (C) dentine bordering sound enamel, (D) dentine bordering non-cavitated lesion.

#### **Sample preparation**

Twelve tooth sections were obtained from 10 extracted human primary molars with an ICDAS score of 0 and 2. Two surfaces were analysed per section so that the total number of surfaces analysed was 24 (4 with an ICDAS score of 0 and 20 with an ICDAS score of 2). Sample preparation involved polishing the tooth surfaces with wet silicon carbide p1500. The quality of the polish was observed under a stereo microscope (Leica Microsystems Ltd. Germany) with a magnification of x15. (Figure 3.26)

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**Figure 3.26** Histological section of primary molar as examined under the stereomicroscope.

#### **Experiment procedure**

The general procedure of the experiment is shown in Figure 3.27. Each sample was placed on the Vickers microhardness tester table with the polished surface facing outwards and positioned under a diamond-tipped indenter and the calibrated optical microscope with integrated imaging. Once in position, the indenter was pressed into the surface. A load of 300g was pressed onto the sample with a dwell time of 11 s in the enamel (Sorkhdini et al., 2021) and 20s in the dentine (Topbaş et al., 2019). The hardness was expressed as Vickers hardness number (VHN). Six measurements were taken at different points on each sample so that the VHN of each sample was the mean of six impressions. The distance between indentations was greater than 100μm.

**Experiment 1**

Microhardness was measured on enamel surfaces with an ICDAS score of 2 before and after the application of SDF +/- KI. Ten surfaces were used for each comparison. An average baseline microhardness value was measured with a 300g load and a dwell time of 11s. Then 38% SDF from the silver capsule (SDI, Bayswater, Victoria) was applied to the same enamel surface using the micro-brush provided in the kit and left for 1 minute. Then the samples were immersed in wells containing 250μL of deionised water (Invitrogen, UK) until re-measured (Topbaş et al., 2019) (Figure 3.27). Further measurement of microhardness was taken alongside the basic indentation (six measurements) and recorded as the final VHN value. When comparing with the use of SDF+KI, the same steps were followed. After the baseline measurement, ten surfaces were treated with SDF (SDI, Bayswater, Victoria) and then KI (SDI, Bayswater, Victoria) was applied from the green capsule with the micro-brush until no white precipitate formed. The blocks were transferred to wells containing 250μL deionised water (Invitrogen, UK), and the final VHN was measured.

**Experiment 2**

In this experiment, the difference between the surface micro-hardness of non-cavitated caries in enamel and sound enamel was investigated. In addition, the difference between the surface micro-hardness of dentine adjacent to an enamel lesion and dentine adjacent to sound enamel was also investigated on eight surfaces (four surfaces with an ICDAS score of 0 and four with an ICDAS score of 2). The average micro-hardness value in enamel was measured with a load of 300g and a dwell time of 11s by averaging six readings on each surface. In dentine, the microhardness value was measured with a load of 300g and a dwell time of 20s.

**Diagram

Description automatically generated**

**Figure 3.27** Flow diagram to outline the main stages in micro-hardness experiments.

## Statistical analysis

All statistical tests were performed using GraphPad Prism version 8 software (GraphPad Software, San Diego, California, USA). Data were entered manually from the data collection sheet, and numbers were rounded to three decimal places.

Before starting the statistical analysis, the data were first checked for distribution (i.e., normality) using the Shapiro-Wilk normality test (Royston, 1995).

Statistical advice throughout the study was obtained from the Statistical Services Department at the University of Sheffield. These tests were used whenever possible as they are generally considered more satisfactory than non-parametric tests (Altman, 1991).

A one-way analysis of variance was used for the analysis of more than two groups. A two-way test (ANOVA) was used to determine the effect of two nominal predictor variables on a continuous outcome variable, followed by a Tukey test to determine if there were significant differences between groups (Girden, 1992). This statistical analysis was applied to the following measurements:

* Differences in MMP expression between different caries lesion stages and depth of the same lesion
* Differences in the amount of collagen degradation in each caries lesion as assessed by ICDAS
* Differences in MMP inhibition between SDF +/-KI
* Differences in the amount of collagen degradation after SDF +/-KI
* Differences in MMP inhibition between different SDF application times

**T-test was used for the analysis of the difference between two variables** (McDonald, 2009)**.** This statistical analysis was applied to the following measurements:

* Agreement of end-point values of MMP activity between serial carious dentine blocks.
* Differences in micro-hardness of non cavitated caries lesion before and after SDF treatment with and without KI.
* Differences in micro-hardness enamel surface of non-cavitated caries lesion and healthy enamel surfaces
* Differences in micro-hardness of dentine bordering healthy enamel and dentine bordering non-cavitated caries lesion

Pearson's correlation coefficients were used to measure how strong a relationship between two variables is (Mukaka, 2012). Table 3.14 shows the interpretation of the size of the correlation coefficient.

**Table ‎3.14** Interpreting the Size of a Correlation Coefficient (Mukaka, 2012).

| **Correlation size** | **Interpretation of results** |
| --- | --- |
| 0.90 to 1.00 | Very high positive (negative) correlation |
| 0.70 to 0.90 | High positive (negative) correlation |
| 0.50 to 0.70 | Moderate positive (negative) correlation |
| 0.30 to 0.50 | Low positive (negative) correlation |
| 0.00 to 0.30 | Negligible correlation |

Pearson correlation coefficients were used in the following measurements:

* Correlation between MMP expression and caries surface appearance as assessed by ICDAS.
* Correlation between MMP expression and the amount of collagen degradation in each caries lesion assessed by ICDAS.
* Correlation between MMP expression and diagnostic tests.
* Evaluation of the repeatability of fluorescence staining.

A p-value <0.05 was considered as statistically significant. Asterisks denote statistical significance (\*p <0.05; \*\*p <0.01; \*\*\*p <0.001; \*\*\*\*p <0.0001, and NS p >0.05).

# CHAPTER 4

OPTIMISATION OF IMMUNOHISTOCHEMICAL STAINING OF MMP IN CARIES

# Chapter 4: Optimisation of immunohistochemical staining of MMPs in caries

# 

## Optimisation protocol for MMP immunohistochemistry staining

Samples of carious teeth were sectioned and prepared for immunohistochemical localisation of MMP9. Sections of varying thicknesses were stained with varying antibody concentrations in order to determine optimal experimental conditions. The immunohistochemical localisation of MMP9 on teeth sections was successful (Figures 4.1, 4.2, 4.3 and 4.4).

### Concentration of MMP9 antibody

Dilutions of MMP9 antibody from 1:100 to 1:1000 were prepared and explored to determine the optimal concentration. Following several trials, a dilution of 1:750 was found to give the best results.

Figures 4.1, 4.2, 4.3, and 4.4 are light micrographs of tooth sections following immunohistochemistry with MMP9 using different antibody concentrations. Positive staining ranged from very intense staining (1:100) to very faint staining (1:1000).

### Thickness of sample sections

The thickness of the sample did not affect the success of the staining; however, it did affect our ability to focus the section under the light microscope. Figure 4.5 shows light microscope images of tooth sections of different thicknesses. Figure 4.5(a) shows 350µm thick sections that did not allow clear focusing. Figure 4.5(b) shows a 150µm thick section that we found acceptable and could be focused under the microscope.

**a**

Dentine-Pulp

Junction

Dentine

**b**

Dentine

**Caries lesion**

Dentine-Pulp Junction

**Figure 0.1** Light microscope images of tooth sections after immunohistochemistry with different concentrations of MMP9 antibody: (a) 1:100 (b) 1:200. The oblique arrow indicates positive staining of MMP9. Both dilutions resulted in very intense staining.

**a**

Dentine-Pulp

Junction

Dentine

**b**

Dentine Caries lesion

Dentine

**Figure 0.2** Light microscope images of tooth sections after immunohistochemistry with different concentrations of MMP9 antibody: (a) 1:300, (b) 1:500. The oblique arrow indicates positive staining of MMP9. Both dilutions resulted in very intense staining.

Dentine Caries lesion

Dentine

|  |
| --- |
| \\stfdata06\home\MD\Mdp13lme\ManW10\Desktop\confirmation review\results picturs for confirmation review\750 in caries.jpg  **a** |

Close-up of a person's skin

Description automatically generated with medium confidence

Dentine

Dentine-Pulp

Junction

**b**

**Figure 0.3** Light micrographs of tooth sections after immunohistochemistry with an MMP9 antibody concentration of 1:750 (a) dentine at 10x magnification and (c) dentine-pulp junction at 10x magnification. The oblique arrow indicates positive staining of MMP9. A dilution of 1:750 was determined to give the best results.

**a**

Dentine

Dentine Caries lesion

**b**

Dentine

Dentine-Pulpal Junction

**Figure 0.4** Light microscope images of tooth sections after immunohistochemistry with a 1:1000 concentration of MMP9 antibody: (a) carious dentine, (b) at the dentine-pulp junction. The oblique arrow indicates positive staining of MMP9. A dilution of 1:1000 resulted in very weak staining.

**a**

Dentine-Pulpal Junction

Dentine

|  |
| --- |
| \\stfdata06\home\MD\Mdp13lme\ManW10\Desktop\confirmation review\results picturs for confirmation review\500,,,.jpg  **b** |

Dentine-Pulpal Junction

Dentine

**Figure 0.5** Light microscope images of tooth sections after immunohistochemistry of tooth sections of differing thicknesses: (a) 350 µm, (b) 150 µm. Sections 150 µm thick produced acceptable images and allowed good focusing under the microscope.

### Evaluation of immunohistochemistry results

Staining was classified as either positive or negative, with the brown chromogen, DAB, defining positive staining. The overall quality of immunostaining was very good throughout the study.

#### **Control**

No immunohistochemical staining was observed in controls, confirming that no non-specific staining or cross-reaction had occurred (Figures 4.6 and 4.7).

#### **Immunohistochemical staining results**

Examination of the immunohistochemical staining with a light microscope revealed that MMP9 was successfully stained in the dentine caries lesion (Figure 4.2b and 4.3b) and at the dentine-pulp junction (Figure 4.3b and 4.4b).

At the dentine-pulp junction, also referred to as odontoblasts in this thesis, MMP9 was stained regardless of the presence of a caries lesion. However, the intensity of the staining differed depending on the presence/absence of caries. The staining was more intense in the odontoblast region where caries lesions were present, while it appeared less intense when no caries lesions were present. Figure 4.8 shows the differences in staining intensity between carious and non-carious areas in the same tooth section.

Dentine Caries lesion

Dentine

|  |
| --- |
| E:\immunostaining pictures 1\control.jpg |

**Figure 0.6** Light microscope image of a tooth section (control) after the immunohistochemical examination. No positive immunohistochemical staining was detected in the dentine caries lesion.

Dentine-Pulpal Junction

Dentine

|  |
| --- |
| **A close up of a person's hair  Description automatically generated with medium confidence** |

**Figure 0.7** Light microscope image of a tooth section (control) after the immunohistochemical examination. No positive immunohistochemical staining was detected at the dentine-pulp junction.

|  |
| --- |
| ***A white garlic on a black surface  Description automatically generated with low confidence***  Dentine-pulp junction  **a** |

**cc**

**b**

**Dentine**

**Dentine**

**Dentine-pulp junction**

**Dentine-pulp junction**

**Figure 0.8** (a) Photographic image of a tooth section with caries lesions and the corresponding microscopic immunostaining images of MMP9 at the dentine-pulp junction at 10x magnification showing the differences in staining intensity between (b) the carious side and (c) the non-carious side of the same tooth section. The oblique arrow indicates positive staining of MMP9.

### Immunohistochemical analysis of MMP2 and MMP9 in tooth sections with carious lesions evaluated according to ICDAS score

Immunohistochemical staining of MMP2 and MMP9 was performed in tooth sections with ICDAS 0 (representing healthy teeth) and ICDAS 5 (clinically representing a cavity with visible dentine). The staining of MMP2 and MMP9 was examined in the dentine and the area of the dentine-pulp junction.

In sections with an ICDAS score of 0, immunohistochemical staining showed that MMP2 was not observed in dentine. In contrast, MMP2 positively stained the dentine-pulp junction (Figure 4.9). In a caries lesion with an ICDAS 5 score, MMP2 staining was seen throughout the dentine of the caries lesion, with higher intensity at the outer surface of the lesion. Increased expression of MMP2 was observed at the dentine-pulp junction (Figure 4.10).

Immunostaining of MMP9 showed a similar trend to that observed for MMP2 in sections with an ICDAS score of 0, with no staining in the dentine and positive staining at the dentine-pulp junction (Figure 4.11). In a caries lesion with an ICDAS score of 5, MMP9 was observed in the caries lesion with increased intensity in the outer layer of caries. MMP9 was highly expressed at the dentine-pulp junction and was detected in the deep layer of dentine, but staining became weaker toward the inner and mid-coronal dentine (Figure 4.12).

|  |  |
| --- | --- |
| **A picture containing mollusk  Description automatically generated**  **a**  Dentine  Enamel  Dentine-pulp junction | |
| **A close-up of a person's skin  Description automatically generated with low confidence**  **b**  Dentine  Enamel | **A close-up of a foot  Description automatically generated with medium confidence**  **cc**  Dentine-pulp junction  Dentine |

**Figure 0.9** (a) Photographic image of a tooth section with ICDAS score 0, caries lesions and the corresponding microscopic images of immunostaining of MMP2 in (b) dentine at 4x magnification and (c) dentine-pulp junction at 10x magnification. MMP2 staining was seen throughout the dentine-pulp junction. No MMP staining was seen in the dentine. The angled arrows indicate positive staining of MMP2.

|  |  |
| --- | --- |
| **a**  Enamel  Dentine  Dentine caries lesion  Dentine-pulp junction | |
| \\stfdata06\home\MD\Mdp13lme\ManW10\Desktop\confirmation review\results picturs for confirmation review\750 caies.jpg  **b**  Enamel  Dentine caries lesion  Dentine | Dentine  \\stfdata06\home\MD\Mdp13lme\ManW10\Desktop\confirmation review\results picturs for confirmation review\750 caries.jpg  **cc**  Dentine-pulp junction |

**Figure 0.10** (a) Photographic image of a tooth section with ICDAS score 5, caries lesions and the corresponding microscopic images of immunostaining of MMP2 in (b) dentine at 4x magnification and (c) dentine-pulp junction at 10x magnification. MMP2 staining was seen throughout the carious lesion in dentine, with intense staining in the outer third of the lesion. Positive MMP2 staining was seen at the dentine-pulp junction. The angled arrows indicate positive staining of MMP2.

|  |  |
| --- | --- |
| **A white paper on a black surface  Description automatically generated with low confidence**  **a**  Enamel  Dentine  Dentine-pulp junction | |
| **A picture containing close  Description automatically generated**  **b**  Enamel  Dentine | **A close-up of a waterfall  Description automatically generated with low confidence**  **cc**  Dentine  Dentine-pulp junction |

**Figure 0.11** (a) Photographic image of a tooth section ICDAS score 0, caries lesions and the corresponding microscopic images of immunostaining of MMP9 in (b) dentine at 4x magnification and (c) dentine-pulp junction at 10x magnification. MMP9 staining was seen throughout the dentine-pulp junction. No MMP staining was seen in the dentine. The oblique arrows indicate positive staining of MMP9.

|  |  |
| --- | --- |
| **A white paper on a black surface  Description automatically generated with low confidence**  **a**  Dentine caries lesion  Dentine  Dentine-pulp junction | |
| **A close-up of a person's skin  Description automatically generated with low confidence**  **b**  Dentine  Dentine caries lesion | **c**  Dentine  Dentine-pulp junction |

**Figure 0.12** (a) Photographic image of a tooth section with ICDAS score 5, caries lesions and the corresponding microscopic images of immunostaining of MMP9 in (b) dentine at 10× magnification and (c) dentine-pulp junction at 10× magnification MMP9 staining was seen throughout the carious lesion in dentine, with intense staining in the outer third of the lesion. Positive staining of MMP9 was seen at the dentine-pulp junction. The angled arrows indicate positive staining of MMP9.

### Co-localisation of MMP2 and MMP9 with the double staining technique

Double immunohistochemical staining showed that MMP2 co-localised with MMP9 in sections with ICDAS scores of 0 and 5. At ICDAS 0, both MMPs were co-localised at the dentine-pulp junction, while no staining was seen in the dentine (Figure 4.14). In ICDAS 5, they were co-localised in carious lesions in dentine and at the dentine-pulp junction (Figure 4.15). The positive staining of MMP2 and MMP9 overlapped, so a clear localisation of the individual MMPs was not possible.

  To investigate the co-localisation of MMP2 and MMP9 using double immunofluorescent staining which would allow each MMP to be examined individually in a separate channel.  Sections with ICDAS 5 caries lesions demonstrated overlapping MMP expression in the caries lesions and at the dentine-pulp junction (Figure 4.16).

|  |  |
| --- | --- |
| **A picture containing white  Description automatically generated**  **a**  Dentine-pulp junction  Enamel  Dentine | |
| **A close-up of a book  Description automatically generated with low confidence**  **b**  Enamel  Dentine | Dentine    **c**  Dentine-pulp junction |

**Figure 0.13** (a) Photographic image of a tooth section with ICDAS score 0 caries lesions and the corresponding microscope double immunostaining of MMP2 and MMP9 in (b) dentine at 4x magnification and (c) dentine-pulp junction at 10x magnification. The double staining of MMP2 and MMP9 was seen throughout the dentine-pulp junction. No positive staining of either enzyme was seen in the dentine. The **black** angled arrows indicate positive staining of MMP2, and the **red** angled arrows indicate positive staining of MMP9.

|  |  |
| --- | --- |
| **A picture containing white  Description automatically generated**  **a**  Dentine  Dentine caries lesion  Dentine-pulp junction | |
| **A picture containing indoor  Description automatically generated**  **b**  Dentine  Dentine caries lesion | **A picture containing half  Description automatically generated**  **c**  Dentine-pulp junction  Dentine |

**Figure 0.14** (a) Photographic image of a tooth section with ICDAS score 5 caries lesions and the corresponding microscopic double immunostaining of MMP2 and MMP9 in (b) dentine at 4x magnification and (c) dentine-pulp junction at 10x magnification. Double staining of MMP2 and MMP9 was seen throughout the carious lesion in dentine, with intense staining in the outer carious lesion. Positive staining of both is seen at the dentine-pulp junction. The **black** angled arrows indicate positive staining of MMP2, and the **red** angled arrows indicate positive staining of MMP9.

|  |  |
| --- | --- |
| **A white object on a black surface  Description automatically generated with low confidence**  Dentine-pulp junction  Dentine caries lesion  Dentine  **a** | |
| **A green light in the dark  Description automatically generated with low confidence**  **b** | **c** |
| **Red lights in the dark  Description automatically generated with medium confidence**  **d** | **A red light in the dark  Description automatically generated with low confidence**  **e** |
| **A picture containing light, colorful, laser  Description automatically generated**  **f** | **g** |

**Figure 0.15** (a) Photographic image of tooth section with ICDAS 5 caries lesion and the corresponding fluorescence microscopic images of MMP2 (red) and MMP9 (green) in (b, d) dentine and (c, e) dentine-pulp junction. The colocalization of MMP2 and MMP9 is shown in (f) dentine and (g) dentine-pulp junction. (Magnification x10).

## Discussion

### Experimental approach

Albert Coons first described immunostaining in 1941 as an antibody-based technique for detecting a specific protein in any sample (Coons et al., 1941). Immunohistochemistry is now the most widely used immunostaining technique. It is used in diagnosis and research to understand the precise localisation and distribution of expressed proteins in biological tissues (Ramos-Vara and Miller, 2014). The aim of this experiment was to localise MMPs in carious dentine. Many types of MMPs are of interest, however, this experiment aimed to develop a successful protocol for MMP staining by using a protein, MMP9, that has already been confirmed to be present in teeth with and without caries.  The protocol can then be used to identify other MMPs.

  Various techniques have been used to study MMPs in dental tissues, such as zymography (Goldberg et al., 2009; Mazzoni et al., 2010; Nui et al., 2011; Mazzoni et al., 2018), ELISA (Nui et al., 2011), scanning electron microscopy (Mazzoni et al., 2007; Shimada et al., 2009 Mazzoni et al., 2018) and immunofluorescence (Toledano et al., 2010; Vidal et al., 2014). Zymography measures the activity of proteolytic enzymes (Leber and Balkwill, 1997), but requires that the dentine is in powder form to measure activity. Although this technique is effective, it does not allow localisation of the MMPs in the tooth section. ELISA can be used to investigate the concentration of the target enzyme, however, obtaining the protein extracts from the tissue is necessary, and localisation of the MMPs in the tooth section is again impossible. Therefore, in our study, immunostaining proved to be the most suitable method for localising MMPs in tooth sections representing varying degrees of caries severity.

  Caries lesions develop over months and even years, undergoing many episodes of demineralisation and remineralisation. Demineralisation of healthy dentine *in vitro* to produce carious lesions has been used in several studies to investigate the function of enzymes. However, demineralised healthy dentine does not mimic the natural progression of a carious lesion, especially the time factor and the role of host factors such as saliva, on the action of collagenolytic enzymes in caries (Tjäderhane et al., 2015).

  In our study, natural carious dentine was used, which provides a more detailed and accurate picture of MMPs and their role in caries development. Previous protocols for staining MMPs in dentine included decalcification steps prior to staining. This may not reflect *in vivo* conditions and may limit the information provided (Mazzoni et al., 2009). Decalcification leads to the denaturation of proteins, which could decrease an enzyme's activity (Mazzoni et al., 2009). For example, EDTA was used in some studies as a decalcifying agent since this solution has a limited denaturing effect on organic structures. However, as decalcification with EDTA requires a longer exposure time, this could still reduce immunostaining (Arana-Chavez and Nanci, 2001). In addition, it has already been reported that some MMPs are soluble in decalcifying EDTA solution and would be lost (Sulkala et al., 2007b; Mazzoni et al., 2009). We prepared the tooth sections without decalcification to investigate the localisation of MMP2 and MMP9 in healthy and carious dentine, avoiding protein loss or histological disruption.

  The main advantage of the decalcification step is that very thin sections (i.e., 4-10 µm) of a tooth can be cut and mounted on slides. This allows better handling of the samples during the immunostaining steps and better imaging under the microscope. Developing a successful immunostaining protocol without decalcification of the samples introduced some particular challenges.

  The first challenge was the sectioning step. Since the decalcification step was excluded from the protocol, the thinnest cut that gave acceptable results was 150µm. The thinner, the more the tooth section falls apart or turns to powder. In addition, the caries lesion was often no longer visible in the sample.

  The next challenge was handling the samples during the immunostaining procedure, as they were not fixed on slides. This procedure was modified using multiwell plates and glass flasks, as discussed in detail in section 3.4.3.

 Although the development of this protocol was challenging, the staining of MMP9 in healthy and carious tooth sections was successful. Due to the limited number of samples available for our study, it was not possible to include all ICDAS scores and so six tooth sections with an ICDAS score of 0, representing healthy teeth, and six tooth sections with an ICDAS score of 5, representing carious lesions (Ismail et al., 2007), were subjected to the protocol. MMP2 and MMP9 were examined in triplicate at the two ICDAS scores.

The gelatinolytic enzymes (MMP2 and MMP9) were the enzymes of interest in our study, as they have been shown to be actively associated with the progression of caries through the degradation of the collagen matrix (Visse and Nagase, 2003) and to affect the interface formed by resin restorative materials when they bond with dentine (Toledano et al., 2010; Apolonio et al., 2017).

### Immunohistochemistry results

In our preliminary protocol study, immunohistochemical staining showed that MMP9 was highly concentrated in the odontoblasts and throughout the caries lesion. The intensity of MMP9 staining in the odontoblasts differed according to the presence of caries. The staining was more intense in the odontoblasts when caries lesions were present, while it appeared less intense when no caries lesion was present. This may be related to the role of this endogenous enzyme in the development of the carious lesion (Leo Tjäderhane et al., 1998). This also suggests that the acidic pH environment that occurs in dental caries induces the production and activation of MMPs (Charadram et al., 2012).

  The results of our study confirm the presence of MMP2 and MMP9 in primary human teeth by immunohistochemistry in healthy and carious teeth. In healthy tooth sections with an ICDAS score of 0, positive staining of MMP2 and MMP9 was detected in the odontoblast region. This is consistent with a previous study in which MMP2 and MMP9 were investigated in healthy human permanent third molars by immunohistochemistry (Niu et al., 2011).

  In tooth sections with an ICDAS score of 5, MMP2 stained positively in caries lesions and odontoblasts. These results are consistent with another study that examined the distribution of MMP2 in caries lesions and found positive staining in caries lesions and odontoblasts (Boushell et al., 2011). However, it is worth noting that Boushell et al used a decalcification step prior to immunostaining and the teeth examined were permanent teeth. Furthermore, the clinical stage of the dentine caries lesion was not considered.

  Our study observed similar results in sections stained with MMP9, where the caries lesion and odontoblasts stained positively. This is in line with previous studies in which MMP9 was detected in its active form in dentine extracts of various carious teeth (Tjaderhane et al., 1998). In our study, we found that the staining of MMP9 in caries lesions was more intense in the superficial layer of caries. The intensity decreased in the mid-coronal dentine to the dentine-pulp junction, where the intensity was more noticeable. This is consistent with a study by Shimada et al. (2009), who investigated the localisation of MMP9 in carious dentine using immunogold labelling. They found that MMP9 labelling was higher in superficial caries than in deep caries lesions. However, the stage of the caries lesion they examined is unclear. Furthermore, the study was conducted on permanent teeth. The increased intensity of MMP9 in the external caries lesion could be explained by the fact that saliva contains several MMPs capable of degrading the collagen matrix in dentine, MMP9 being the predominant enzyme as it is secreted by the salivary glands and found in the GCF (Tjaderhane et al., 1999). Tjaderhane et al (1998) evaluated the degradation activity of the sterilised salivary hosts MMP2, MMP9 and MMP8 on demineralised human dentine and found that these enzymes degraded demineralised organic dentine matrix in saliva in vitro. This suggests that salivary MMP9 may indeed be involved in the pathogenesis of dental caries.

  Another finding of our study was that MMP9 was detected in the deep layer of dentine near the dentine-pulp junction, but the staining became weaker towards the inner and middle dentine. This was previously explained when Mazzoni et al. (2009) demonstrated MMP9 to be an intrinsic component of the fibrillar network of the organic matrix in human dentine (Mazzoni et al., 2009). Odontoblasts synthesise MMP9 after tooth formation is complete (Tjaderhane et al., 1998). MMP9 production is thought to be primarily related to the formation of pre-dentine. Once secreted by the odontoblasts, the protein diffuses into the dentine matrix and becomes tightly bound to the collagen in the dentine matrix (Boushell et al., 2008).

### Co-localisation of MMP2 and MMP9 with the double staining technique

Immunohistochemical double staining allows the detection of two different antigens in one tissue sample. An advantage of IHC double staining is that this technique can save tissue samples and time (Chen et al., 2010). Due to the limited number of samples, double staining of the MMP2 and MMP9 in the same tooth section was investigated.

  The double immunohistochemical staining technique successfully co-localised MMP2 and MMP9, showing that both MMPs were present in the caries lesion and at the dentine-pulp junction. However, the examination and analysis of each MMP were impossible because the two MMPs overlapped, and it is not possible to separate the staining, or localisation, of each enzyme. In order to better understand the staining pattern of the two MMPs we used immunofluorescence.

  Double immunofluorescence staining allows the detection of two biomarkers in the same sample. Reliable and reproducible results can therefore be obtained in tissue sections where the two target antigens overlap (co-localise) in the same structure. In our study, MMP2 and MMP9 were positively stained in the caries lesion and at the dentine-pulp junction using double immunofluorescence staining. This technique allowed us to study the co-localisation of the two MMPs and to examine each MMP in a separate channel under the fluorescence microscope. Therefore, this technique proved to be the most effective staining protocol to achieve the objectives of this study.

## Summary

* MMP2 and MMP9 were detected in odontoblasts in carious and non-carious teeth with increased intensity in odontoblasts from carious lesions.
* MMP2 and MMP9 were detected in the carious dentine of primary teeth.
* Double immunohistochemical staining was successful in co-localising MMP2 and MMP9. However, it was not possible to examine each MMP individually as the two MMPs overlapped in the tooth tissue.
* Immunocytochemistry using the immunofluorescence technique proved to be the most appropriate method to double stain and co-localise MMP2 and MMP9 without overlap. The application and results of this technique in staining MMP2 and MMP9 were discussed in detail in Chapter 5.

# CHAPTER 5

CORRELATION BETWEEN MMP PRESENCE AND CARIES SURFACE APPEARANCE

# Chapter 5: Correlation between MMP presence and caries surface appearance

## Introduction

Dental caries is a common global disease that negatively affects the quality of life. It is caused by cariogenic bacteria that ferment carbohydrates from food, releasing lactic acid that dissolves tooth enamel and dentine. Matrix metalloproteinases (MMPs) play a key role in the degradation of the collagen matrix, which compromises remineralisation, and several MMPs have been identified in carious lesions. The correct diagnosis of caries and its stage is crucial for prevention and management. The International Caries Detection and Assessment System (ICDAS) was developed to provide an evidence-based system for caries diagnosis and assessment. The aim of this research project is to investigate the presence of gelatinolytic MMPs in carious dentine at different caries stages and depths in human primary teeth, as well as the correlation between MMPs and the appearance of caries' surface, as recorded by ICDAS. The ultimate goal is to improve diagnosis and personalised care planning for dental caries.

## Aim and objectives

The aim of the present study was to investigate the localisation of Gelatinolytic MMPs (MMP2 and MMP9) and their correlation with caries lesions assessed by the ICDAS score. The specific objectives were as follows:

* To identify and characterise the location and distribution of MMP2 and MMP9 at different depths of human primary carious dentine.
* Correlate expression of MMPs and carious lesions as assessed by ICDAS scores.
* Quantify the amount of denatured collagen in dentine with varying degrees of caries severity as assessed by ICDAS.
* Relate MMP expression to the degree of collagen degradation in caries lesions of different ICDAS scores.
* To investigate whether the MMPs in caries lesions show a correlation with diagnostic tests: laser fluorescence pen, histological score, and radiological scores.

### Null hypothesis

Matrix Metalloproteinases do not correlate with the stage of caries and do not differ with the depth of caries.

## Methods

Details regarding the overall experiments carried out have been previously outlined in the Material and Method Chapter 3. Sample preparation was outlined in section 3.3.2. The staining technique used in this experiment was the immunofluorescence technique which has been explained in detail in section 3.6. The HYP assay was used to quantify any denatured collagen. Sample preparation for this assay is described in section 3.3.3.1, and the detailed protocol for this experiment is outlined in section 3.7.

## Statistical analysis

A one-way ANOVA test was used to determine the effect of two nominal predicated variables on a continuous outcome variable.

Pearson correlation was used to measure the correlation between two variables.

A p-value <0.05 was considered statistically significant. Asterisks denote statistical significance (\*P <0.05; \*\*P <0.01; \*\*\*P <0.001; \*\*\*\*P <0.0001, and NS P >0.05).

## Results

### Evaluation of Immunofluorescence results

#### **Controls**

***Control of the endogenous tissue background***

  No endogenous background staining was found in the sections which confirm that no signal originated from the tooth tissue itself.

***Negative / No-primary antibody control***

The control sections showed no immunoreactivity staining in the "no antibody control" (Figure 5.1), confirming that no cross-reactions had occurred.

#### **Quality of staining**

The overall quality of immunostaining was excellent throughout the study. Two sections, one from a caries lesion with ICDAS 2 and one with ICDAS 5, were of poor quality and had very weak staining. These sections were replaced by two spare tooth sections from the same ICDAS severity score.

|  |  |
| --- | --- |
| **(a)**  A white object on a black surface  Description automatically generated with low confidence | **(b)**  A picture containing green  Description automatically generated |
| **(c)** |

**Figure 5.1** (a) Photographic image of tooth section with caries lesions and the corresponding fluorescence microscopic images of (b) MMP9 and (c) MMP2 controls with no specific labelling (magnification x10).

#### **Reproducibility assessment of the quantification of the mean intensity of areas with positive expression**

The data for the initial and repeat measurements of MMP9 are shown in Table 5.1. One section of each caries lesion recorded with each ICDAS score was re-analysed, and the results are presented separately for each of the five different fields of analysis. The mean percentage difference and Pearson correlation coefficient between the initial and repeat measurements are also given. Overall, it can be seen that there were only minor differences between the two measurements. These differences ranged from 0.15% to 4.59%. In general, reproducibility appeared to be optimal in the analysis of field 1 and the poorest reproducibility was found in the analysis of field 3.

**Table ‎5.1** data for initial and repeat measurements of the mean intensity of areas with positive expression according to ICDAS score and field.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Caries lesion assessed by ICDAS/** Field | | Initial intensity mean (±SD) | repeat intensity mean (±SD) | % Mean difference | Pearson correlation coefficient |
| **ICDAS 0** | Field 1  Field 2  Field 3  Field 4  Field 5 | 2.695 (±0.23)  3.426 (±0.41)  2.829 (±0.19)  1.641 (±0.56)  4.137 (±1.01) | 2.724 (±0.31)  3.401 (±0.45)  2.702 (±0.31)  1.652 (±0.41)  4.213 (±1.21) | ±1.07%  ±0.73%  ±4.59%  ±0.65%  ±1.82% | 0.81  0.92  0.98  0.90  0.96 |
| **ICDAS 1** | Field 1  Field 2  Field 3  Field 4  Field 5 | 3.635 (±0.32)  3.016 (±0.24)  3.029 (±0.52)  1.701 (±0.14)  4.437 (±0.24) | 3.651 (±0.35)  3.125 (±0.23)  3.103 (±0.45)  1.721 (±0.18)  4.412 (±0.23) | ±0.44%  ±3.54%  ±2.41%  ±1.72%  ±0.56% | 0.76  0.98  0.98  0.87  0.98 |
| **ICDAS 2** | Field 1  Field 2  Field 3  Field 4  Field 5 | 4.321 (±0.55)  3.332 (±0.26)  2.312 (±0.16)  1.231 (±0.03)  3.432 (±0.24) | 4.392 (±0.45)  3.287 (±0.21)  2.318 (±0.12)  1.233 (±0.08)  3.398 (±0.22) | ±1.62%  ±1.35%  ±0.25%  ±0.16%  ±0.99% | 0.96  0.98  0.96  0.99  0.94 |
| **ICDAS 3** | Field 1  Field 2  Field 3  Field 4  Field 5 | 29.845 (±2.57)  9.762 (±1.35)  3.756 (±0.14)  7.654 (±0.92)  13.634 (±1.04) | 29.712(±2.34)  9.612 (±1.39)  3.750 (±0.12)  7.561 (±0.84)  13.331(±1.10) | ±0.44%  ±1.54%  ±0.15%  ±1.22%  ±2.24% | 0.97  0.93  0.99  0.98  0.98 |
| **ICDAS 4** | Field 1  Field 2  Field 3  Field 4  Field 5 | 50.664 (±2.65)  58.712 (±2.98)  60.299 (±3.17)  20.865 (±1.62)  40.251 (±2.12) | 51.042(±2.42)  57.413(±2.21)  62.019(±3.20)  20.417(±1.41)  40.982(±2.32) | ±0.74%  ±2.23%  ±2.81%  ±2.17%  ±1.79% | 0.97  0.92  0.80  0.88  0.81 |
| **ICDAS 5** | Field 2  Field 3  Field 4  Field 5 | 67.883 (±3.18)  67.387 (±2.98)  25.774 (±1.18)  34.385 (±1.92) | 66.234 (±3.28)  68.121 (±2.28)  25.164 (±1.31)  34.911 (±1.41) | ±2.45%  ±1.89%  ±2.39%  ±1.51% | 0.97  0.97  0.98  0.91 |
| **ICDAS 6** | Field 2  Field 3  Field 4  Field 5 | 74.522 (±3.12)  68.771 (±3.01)  22.871 (±2.19)  35.775 (±1.99) | 73.422 (±2.98)  67.211 (±3.21)  22.412 (±2.20)  34.995 (±1.48) | ±1.48%  ±2.29%  ±2.02%  ±2.21% | 0.87  0.89  0.93  0.97 |

### Presence of MMP2 and MMP9 in the dentine of carious lesions and their expression in odontoblasts at each ICDAS score

Staining was assessed for positivity and intensity in the caries lesions and odontoblasts, with the presence of fluorescence defining positive staining. Table 5.2 summarises the results of MMP2 and MMP9 in the dentine and Dentine-pulpal region according to positivity in each caries lesion assessed by ICDAS.

**Table ‎5.2** immunolabelling results for MMP2 and MMP9 in dentine and Dentine-pulpal region according to positivity in each cares lesion assessed by ICDAS.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Caries lesion assessed by ICDAS** | **Presence of MMPs in**  **dentine** | | **Expression of MMPs in dentine-pulpal region** | |
| MMP2 | MMP9 | MMP2 | MMP9 |
| ICDAS 0 | Close with solid fill | Close with solid fill | Tick with solid fill | Tick with solid fill |
| ICDAS 1 | Close with solid fill | Close with solid fill | Tick with solid fill | Tick with solid fill |
| ICDAS 2 | Close with solid fill | Close with solid fill | Tick with solid fill | Tick with solid fill |
| ICDAS 3 | Tick with solid fill | Tick with solid fill | Tick with solid fill+ | Tick with solid fill+ |
| ICDAS 4 | **Tick with solid fill**++ | Tick with solid fill++ | Tick with solid fill++ | Tick with solid fill++ |
| ICDAS 5 | Tick with solid fill++ | Tick with solid fill++ | **Tick with solid fill**++ | Tick with solid fill++ |
| ICDAS 6 | Tick with solid fill++ | Tick with solid fill++ | Tick with solid fill++ | Tick with solid fill++ |

The intensity of the staining was measured with the software ImageJ, as explained in section 3.6.5. Tables 5.3 and 5.4 show the mean (SD) intensity of positive staining of MMP2 and MMP9 in each tooth section according to the stage of caries assessed by ICDAS and the depth of the lesion.

   Immunofluorescence examination of human primary tooth tissue showed that MMP2 and MMP9 were present in both sound and carious dentine. However, immunoreactivity varied significantly within the dentine region and with the presence of caries. Furthermore, although with varying intensity, immunoreactivity was predominantly observed in the odontoblasts regardless of the presence of a carious lesion.

  Regarding the type of MMPs, relatively similar immunoreactivity was observed for MMP2 and MMP9 in all tooth sections; although MMP2 showed weaker staining in all sections, the difference was not significant (p= 0.998).

  In sections with an ICDAS score of 0, representing a sound tooth, very low immunoreactivity was observed for MMP2 (1.484±1.092) and MMP9 (3.853±1.492) in the dentine and at the enamel-dentine junction. In contrast, their expression (4.627±0.867, 8.588±0.277 respectively) was more intense in odontoblasts at the dentine-pulpal junction region (Figure 5.2). A similar trend of very low MMP2 and MMP9 immunoreactivity was observed in dentine (1.486±1.874, 4.290±1.610 respectively) and at the dentine-pulp junction (6.381±3.684, 9.962±0.8883 respectively) in tooth sections with an ICDAS score of 1, representing the first visible change in enamel after air-drying (Figure 5.3).

  In sections with caries lesions scored ICDAS 2, representing a distinct visual change in enamel without air-drying; the immunoreactivity of both MMPs in the dentine was similar to ICDAS 0 and 1; however, the noticeable intensity in MMP2 and MMP9 (5.022±1.493, 8.675±0.568 respectively) was observed in the odontoblasts when compared to dentine (P<0.001). This indicates a very early response of the dentine pulp complex to early caries lesions (Figure 5.4).

  In the tooth sections with microcavities (ICDAS 3), representing localised enamel breakdown with no visible dentine, immunoreactivity of MMP2 and MMP9 was seen throughout the outer third of the dentine (8.227±3.214, 16.04±9.820 respectively), with more intense staining near the enamel-dentine junction (20.480±12.98, 24.43±8.469 respectively) and weaker staining in the middle third of the dentine (3.214±1.049, 4.415±1.550 respectively). The increased immune activity in MMP2 and MMP9 was also seen in the dentine-pulpal junction (11.61±1.987, 14.51±5.052 respectively) (Figure 5.5).

  In tooth sections with caries lesions with an ICDAS score of 4, representing the underlying dark shade of the dentine without visible cavitation, significantly higher immunoreactivity of MMP2 and MMP9 (57.03±11.78, 65.81±16.94 respectively) was observed in the enamel-dentine junction and in the outer (61.61±3.042, 70.57±8.834) and middle thirds (58.47±4.899, 66.53 ±16.35) of the dentine, while it became weaker in the inner third of the dentine (22.63±3.689, 26.83±11.09). A significant increase in intensity was observed in odontoblasts (36.57±3.801, 44.84±12.77) compared to caries lesions with ICDAS 3 score (P= 0.001) (Figure 5.6).

  In a caries lesion with an ICDAS 5 score, which clinically represents a cavity with visible dentine, a significant increase in the intensity of MMP2 and MMP9 staining was noted in the outer third (70.58±2.660, 80.90±6.167, respectively), the middle third of dentine (60.38±12.00, 76.72±3.601) and odontoblasts (29.80±4.820, 36.80±7.781), with this reactivity, also becoming weaker in the inner third of dentine (22.87±2.626, 27.71±5.402 respectively) (Figure 5.7). A similar trend was observed in caries with an ICDAS score of 6, representing an extensive cavity with visible dentine. A noticeable intensity was seen throughout the carious dentine and odontoblasts (Figure 5.8).

**Table ‎5.3** Mean (±SD) intensity of areas of staining for MMP2 according to ICDAS score and field.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Caries lesion assessed by ICDAS | Location of MMP2 presence and expression | | | | |
| Dentine-enamel junction | Outer  third dentine | Middle third dentine | Inner  third dentine | Dentine-pulpal Junction  Odontoblasts |
| ICDAS 0 | 1.484 (±1.092) | 1.782  (±1.425) | 0.9583  (±1.620) | 2.174  (±0.7976) | 4.627  (±0.8675) |
| ICDAS 1 | 1.486  (±1.874) | 1.769  (±1.111) | 1.577  (±0.8449 | 2.631  (±0.8731 | 6.381  (±3.684) |
| ICDAS 2 | 2.996  (±1.459) | 1.702  (±1.453) | 1.577  (±0.8449) | 2.290  (±1.006) | 5.022  (±1.493) |
| ICDAS 3 | 20.48  (±12.98) | 8.227  (±3.214) | 3.214  (±1.049) | 7.212  (±0.654) | 11.61  (±1.987) |
| ICDAS 4 | 57.03  (±11.78) | 61.61  (±3.042) | 58.47  (±4.899) | 22.63  (±3.689) | 36.57  (±3.801) |
| ICDAS 5 | - | 70.58  (±2.660) | 60.38  (±12.00) | 22.87  (±2.626) | 29.80  (±4.820) |
| ICDAS 6 | - | 81.71  (±6.295) | 67.94  (±0.7182) | 27.86  (±5.882) | 40.46  (±4.836) |

**Table ‎5.4** Mean (±SD) intensity of areas of staining for MMP9 according to ICDAS score and field.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Caries lesion assessed by ICDAS | Location of MMP9 presence and expression | | | | |
| Dentine-enamel junction | Outer  third dentine | Middle third dentine | Inner  third dentine | Dentine-pulpal Junction  Odontoblasts |
| ICDAS 0 | 3.853  (±1.492) | 3.360  (±1.171) | 1.901  (±0.989) | 4.952  (±2.952) | 8.588  (±0.2770) |
| ICDAS 1 | 4.290  (±1.610) | 3.724  (±2.189) | 1.474  (±1.471) | 5.128  (±2.620) | 9.962  (±0.8883) |
| ICDAS 2 | 3.371  (±2.009) | 1.648  (±1.596) | 1.474  (±1.471) | 4.773  (±0.9429) | 8.675  (±0.5688) |
| ICDAS 3 | 24.43  (±8.469) | 16.04  (±9.820) | 4.415  (±1.550) | 7.481  (±4.662) | 14.51  (±5.052) |
| ICDAS 4 | 65.81  (±16.94) | 70.57  (±8.834) | 66.53  (±16.35) | 26.83  (±11.09) | 44.84  (±12.77) |
| ICDAS 5 | - | 80.90  (±6.167) | 76.72  (±3.601) | 27.71  (±5.402) | 36.80  (±7.781) |
| ICDAS 6 | - | 82.59  (±2.399) | 79.07  (±5.005) | 28.01  (±2.308) | 35.93  (±6.686) |

|  |  |  |
| --- | --- | --- |
| **Photographic image of tooth sections with caries recorded with the ICDAS score 0** | **Gelatinolytic MMP expressions** | |
| **MMP2** | **MMP9** |
| (c)  (b)  (a)  **Dentine**  (e)  (d)  **Odontoblasts** | | |
| (f) | | |

**Figure 5.2 (**a) Photographic image of tooth section with caries lesions recorded by ICDAS score 0 and the corresponding fluorescence microscopic images of MMP2 (red) and MMP9 (green) in (b, c) dentine and (d, e) dentine-pulp junction. (f) Bar graphs show the mean (±SD) expression of MMPs across dentine depth. Data are from triplicate, Statistical analysis using one-way ANOVA followed by Tukey`s post-hoc analysis (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, \*\*\*\*p-value < 0.0001).

(Magnification x10).

|  |  |  |
| --- | --- | --- |
| **Photographic image of tooth sections with caries recorded with the ICDAS score 1** | **Gelatinolytic MMP expressions** | |
| **MMP2** | **MMP9** |
| (b)  (c)    (a)  (a)    **Dentine**  **Odontoblasts**    (e)  (d) | | |
| (f) | | |

**Figure 5.3** (a) Photographic image of tooth section with caries lesions recorded by ICDAS score 1 and the corresponding fluorescence microscopic images of MMP2 (red) and MMP9 (green) in (b, c) dentine and (d, e) dentine-pulp junction. (f) Bar graphs show the mean (±SD) expression of MMPs across dentine depth. Data are from triplicate, Statistical analysis using one-way ANOVA followed by Tukey`s post-hoc analysis (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, \*\*\*\*p-value < 0.0001).

(Magnification x10).

|  |  |  |
| --- | --- | --- |
| **Photographic image of tooth sections with caries recorded with the ICDAS score 2** | **Gelatinolytic MMP expressions** | |
| **MMP2** | **MMP9** |
| (b)  (c)  (a)  **Dentine**  (a)  **Odontoblasts**  (e)  (d) | | |
| (f) | | |

**Figure 5.4** (a) Photographic image of tooth section with caries lesions recorded by ICDAS score 2 and the corresponding fluorescence microscopic images of MMP2 (red) and MMP9 (green) in (b, c) dentine and (d, e) dentine-pulp junction. (f) Bar graphs show the mean (±SD) expression of MMPs across dentine depth. Data are from triplicate, Statistical analysis using one-way ANOVA followed by Tukey`s post-hoc analysis (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, \*\*\*\*p-value < 0.0001). (Magnification x10).

|  |  |  |
| --- | --- | --- |
| **Photographic image of tooth sections with caries recorded with the ICDAS score 3** | **Gelatinolytic MMP expressions** | |
| **MMP2** | **MMP9** |
| (b)  (c)  (a)  **Dentine**  (a)  **Odontoblasts**  (e)  (d) | | |
| (f) | | |

**Figure 5.5** (a) Photographic image of tooth section with caries lesions recorded by ICDAS score 3 and the corresponding fluorescence microscopic images of MMP2 (red) and MMP9 (green) in (b, c) dentine and (d, e) dentine-pulp junction. (f) Bar graphs show the mean (±SD) expression of MMPs across dentine depth. Data are from triplicate, Statistical analysis using one way ANOVA followed by Tukey's post-hoc analysis (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, ​​\*\*\*\*p-value < 0.0001).

(Magnification x10).

|  |  |  |
| --- | --- | --- |
| **Photographic image of tooth sections with caries recorded with the ICDAS score 4** | **Gelatinolytic MMP expressions** | |
| **MMP2** | **MMP9** |
| (c)  (b)  (a)  **Dentine**  (e)  (d)  **Odontoblasts** | | |
| (f) | | |

**Figure 5.6** (a) Photographic image of tooth section with caries lesions recorded by ICDAS score 4 and the corresponding fluorescence microscopic images of MMP2 (red) and MMP9 (green) in (b, c) dentine and (d, e) dentine-pulp junction. (f) Bar graphs show the mean (±SD) expression of MMPs across dentine depth. Data are from triplicate, Statistical analysis using one-way ANOVA followed by Tukey's post-hoc analysis (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, ​​\*\*\*\*p-value < 0.0001).

(Magnification x10).

|  |  |  |
| --- | --- | --- |
| **Photographic image of tooth sections with caries recorded with the ICDAS score 5** | **Gelatinolytic MMP expressions** | |
| **MMP2** | **MMP9** |
| (c)  (b)    (a)    **Dentine**    (e)  (d)  **Odontoblasts** | | |
| (f) | | |

**Figure 5.7** (a) Photographic image of tooth section with caries lesions recorded by ICDAS score 5 and the corresponding fluorescence microscopic images of MMP2 (red) and MMP9 (green) in (b, c) dentine and (d, e) dentine-pulp junction. (f) Bar graphs show the mean (±SD) expression of MMPs across dentine depth. Data are from triplicate, Statistical analysis using one way ANOVA followed by Tukey's post-hoc analysis (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001​​, \*\*\*\*p-value < 0.0001).

(Magnification x10).

|  |  |  |
| --- | --- | --- |
| **Photographic image of tooth sections with caries recorded with the ICDAS score 6** | **Gelatinolytic MMP expressions** | |
| **MMP2** | **MMP9** |
| (c)  (b)    (a)    **Dentine**    (e)  (d)  **Odontoblasts** | | |
| (f) | | |

**Figure 5.8** (a) Photographic image of tooth section with caries lesions recorded by ICDAS score 6 and the corresponding fluorescence microscopic images of MMP2 ((red) and MMP9 (green) in (b, c) dentine and (d, e) dentine-pulp junction. (f) Bar graphs show the mean (±SD) expression of MMPs across dentine depth. Data are from triplicate, Statistical analysis using one-way ANOVA followed by Tukey's post-hoc analysis (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001​​, \*\*\*\*p-value < 0.0001).

(Magnification x10).

### Localisation of MMP2 and MMP9 in different depths of carious dentine at each ICDAS score

To understand whether the presence of MMP2 and MMP9 at different caries stages correlates with the depth into dentine (i.e., from the enamel-dentine junction to the dentine-pulp junction), expressions were quantified using ImageJ software and data were analysed in each area of the five fields of interest across each caries lesion assessed with ICDAS. Staining was scored for positivity and intensity.

  The data show that immunoreactivity of MMP2 and MMP9 was predominant in the dentine-pulp region (odontoblasts) regardless of the presence of caries lesions but to varying degrees. The intensity was similar between ICDAS 0 and 3; although it was higher in ICDAS 3, there was no statistically significant difference (p=0.605). From ICDAS 4 onwards, there was a statistically significant increase in intensity (p<0.001), followed by an almost identical trend in ICDAS 5,6 (Figure 5.9.a).

  Interestingly, the immunoreactivity of MMPs in the inner third of the dentine was very low in all tooth sections with varying degrees of caries severity. Increased intensity in the inner dentine was seen from ICDAS 4 to ICDAS 6 scores, with no significant difference between these scores (p=0.945). Although this increase in intensity was seen in the inner dentine, it was statistically significantly lower than the intensity in the middle and outer dentine of the same lesion (p= 0.01). (Figure 5.9.b).

  In the middle third of the dentine, very weak MMPs immunoreactivity was observed in lesions from ICDAS 0 to 3. In contrast, an intensity increase was observed in ICDAS 4 to ICDAS 6 (Figure 5.9.c).

  In the outer third of the dentine, MMPs immunoreactivity was first detected in ICDAS 3, with non-statistically significant (p= 0.873) higher intensity of MMP9 (24.43±8.469) compared to MMP2 (20.48±12.98). From ICDAS 4 to ICDAS 6, the immunoreactivity of both MMP2 and MMP9 was significantly increased (p<0.001). (Figure 5.9.d)

Immunoreactivity of MMPs at the enamel-dentine junction was analysed at ICDAS 0 to ICDAS 4 and excluded at ICDAS 5 and 6 as the caries lesion expanded and the region of the enamel-dentine junction was lost. From ICDAS 0 to ICDAS 2, a weak intensity was observed. In ICDAS 3, the intensity of MMPs staining was stronger but not statistically significant compared to ICDAS 0-2 (p=0.061). This intensity was significantly lower than ICDAS 4 (p < 0.001). (Figure 5.10. e)

|  |  |
| --- | --- |
| (c)  (a) | (b) |
|  | (d) |
| (e) | |

**Figure 5.9** Bar graphs showing mean (±SD) expression of MMP2 and MMP9 in primary carious teeth according to the localisation of MMPs in each ICDAS code (a) MMPs expressions in the dentine-pulp junction, (b) in the inner third of the dentine, (c) in the middle third of the dentine, (d) in the outer third of the dentine and (e) at the dentine-enamel junction. Statistical analysis using one way ANOVA followed by Tukey's post-hoc analysis (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001​​, \*\*\*\*p-value < 0.0001).

### Correlation between the immunoreactivity of MMP2 and MMP9 and caries surface appearance assessed by ICDAS

To understand whether the MMPs in the dental tissue correlate with the surface appearance of caries, Pearson correlation coefficients were calculated. There was a very high positive correlation between the ICDAS score and MMP2, [r (5) = 0.90, p=.002] and also between ICDAS and MMP9, [r (5) = 0.92, p=.004]. (Figure 5.10)

|  |
| --- |
| **(a)** |
| **(b)** |

**Figure 5.10** Scatter plots of Pearson’s correlation coefficient between the surface appearance of caries assessed by ICDAS and (a) MMP2 and (b) MMP9.

### Quantification of collagen degradation

#### **Collagen degradation analysis**

The results of the HYP assay are shown in Figure 5.11. A one-way AVOVA statistical test followed by Tukey post hoc analysis showed that collagen degradation increased with increasing MMP presence across different caries depths. Although the amount of degradation was statistically significantly (p=0.015) larger for ICDAS 3 compared with non-cavitated lesions (ICDAS 0-2), the amount of HYP was higher in carious lesions with ICDAS 5 compared with ICDAS 3 (p=0.004).

|  |
| --- |
|  |

**Figure 5.11** HYP assay showing quantity of denatured collagen from carious dentine specimen. Statistical analysis using one-way ANOVA followed by Tukey's post-hoc analysis. Data are from triplicate (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001).

#### **Correlation between ICDAS and collagen degradation**

The Pearson correlation coefficient was used to calculate the correlation between the presence of MMPs in dental tissues with different degrees of caries severity and the extent of collagen degradation recorded in each caries lesion by the ICDAS score. There was a high positive correlation between the MMPs in each ICDAS score and the amount of collagen degradation. The Pearson correlation between collagen degradation and MMP2 was [r (3)=0.86, P=0.005] , and MMP9 was [r (3)=0.82, P=0.004] (Figure 5.12).

|  |
| --- |
| **(a)** |
| **(b)** |

**Figure 5.12** Scatter plots of Pearson’s correlation coefficient between the quantity of denatured collagen presented as HYP concentration and (a) MMP2 and (b) MMP9 in each caries lesion assessed by ICDAS score.

### Correlation between MMPs and laser pen scores, radiographic scores, and histology scores

Tooth samples used in this project are unique dental specimens because descriptive data from four diagnostic methods were collected for each specimen, *in vivo*. The four methods were: visual diagnosis with ICDAS, laser pen scores, radiographic scores, and histology scores. To understand whether there was a correlation between the presence of MMPs in each sample and the other three measurements, the Pearson correlation was calculated.

For the laser pen score data, there was a low positive correlation with MMP2 [r (22) =0.46, p < 0.001] (Figure 5.13.a), and with MMP9 [r (22) =.40, p= 0.001] (Figure 5.13.b).

  A highly positive correlation between the presence of MMP2 in dental tissue and the radiographic score was noted [r (22) =0.76, p<0.001] (Figure 5.14.a), with a similar correlation with MMP9 [r (22) =0.73, p<0.001] (Figure 5.14.b). A moderately positive correlation between MMP2 and histology score of [r (22) =0.65, p< 0.001] was seen and again a similar correlation with MMP9 [r (22) =0.64, p= 0.001] (Figure 5.15).

|  |
| --- |
| **(a)** |
| **(b)** |

**Figure 5.13** Scatter plots of Pearson’s correlation coefficient between laser pen scores and (a) MMP2 (n=20) and (b) MMP9 (n=20) in each caries lesion assessed by ICDAS score.

|  |
| --- |
| **(a)** |
| **(b)** |

**Figure 5.14** Scatter plots of Pearson’s correlation coefficient between radiographic scores and (a) MMP2 (n=20) and (b) MMP9 (n=20) in each caries lesion assessed by ICDAS score.

|  |
| --- |
| **(a)** |
| **(b)** |

**Figure 5.15** Scatter plots of Pearson’s correlation coefficient between histology scores and (a) MMP2 (n=20) and (b) MMP9 (n=20) in each caries lesion assessed by ICDAS.

## Discussion

Cariogenic bacterial acids are known to dissolve the inorganic elements in dentine caries and expose the dentine matrix (Chaussain-Miller et al., 2006). Host-derived matrix metalloproteinases (MMPs) play an essential role in caries progression (Tjaderhane et al., 1998) by significantly regulating extracellular matrix turnover and degrading exposed collagen, triple-helical collagen molecules and the terminal non-helical ends of collagen, leading to a gradual loss of collagen matrix and preventing interfibrillar remineralisation (Hannas et al., 2007). The gelatinolytic MMPs (MMP2 and MMP9) and their role in collagen alteration and caries progression have long been the focus of biomedical research. The discovery of their presence and the assessment of their functional activity have opened the door for further research in this area. However, a more detailed picture of these proteases will further improve our understanding of their role in the caries process and their presence at different depths of the lesion. This, in turn, will help clinicians to make the correct diagnosis with regard to the extent of a lesion and the level of activity, which is crucial in determining whether invasive treatment or a conservative approach is indicated; the treatment of a carious lesion should be based on the principles and techniques of minimally invasive dentistry (Pitts, 2004).

  In this study, we investigated the presence of gelatinolytic MMPs (MMP2 and MMP9) in natural carious dentine at different caries stages and depths in human primary teeth. The correlation between the MMPs in the carious lesion and the appearance of the caries surface has not previously been studied. Therefore, a further aim was to correlate the diagnosis and surface appearance of different caries lesions, as recorded by ICDAS, with the level of expression of MMP2 and MMP9 in the lesions to provide clinicians using ICDAS as a clinical scoring system with a more detailed picture of caries and thus avoid over- or underestimation of the caries lesion, which subsequently improves diagnosis and helps in personalised care planning.

This discussion section will now discuss the experimental approach used in this study and the strengths, limitations and challenges encountered during the study. The main results are assessed and compared with those of previous studies. Finally, conclusions and the clinical relevance of the study is outlined.

### Experimental approach

To achieve the aims of our study, we had to consider that MMPs should be studied in or close to their natural environment in caries lesions using a natural substrate, as when studying enzymes, especially in dentine, the choice of substrate can be as important as the enzyme being studied (Tjaderhane et al., 2015). Therefore,

the use of human teeth and natural caries lesions was considered essential to enable the correct interpretation of the results.

  Caries lesions develop over months and even years after several episodes of demineralisation and remineralisation. Demineralisation of healthy dentine *in vitro* to produce caries lesions has been used in several studies to investigate the function of enzymes. However, this demineralised healthy dentine would not mimic the natural progression of a carious lesion, especially the time factor and the role of host factors, such as saliva, on the action of collagenolytic enzymes in caries (Tjaderhane et al., 2015). Therefore, the rapid demineralisation cycles in experimental studies do not consider the potential temporal changes in the organic dentine matrix that may occur in a natural caries lesion. Quantitative studies that have characterised the differences between natural and artificial caries lesions confirm that there are major differences between them and that they are far from identical. Natural caries lesions are partially demineralised/partially remineralised dentine and the tubules of which are partially sealed with acid-resistant mineral while artificial caries lesion is fully demineralised lesions whose tubules were empty (Ito et al., 2005; Tjaderahane et al., 2015).

  The study of MMPs in relation to the depth of caries in lesions and how their presence relates to inner and outer dentine is an interesting area that was explored in this study. It was not possible to do this with artificial caries, as creating natural caries lesions with caries-affected and infected dentine by demineralising natural healthy dentine has not been very successful (Tjaderahane et al., 2015). In this study, natural human carious dentine was used, which provides a more detailed and accurate picture of the MMPs and their role in caries formation and largely mimics the environment *in vivo*.

  As explained in Chapter 4, after the preliminary study to select the most effective staining protocol to achieve the objectives of this study, immunocytochemistry using the immunofluorescence technique proved to be the most appropriate method to double stain and colocalise MMP2 and MMP9 without overlap. This technique can detect the presence of two different antigens in the tissue with very high sensitivity by using two antibodies labelled with different fluorophores. This technique was used by Vidal et al. (2014) to compare the abundance of MMP2 and MMP9 in healthy and caries-affected dentine in human permanent molars.

 Previous protocols for staining MMPs in dentine have involved decalcification steps prior to staining. This does not correspond to *in vivo* conditions and may limit the validity (Mazzoni et al., 2009). Decalcification leads to the denaturation of proteins, which could reduce an enzyme's activity and have a detrimental effect on antigenicity (Frank et al., 1993; Mazzoni et al., 2009). To exclude the effect of decalcification and overcome the challenges associated with this, the protocol in this study was performed without decalcification of the samples by making some modifications to the protocol, as already described in Chapter 3 Section 3.5.6 and Chapter 4 Section 4.5.4.

  Although omitting the decalcification step has advantages, as mentioned in the previous paragraph, it limits the number of sections that can be obtained from each tooth. It should be noted that the samples used in this study are from a previous study. As the samples had already been cut to 500μm thickness, the middle section of each tooth was included in the study as the lesion, especially the non-cavitated (ICDAS 1 and 2) and the micro-cavitated lesions (ICDAS 3), will not be present in other sections of the same tooth. Therefore, it was decided to use only one section per tooth for immunostaining to ensure that the desired caries lesion was stained. This was one of the main reasons why double staining by immunofluorescence was necessary.

  A total of 65 teeth were included from the previous study. The samples were ordered and sorted according to their original ICDAS score (as previously assessed by Subka et al., 2019). The number of surfaces examined for each ICDAS was found to vary considerably. For example, three samples diagnosed with ICDAS 4 were examined, and 22 surfaces with ICDAS 5 were examined. It was not possible to obtain a large and equal number of surfaces for all ICDAS scores. Therefore, to ensure similar samples were used, three from each ICDAS score were included in this study, making the total of surfaces 24. It is worth noting that despite the small sample size, the variance between samples was minimal as all samples were from primary teeth of children aged 5-10 years, and all were molars with coronal proximal caries lesions.

  After immunofluorescence staining, both qualitative and quantitative data were measured. The overall immunoreactivity of MMP2 and MMP9 in positivity and localisation was assessed by directly examining the stained samples under the microscope. However, the intensity of the staining also provided important data. Image analysis was performed using ImageJ software, which allows objective analysis of fluorescence staining in the area of interest (section 3.6.5). Although measurement with software analysis is more precise and avoids the subjectivity of human variables, it was important to measure the repeatability of the measurement. Therefore, an assessment of the quantification reproducibility of the mean intensity of the areas of positive expression was performed, and the results showed a high level of agreement between them (p < 0.05).

  This study was preceded by an immunohistochemical study in which a successful protocol for staining MMPs in dental hard tissue by immunohistochemistry was developed. The immunohistochemical investigation of the positivity and localisation of MMPs in dental tissue was very close to the immunofluorescence staining results, which increased the validity of the results (Chapter 4).

### Presence of MMP2 and MMP9 in the dentine of carious lesions and their expression in odontoblasts at each ICDAS score

MMPs are thought to be involved in various physiological processes in dentine, such as the formation and calcification of intra- and intertubular dentine, as well as pathological processes, such as caries formation (Hannas et al., 2007). In our study, MMP2 and MMP9 were observed in human primary coronal dentine in varying amounts, patterns and distributions. These gelatinolytic proteins were assessed in each caries lesion by ICDAS in the enamel-dentine junction region, in three dentine regions (outer, middle and inner dentine) and in the dentine-pulp junction region (expression in odontoblasts).

**ICDAS 0**

  The recording of ICDAS 0 means that the tooth surface is healthy (Ismail et al.,2007).  Previous studies have reported the presence of MMPs in healthy dentine (Sulkala et al., 2007), and in this study, very low expression of MMP2 was observed at the enamel-dentine junction and remained unchanged in the three dentine regions. This is consistent with the results of a previous study investigating the presence of MMP2 in healthy human coronal dentine using immunohistochemistry (Boushell et al., 2011) and another study analysing human dentine extracts from healthy human permanent teeth (Martin-de Las Heras et al., 2000). These two studies found that MMP2 was present in the protein matrix of dentine prior to demineralisation, suggesting that this enzyme is incorporated into the mineralised matrix during mineralisation. However, they contradict the results of Golderg et al. (2003), who found that MMP2 is strongly localised in the enamel-dentine junction compared to dentine. This may be due to the fact that their study was conducted on rats and not on human teeth, which may have a different structure.

  This study confirms the presence of MMP9 in healthy human dentine, which is consistent with the results of previous studies (Boushel et al, 2008; Mazzoni et al, 2009). This suggests that MMP9 is an intrinsic component of the fibrillar organic matrix network in human dentine. My results show that MMP9 expression was higher at the enamel-dentine junction than in the other dentine regions, which is consistent with the results of a previous study (Niu et al., 2011). However, in Boushel et al and Mazzoni et al studies, no increase at the enamel-dentine junction was found.

  MMP9 immunoreactivity showed a decreasing intensity from the deep to the superficial dentine layer; this result is in agreement with a previous study (Boushell et al. 2008), suggesting that MMP9 is mainly related to the pre-dentine formation and is bound to collagen in the dentine matrix once it is expressed by odontoblasts, where the subsequent deposition of appetite crystals occurs during pre-dentine mineralisation.

MMPs, especially MMP2 and MMP9, are mainly expressed by human odontoblasts (Tjadenhane, 1998; Palosaari et al, 2003). In this study, the immunoreactivity of MMP2 and MMP9 was high in the dentine-pulp transition region. This was consistent with the results of a previous study (Niu et al., 2011).

**ICDAS 1/ ICDAS 2**

  ICDAS 1 means that the first visible change in enamel was noted after air-drying, and ICDAS 2 means that a marked change in enamel was noted without air-drying (Ismail et al.,2007).  Although the extent of enamel caries is different for the two codes, both are considered non-cavitated caries lesions. The study of MMPs in dentine in relation to non-cavitated lesions has not previously been investigated. Therefore, our study is the first study to investigate and report this.  In my study, a similar trend to ICDAS 0 was observed in MMP2 and MMP9 immunoreactivity in dentine and at the dentine-pulpal junction in tooth sections with ICDAS scores of 1 and 2.

  Interestingly, however, a marked increase in intensity was observed in the dentine-pulp region at ICDAS 2 compared to ICDAS 0, with MMP9 more strongly expressed than MMP2. This suggests a very early response of the dentine-pulp complex to early caries lesions. In an early caries lesion and when the enamel begins to demineralise, this leads to the appearance of a carious lesion characterised by a microscopic cavity in which "cariogenic" bacteria proliferate and release additional acids that gradually deepen the lesion. When the enamel barrier is disrupted, the metabolic activity of these microorganisms leads to the release of bacterial components into the dentinal tubules, where the permeability of the dentine plays an important role in the penetration of potential irritants into the pulp (Love and Jenkinson, 2002). In addition, the enamel of primary teeth is thinner and less mineralised than that of permanent teeth; therefore, the caries progression is higher in primary teeth (Koutsi et al., 1994).

Several micro-invasive procedures have been proposed for the treatment of proximal non-cavitated lesions, such as therapeutic sealants (Gomez et al., 2005; Martignon et al., 2006) and resin infiltration (Paris et al., 2007; Meyer-Lueckel and Paris, 2008), which aim to transform the lesion from active to inactive by creating a diffusion barrier within the lesion. Ekstrand et al., (2010) investigated the effectiveness of treating proximal caries lesions with an ICADS score of 0-2 with resin infiltration in the primary dentition. Using the split-mouth technique, they evaluated the therapeutic effect of resin infiltration with fluoride varnish compared to fluoride varnish alone (control) in 42 children with a follow-up of 12 months. They found that 67% of the control group and 31% of the resin infiltration group had progressed. The therapeutic effect of resin infiltration was 35% higher than that of the control group. Similar results were obtained in another study comparing the effectiveness of resin infiltration with sealing in the treatment of proximal caries lesions in permanent teeth. Both treatments were found to have a statistically significant higher therapeutic effect compared to placebo (Martignon et al., 2012). These studies support the use of resin infiltration to arrest early non-cavitated carious lesions in proximal surfaces in primary and permanent teeth.

  The understanding of non-cavitated lesions and the novel addition in this study showing their early effect and the resultant tendency for odontoblasts to increase MMP expression demonstrates the importance of early detection of caries lesions and implementation of non-invasive/micro-invasive measures that limit the progression of the lesion to an irreversible lesion because early dental caries is a reversible disease that can be controlled at any time as long as the biofilm can be removed (Featherstone, 2004; Fejerskov et al., 2015). The progression of non-cavitated lesions appears to be slower than cavitated lesions (Baelum et al., 2006), so non-invasive/micro-invasive strategies can be used at a time when lesions have the greatest chance of being arrested, provided more sensitive methods are used to improve caries diagnosis and help clinicians monitor non-surgical treatments.

**ICDAS 3**

  ICDAS 3 is described as localised enamel loss without visible dentine and has also been referred to as micro-cavitated lesions (Ismail et al.,2007). To date, no previous studies have investigated the presence of MMPs in dentine at this particular stage of caries.

  In this study, increased immunoreactivity of MMP2 and MMP9 was found throughout the outer third of the dentine, with more intense staining near the enamel-dentine junction. This could be explained, as mentioned earlier, by the fact that enamel degradation allows greater bacterial penetration into the dentine, causing odontoblasts to express more MMPs and attack the dentine matrix in this area. It was also found that the immunoreactivity of MMP2 and MMP9 was weaker in the middle and inner third of the dentine. Their expression in the dentine-pulp junction was increased compared to ICDAS 2, but the increase was not statistically significant.

  Dental caries remains one of the most prevalent diseases and a significant burden on health systems. In recent years, the restorative approach has been shown to be applicable, but it has disadvantages in treating dental caries. The current biological understanding of the caries process has led to the development of new philosophies based on early detection, preventive management, and tooth structure preservation (Pitts 2004). Of all caries lesions, the ICDAS 3 code is probably of the greatest importance to dentists. Non-cavitated lesions with ICDAS 1 and 2 are reversible, while cavitated lesions will most likely require operative intervention. However, for micro-cavitated lesions (ICDAS 3), the best treatment approach is always questioned.

  Several studies have evaluated different treatment approaches for micro-cavitated lesions (ICDAS 3) (Hesse et al., 2014; Monoz-Sandoval et al., 2019; Lindquist and Emilson, 2020). Hesse et al. conducted a clinical study to assess the efficacy of sealants in arresting caries lesions that extended to the outer third of the dentine compared with partial excavation and restorative treatment in primary molars with clinical and radiographic evaluation at 6, 12 and 18 months. They found that sealing had similar efficacy in arresting caries compared to partial excavation. They concluded that it is not necessary to remove carious tissue to treat micro-cavitated lesions in primary teeth (Hesse et al., 2014). Another study investigated sealants on first permanent molars as a treatment to arrest the progression of caries in lesions with ICDAS 3. It concluded that the use of a therapeutic sealant was effective in stopping the progression of micro-cavitated lesions (Muñoz-Sandoval et al., 2019). Lindquist and Emilson conducted a randomised control trial to evaluate the effectiveness of proximal sealing in stopping micro-cavitated caries lesions in permanent molars after 2 years. They concluded that caries sealing is a clinically acceptable technique that can preserve tooth structure and delay and minimise the need for restorative treatment (Lindquist and Emilson, 2020). These studies demonstrated that micro-cavitated lesions with an ICDAS 3 score responded well to sealing, which is consistent with the concept of minimally invasive dentistry (Ericson et al., 2003; Banerjee et al., 2020). These results may be attributed to the low MMP expression in caries lesions with ICDAS 3, as shown in our study.

**ICDAS 4**

The underlying dark shading in the dentine without visible cavitation is referred to as ICDAS 4 (Ismail et al.,2007). This lesion occurs when caries has spread to the dentine but has not yet weakened the enamel enough to cause its degradation. The relationship between ICDAS 4 and histological features has been investigated in previous *in vitro* studies (Ekstrand et al., 2007; Diniz et al., 2009) which showed that these lesions have substantial dentine involvement. Histological examination is considered the gold standard for assessing the extent of disease in a tissue (Mitropoulos et al., 2010). However, in a clinical setting, histological validation is not possible and radiographic examination, in combination with clinical examination, provides an effective tool for assessing the lesion and deciding on treatment (Mejàre, 1999). Furthermore, Ekstrand et al., (1997) suggested that once the lesion has reached dentine, radiographic assessment of the extent of the lesion is similar to histological validation.

 The correlation between ICDAS 4 and the radiographic assessment was investigated in two Brazilian studies (Bertella et al., 2013; Marquezan et al., 2019). Bertella et al. (2013) investigated the relationship between radiographic patterns and ICDAS 4 caries lesions in 95 permanent teeth. They found that the vast majority of lesions (67%) demonstrated no radiolucency, 17% had radiolucency confined to the enamel-dentine junction and 13% extended to the outer third of the dentine. This is consistent with Marqueza et al. (2019), who investigated the relationship between radiographic patterns and ICDAS 4 in 142 permanent teeth and found that approximately 78% had no radiolucency, 20% had radiolucency that extended to the enamel-dentine junction and only 0.7% extended to the outer third of the dentine. Both studies concluded that caries lesions with an ICDAS score of 4 did not correlate with the radiographic pattern and recommended that radiographic assessment at this particular stage of caries is required before a treatment decision is made, as the majority of these lesions did not show radiolucency on radiographic assessment. These differences between the histological and radiographic patterns in ICDAS 4 can be attributed to the different sample sizes. The in vivo studies mentioned above used large sample sizes, whereas the *in vivo* studies used fewer samples, ranging from three to eight extracted teeth. In addition, the teeth used in the *in vitro* studies could have more advanced caries leading to extraction than is the case in the epidemiological setting. It is worth noting that all previously mentioned studies were conducted on occlusal surfaces of permanent teeth, in contrast to our sample, which included proximal caries lesions with ICDAS 4 on primary teeth. All carious teeth that we included in the ICDAS 4 analysis had a radiolucency extending to the outer and middle thirds of the dentine.

  In our study, significantly higher immunoreactivity of the two MMPs in ICDAS 4 was observed at the enamel-dentine junction and in the outer and middle thirds of the dentine, whereas it was weaker in the inner third of the dentine. Compared to caries lesions with an ICDAS 3 score, a significant increase in intensity was observed in the odontoblasts (p<0.001) (section 5.5.3). The high MMP immunoreactivity in ICDAS 4 is consistent with previous studies that found a high abundance of MMP2 and MMP9 in caries lesions in permanent teeth (Sulkala et al., 2002; Goldberg et al., 2003; Sulkala et al., 2007; Charadram et al., 2012). However, these studies did not consider the clinical diagnosis or stage of caries when comparing MMP expression in healthy and carious lesions.

  This increase in MMP immunoreactivity compared to ICDAS 3 reflects that the progression of the carious lesion and the extent of collagen degradation are likely to be greater, which raises the question of whether cavity sealing alone would result in the arrest of the carious lesion with ICDAS 4 compared to an ICDAS 3 lesion where MMPs have lower immunoreactivity. Meyer-Lueckel and Paris, (2016) recommend in their article that active lesions in proximal surfaces with an ICDAS score of 3-6 require restorative treatment with selective removal of carious enamel and dentine in most cases to achieve better results. The International Caries Classification and Management System (ICCMS™) classifies caries lesions with an ICDAS score of 4 as "moderate stage" and recommends that active moderate lesions should be treated with tooth-preserving surgical treatment (Ismail et al., 2015).

  Banerjee et al. (2020) highlighted the three levels of invasiveness in intervention strategies based on the degree of carious tissue removal: non-invasive, micro-invasive and minimally invasive. No removal of dental tissue with biofilm control measures and chemical agents that balance minerals are referred to as a non-invasive strategy. Removal of dental tissue at the micrometre level, e.g., sealants and resin infiltration treatments, is referred to as a micro-invasive strategy. Selective removal of the caries lesion with or without placement of restoration is called a minimally invasive strategy. They emphasised that the decision to intervene should be based on the assessment of the activity and cavitation status of the lesion, taking into account the radiographic findings.

  Several studies have evaluated different treatment approaches for caries lesions with an ICDAS score of 4 (Paris et al., 2011; Kasemkhun et al., 2020; Lunkes et al., 2022). In their in vitro study, Paris et al., (2011). investigated the pattern of resin infiltration in proximal caries lesions of permanent teeth with ICDAS scores 2-5. After infiltration of the lesion, the teeth were sectioned and the section with the deepest lesion was used for analysis. The depth of infiltration was assessed using a fluorescence staining technique and laser scanning microscope. They found that in lesions with ICDAS 3, 4 and 5, resin infiltration was observed in 8%, 50% and 41% of lesions, respectively. In ICDAS 4 lesions, the depth of infiltration was significantly less compared to ICDAS 2 lesions and insignificantly less than in ICADS 3 lesions. This study concluded that the effectiveness of resin infiltration in ICDAS 4 might be hindered by the little infiltrated enamel. However, this study was conducted on permanent teeth. Whether the same pattern can be expected on the proximal surface of primary teeth requires further investigation. Although ICDAS 4 showed low enamel infiltration, whether this is sufficient to arrest caries lesions in vivo needs further investigation.

  Two studies (Meyer-Lueckel et al., 2012; Peters et al., 2019) investigated the efficacy of resin infiltration of non-cavitated lesions on the proximal surfaces of permanent teeth with radiographic extension to the inner enamel and outer third of the dentine with a three-year follow-up. They concluded that resin infiltration is effective in arresting non-cavitated caries lesions. However, it is worth noting that Peters et al (2019) found that none of the infiltrated lesions whose radiographic extent was limited to the inner enamel showed progression, whereas the resin-infiltrated lesions that showed progression were lesions that extended to the outer and middle thirds of the dentine. They suggested that deeper dentine lesions may not be reliable candidates for resin infiltration. However, both studies should be interpreted with caution as none of them included the clinical diagnosis of caries.

  Kasemkhun et al., (2021) investigated the efficacy of sealant placement in arresting carious lesion progression with and without bonding agents on occlusal lesions of 240 permanent teeth in Thai children aged 7-9.9 years with ICDAS scores of 2-4 with 24-month follow-up. They found no lesion progressed as long as the sealant was maintained. This study investigated the sealing of occlusal surfaces of permanent teeth. ICDAS 4 lesions on proximal surfaces present a diagnostic and therapeutic challenge and may not produce the same results as occlusal surfaces, since proximal caries have a relatively higher rate of progression in primary molars and permanent teeth in adolescence (Vanderas et al., 2006). Considering the difference between the treatment of primary teeth and permanent teeth, sealing proximal lesions in primary teeth with ICDAS 4 using a preformed metal crown has been shown to be effective in arresting the carious lesion (Innes et al., 2007), but this may not be the ideal treatment for the same lesion in permanent teeth.

  Lunkes et al., (2022) assessed three levels of invasiveness in the treatment of occlusal caries lesions with ICDAS 4. Their retrospective study assessed the need for reintervention in the patient records of 81 lesions in primary first molars and 30 lesions in permanent first molars. They found that lesions treated with non-invasive treatment were more likely to progress compared to lesions treated with sealants and restorations.

  Previous studies have shown inconsistent results in treating ICDAS 4 with micro-invasive strategies. Therefore, a randomised controlled trial is needed to determine the best treatment option for lesions with approximal ICDAS 4 caries in the primary and the permanent dentition.

**ICDAS 5/ ICDAS 6**

  The clinical description of a lesion with ICDAS 5 is a caries lesion with visible dentine, and ICDAS 6 is an extensive caries lesion with visible dentine (Ismail et al.,2007). Both lesions are described as cavitated caries lesions.

   In a caries lesion with an ICDAS 5 score, there was a significant increase in the intensity of staining in the enamel-dentine junction, outer third and middle third of the dentine and odontoblasts, with this reactivity becoming weaker in the inner third of the dentine. A similar trend was observed in caries with an ICDAS score of 6, where noticeable staining intensity was observed throughout the carious dentine and odontoblasts. This finding suggests that the caries lesion stimulates the expression of MMP2 and MMP9, leading to increased expression by the odontoblasts and presence in the carious dentine. This has been reported in previous work by Tjaderhane and colleagues (1998), where MMP9 in its active form was detected in dentine extracts of various decayed teeth by the use of zymography analysis. This is also consistent with a previous study that found MMP2 to be highly expressed in carious lesions (Toledano et al., 2010) and with other studies that found MMP9 and MMP2 to be significantly increased in carious lesions (Sulkala et al., 2002; Goldberg et al., 2003; Sulkala et al., 2007; Charadram et al., 2012). However, it is worth noting that these previous studies were conducted on demineralised tooth sections, not a natural caries lesion, on rat teeth and on carious lesions without measuring the stage of caries severity.

  The primary goal of restoring cavitated lesions is to restore tooth form and function so that patients can maintain effective plaque control (Kidd & Fejerskov, 2004). The results of my study show that high MMP immunoreactivity indicates an irreversible caries lesion, and a high degree of collagen degradation has occurred, suggesting restorative treatment as an option for this stage of caries, bearing in mind the importance of selective removal of caries tissue (Meyer-Lueckel, 2013; Banerjee et al. 2020). However, when restorative treatment is not an option due to the limited cooperation of the young patients, non-invasive treatment such as SDF is an alternative treatment to arrest caries lesion and support the concept of minimally invasive dentistry (Gao et al., 2016; Schmoeckel et al., 2020). Minimally invasive methods should be used as often as possible, as this treatment option is no worse than conventional treatment and gives better results (Schwendicke et al., 2021).

  I also noted a decreasing distribution of MMP2 and MMP9 from the superficial to the deep dentine layer. This could be due to the significant role MMPs play in denaturing the dentine matrix. This was also noted in previous work by Nascimento et al. (2011), who observed a decrease in MMP activation with increasing carious depth in human permanent teeth; however, Nordbo et al.(2003) found a significant increase in MMPs, especially MMP8, in the saliva of individuals suffering from a carious lesion which is consistent with the study of Shimada et al. (2009) in which a significant level of MMP8 and MMP9 was found in the outer layer of the carious lesion compared to the inner layer in human permanent teeth. All previous studies suggest that salivary MMPs may indeed be involved in the pathogenesis of dental caries, as the extremely damaged tooth allows molecular penetration of salivary MMPs into the pulp (Byers and Lin, 2003).

### Localisation of MMP2 and MMP9 in different depths of carious dentine at each ICDAS score

The carious dentine lesion consists of two layers differing in chemical and morphological structure; the inner (affected dentine) and outer layer (infected dentine) (Fusayama and Terachima, 1972). Caries-affected dentine is present near the normal dentine and is partially demineralised with most of the collagen matrix intact. The clinical significance of this layer is that it can be remineralised and could act as a suitable substrate for dentine restorative adhesion. However, the presence of endogenous MMPs in this layer has been confirmed in several studies (Van Strijp et al., 2003; Boushell et al., 2008; Shimada et al., 2009; Toledano et al., 2010; Charadram et al., 2012, Vidal et al., 2014) and could compromise remineralisation with the organic matrix of the caries-affected dentine undergoing self-degradation.

  In this study, MMPs were assessed in each caries lesion by ICDAS in the enamel-dentine junction region, in three dentine regions (outer, middle and inner dentine) and in the dentine-pulp junction region.  The immunoreactivity of MMPs in the inner third of the dentine, which represents to a great extent the caries-affected dentine, was very low in all tooth sections with varying degrees of caries severity. Although increased intensity was recorded from ICDAS 4 to ICDAS 6, the intensity was insignificant (p>0.05) compared to the intensity in the middle and outer dentine in the same lesions, which represents caries-infected dentine (section 5.5.3).   In the middle third of the dentine, very weak MMP immunoreactivity was observed in lesions from ICDAS 0 to 3. In contrast, a statistically significant increase in intensity was observed in ICDAS 4 to ICDAS 6 (p< 0.001).

  Shimada et al. (2009) demonstrated the localisation of MMPs through different layers of carious dentine, i.e., healthy, affected and infected dentine, using immunogold labelling. The presence of MMP2 was confirmed in all layers but showed no significant differences between them. However, MMP9 was detected at a lower concentration in the affected dentine layer compared to infected dentine. This is in agreement with another study, which found that MMP9 concentration in dentine fluid from deep cavities is lower than that from shallow counterparts and that MMP2 concentration remains the same (Ballal et al., 2017). On the other hand, using immunofluorescence microscopy, Toledano et al. (2010) found that the intensity of MMP2 was lower in the affected dentine than in the infected layer which agrees with our results.

Vidal et al. (2014) compared the presence of MMP2 and MMP9 in healthy (n=5) and caries-affected dentine (n=5) of extracted permanent molars. The tooth samples were sectioned and subjected to immunofluorescence staining. They found that MMPs were more intense in caries-affected dentine than in healthy dentine, with MMP9 showing higher intensity than MMP2, which is consistent with our results.

  The results of our study revealed that expression levels of MMP2 and MMP9 are lower in the inner third of the dentine, suggesting and supporting the selective removal of caries tissue from cavities, as the treatment of caries lesions should be based on the principle and techniques of minimally invasive dentistry (Pitts, 2004). Furthermore, the complete removal of caries increases the risk of exposing the pulp, which in turn leads to more pulpal complications (Ricketts et al., 2013).

  Although low MMP immunoreactivity has been found in the inner dentine, their presence in this area has been shown to potentially impact the integrity of the resin-dentine bond. Once activated by acid etching, they degrade the hybrid layer's collagenous matrix (Pashley et al., 2004). Understanding the role of these endogenous enzymes in the hydrolysis of the hybrid layer has led to different approaches to maintaining the integrity of this layer (Tjäderhane et al., 2013). Therefore, this study suggests that lower expression of MMP2 and MMP9 in carious inner (affected) dentine indicates less collagen degradation and degradation of the hybrid layer, and better survival of the restorative material can be expected.

### Correlation between the immunoreactivity of MMP2 and MMP9 and caries surface appearance assessed by ICDAS

Determining the presence and stage of dental caries is essential for preventing and treating the disease (Pitts, 2004). It is also essential for risk assessment, treatment planning, and for research related to outcomes (Ismail, 2004; Kagihara et al., 2009). ICDAS as a scoring system provides researchers and clinicians with diagnostic criteria that show clear stages of the caries process, from non-cavitated to micro-cavitated to cavitated lesions, so that they can decide at what stage of disease and severity to measure caries (Ismail et al., 2007). Several studies have been conducted to validate the ICDAS system and have shown its high validity and reproducibility. Shoaih et al. (2009) found ICDAS a suitable system for proximal and occlusal caries diagnosis. Martignon et al. (2007) demonstrated a significant correlation between ICDAS scores and histological changes in caries in both primary and permanent teeth. Furthermore, Jablonski-Momeni et al., (2008) found that the ICDAS system is an optimal method for caries diagnosis in primary and permanent teeth.

  The vital role of endogenous enzymes in physiological and pathological processes has already been explained in detail. Although MMP localisation in the sound tooth and the caries lesion has been investigated in many studies, no study has previously related MMP expression to the stage of the caries lesion or the surface appearance of the lesion. This study represents a new addition to the field by relating caries diagnosis to MMPs in the dentine substrate.

  My study found a significantly positive correlation between the ICDAS score and MMP2 and MMP9. MMP expression in odontoblasts and their presence in dentine and caries lesion increase with increasing caries stage and severity. This is of clinical importance as it provides more knowledge about the enzymes and their function during the caries process. Clinicians using ICDAS as a clinical scoring system can thus obtain a more detailed picture of caries to avoid over- or underestimation of the caries lesion, which improves diagnosis and helps in treatment planning.

### Quantification of collagen degradation

The degradation of type 1 collagen in dentine is considered to be one of the major pathological functions of the endogenous enzymes collagenase (MMP8) and gelatinase (MMP2 and MMP9) (Kleter et al. 1998; Tjaderhane et al., 1998). While helical collagen degradation by collagenases causes the final destruction of collagen, MMP2 and MMP9 have telopeptide activities that cause cleavage of the non-helical C- and N-terminal ends of collagen, resulting in irreversible denaturation of collagen that limits intrafibrillar remineralisation (Buzalaf et al., 2015).

  In biomedical studies, the HYP assay is considered the standard experiment for quantifying collagen (Bornstein and Sage, 1980). It quantifies hydroxyproline amino acids in tissue lysates and biological fluids and measures the percentage of dissolved collagen fragments in the tissue, reflecting the extent of collagen degradation. It is estimated that 90% of the dry mass of demineralised dentine contains type I collagen (Linde et al., 1980). In addition, hydroxyproline accounts for 9.6% of the mass of type I collagen fibrils (Goldberg et al., 2011). Since HYP is restricted to collagen, the amount of HYP can be equated with the amount of denatured collagen.

  The results of the HYP assay indicate that the collagen in the carious dentine was denatured. It also showed that collagen degradation increased with increasing MMP presence at different caries depths. Although the extent of degradation was significant in ICDAS 3 compared to non-cavitated lesions (ICDAS 0-2), the amount of hydroxyproline was high in carious lesions with ICDAS 5 compared to ICDAS 3. This could be explained by the fact that carious lesions, especially in ICDAS 3, may have a combination of denatured and non-denatured collagen. Collagen consists of band patterns and bonds between these band patterns. Denaturation can occur either in the band pattern, in the bonds, or both. Therefore, the significant amount of HYP recorded in ICDAS 3 may be mainly due to denaturation in the bonds, indicating that some degree of denaturation has occurred in the collagen. while maintaining its cross-links and banding pattern.

   It should be noted that not all caries lesions with ICDAS scores were included in our study of collagen denaturation; this was due to the limitation of the number of samples, especially for ICDAS 4. Therefore, the analysis was performed to compare the healthy tooth (ICDAS 0), the non-cavitated lesion (ICDAS 1-2) and the micro-cavitated lesion (ICDAS 3) with the cavitated lesion (ICDAS 5). It would be interesting to see how much collagen was degraded in ICDAS 4. However, the results of this study show that the immunoreactivity of MMP2 and MMP9 was comparable in ICDAS 4 to ICDAS 6, suggesting that the extent of collagen degradation may be almost the same. However, this needs to be further investigated and confirmed.

  The correlation between the presence of MMPs in dental tissues with different degrees of caries severity and the extent of collagen degradation recorded in each caries lesion by the ICDAS score was calculated. There was a significantly positive correlation between the MMPs in each ICDAS score and the extent of collagen degradation.

### Correlation between MMPs and laser pen scores, radiographic scores, and histology scores

The dental specimens used in this project are unique because descriptive data was collected for each specimen using four methods in vivo. The four methods were: visual diagnosis with ICDAS, Laser Pen scores, and radiographic and histology scores. It was interesting to know if there was a correlation between the presence of MMPs in each sample and the other three methods.

   The Laser Fluorescence Pen is a commercially available detection technology that helps to distinguish between healthy and carious tissue and to detect carious lesions early. The DIGNOdent pen 2190 was used for these samples, and the Laser Pen Score data was retrieved for the specific samples on which the MMP analysis was performed. The laser pen detects the fluorescence intensity caused by a carious lesion, which is higher than that of healthy tooth tissue. When the laser irradiates the tooth, the light is absorbed by organic and inorganic substances in the tooth tissue and metabolites from oral bacteria. These metabolites include porphyrins produced by different types of oral bacteria (Lussi et al., 2004).

  In this study, a low positive correlation was found between the Laser Pen score data and the immunoreactivity of MMP2 and MMP9 (r (5) = 0.90, p=.002, r (5)= 0.92, p=.004 respectively). This could be because the Laser Pen measures fluorescence and bacterial metabolites in both enamel and dentine, whereas MMPs are localised in dentine. Therefore, the initial values of the Laser Pen are very high compared to the MMP data. This leads to a low correlation between the two parameters.

  Bitewing radiographs have been shown to add diagnostic value to clinical examinations diagnosing dental caries in children (Kidd and Pitts, 1990). This study found a high positive correlation between the presence of MMP2 and MMP9 in dental tissue and radiographic findings (r (22) =0.76, p < 0.001, r (22) =0.73, p < 0.001 respectively). Since MMPs correlate strongly with the severity and stage of caries lesion and collagen degradation, it is expected that MMPs correlate with radiographic scores. This study found a moderately positive correlation between MMP2 and MMP9 and histology scores (r (22) =0.65, P< 0.001, r (22) =0.64, P= 0.001 respectively). This is possibly due to the fact that ICDAS 4-6 had the same histological score, as shown in Figure 5.16.

## Summary

The study of MMPs and their role in dental caries at different stages has provided a more detailed picture of the pathogenesis of caries. Understanding and identifying specific MMPs in carious lesions will open up therapeutic options for caries prevention and treatment by possibly using MMP inhibitors to inhibit the progression of dental caries. Improving knowledge of the role of MMPs in the affected dentine layer will support the selective removal of carious tissue. This may help to develop clinical strategies for the fabrication of durable restorations in which the affected dentine layer serves as a suitable substrate for the adhesion of dentin restorations. The correlation between the presence of MMPs and surface appearance gives dentists using ICDAS as a visual diagnostic method a more detailed picture to avoid over- or underestimating carious primary teeth based on the surface appearance of the carious lesion. The main findings of this study are summarised below:

* The expression of MMP2 and MMP9 and the degree of collagen degradation correlate strongly with caries diagnosis assessed by ICDAS. The use of ICDAS to assess the severity of caries lesions and the correlation with the presence and expression of MMP in these lesions validates the modern approach to caries treatment with minimally invasive concept.
* Low MMP expression in ICDAS 0-2 and statistically significant higher expression in caries with ICDAS 4-6 compared to ICDAS 3.
* Caries with an ICDAS 3 score should be treated with micro invasive treatments where MMP expression and collagen degradation are significantly lower compared to ICDAS 4-6 and slightly higher compared to ICDAS 1-2.
* Teeth responded to early caries lesions (ICDAS 2) with early expression of MMPs in odontoblasts.
* MMP expression was significantly reduced in the inner carious dentine.
* Low positive correlation was found between the Laser Pen score data and the immunoreactivity of MMP2 and MMP9.
* High positive correlation between the presence of MMPs in dental tissue and radiographic findings.
* Moderately positive correlation between MMPs and histology scores.

# CHAPTER 6

CARIES-INHIBITORY EFFECTS OF SILVER DIAMINE FLUORIDE: INFLUENCE OF TIME AND POTASSIUM IODIDE

# Chapter 6: Caries-Inhibitory Effects of Silver Diamine Fluoride: Influence of Time and Potassium Iodide

## Introduction

Various chemical agents are used in dentistry to arrest and/or control the progression of caries in the dentine, without the need for surgical removal of the lesion. These agents, and their topical application to active caries lesions, represent an effective conservative approach to the treatment of dental caries.

 Silver diamine fluoride (SDF) is an alkaline solution containing fluoride, silver and ammonia. It has an anti-cariogenic effect that combines fluoride's remineralising effect and silver's antibacterial effect (Suzuki et al., 1976; Mei et al., 2013a). In a previous study, SDF was also shown to have an inhibitory effect on MMPs (MMP2, MMP8 and MMP9) (Mei et al., 2012).

 There are two critical clinical aspects of applying SDF to carious lesions that have not yet been fully investigated: firstly, the application of potassium iodide after SDF treatment, which was introduced to overcome the side effect of discolouration caused by SDF, and secondly, the application time, as current clinical recommendations suggest of SDF to caries for 3 minutes, however, this can be challenging in young patients.

  While several studies have reported the clinical success of SDF in preventing and arresting dental caries, laboratory studies have focused on SDF's mineral density and antibacterial effects. Moreover, only the short-term inhibitory effect of SDF on MMPs has been investigated, using either recombinant MMPs or artificial demineralised dentine. The modifying influence of potassium iodide and application time on MMP inhibition has not yet been investigated. Therefore, this study aims to investigate the long-term inhibitory effect of MMPs on SDF with and without the application of potassium iodide (KI) with different application times in natural carious dentine.

## Aim and objectives

This *in vitro* study aimed to investigate the long-term inhibitory effect of SDF on MMP activity in relation to two important clinical variables: the presence of KI and the application time of SDF. The specific objectives were as follows:

* To investigate the long-term effect of SDF on total MMP activity in caries lesions.
* To evaluate the effects of Potassium Iodide (KI) application following SDF on the anti-MMP activity potential of SDF in caries lesions.
* To assess and quantify the effect of SDF and SDF with KI on collagen degradation.
* To investigate different application times of SDF and any effect on MMP activity.
* To evaluate the effect of SDF and SDF with KI on the surface micro-hardness of non-cavitated caries lesions.
* To evaluate the differences in micro-hardness between sound and non-cavitated caries lesions.

### Null hypothesis

The use of KI after SDF does not affect the anti-MMP potential of SDF.

## Methods

Details regarding the overall experiment have previously been outlined in Chapter 3. A general overview of the methods used is shown in Figure 6.1. All details of the distribution of the samples are described in section 3.2.2.3. Sample preparation was described in section 3.3.3. The assays used in this experiment were the Generic MMP activity assay, detailed in section 3.8, and the hydroxyproline assay, detailed in section 3.7. The collection and preparation of saliva as an incubation medium was explained in detail in section 3.3.3.2.

63 carious dentine blocks

Collection and preparation of saliva

**Figure 6.1** Schematic diagram illustrating the general overview of the method. Extracted teeth with ICDAS scores of 5 and 6 were used to obtain 63 carious dentine blocks (3mm x 3mm x 500um). 45 blocks were classified into 4 categories: Baseline activity, SDF, SDF+K and Control. They were then incubated in natural saliva for 1, 4 and 12 weeks. After each incubation, total MMP activity was determined with a colorimetric MMP assay and collagen degradation was measured with a hydroxyproline assay. To investigate the duration of the SDF application, 18 blocks were examined.

### Statistical analysis

A one-way ANOVA test was used to determine the effect of two nominal predicated variables on a continuous outcome variable. **T-test was used for the analysis of the difference between two variables**

A p-value <0.05 was considered statistically significant. Asterisks denote statistical significance (\*p <0.05; \*\*p <0.01; \*\*\*p <0.001; \*\*\*\*p <0.0001, and NS p >0.05).

## Results

### Agreement between MMP activity in serial sections

This preliminary test was conducted to measure MMP activity in the same carious lesion in three serial sections in order to determine the agreement between the three measurements (section 3.8.2.4). Table 6.1 shows the endpoint values of MMP activity in three serial carious dentine blocks. The paired t-test showed that the measurement of MMP activity in each carious lesion was very similar, and the difference in MMP activity between the blocks was not statistically significantly different (p= 0.825).

**Table ‎6.1** End-point values of the MMP activity in three serial carious dentine blocks (p= 0.825).

|  |  |  |  |
| --- | --- | --- | --- |
| **Samples** | **Block 1** | **Block 2** | **Block 3** |
| **1** | 0.029 | 0.026 | 0.027 |
| **2** | 0.025 | 0.028 | 0.024 |
| **3** | 0.035 | 0.034 | 0.037 |

### Percentage of inhibition of total MMP activity in caries lesions after SDF and SDF+KI application

The activity of MMPs was measured with the Generic MMP activity assay, (section 3.8). The endpoint values (residual activity of the MMPs) of the experimental solutions and the controls at the three incubation periods (1, 4 and 12 weeks) are listed in Table 6.2. A low endpoint value indicates high MMP inhibition. The percentage of inhibition was also calculated.

 After one week of incubation, the mean endpoint values were statistically significantly lower in the SDF experimental group compared to the baseline (before treatment) (p < 0.001). The percentage of inhibition was 93%. MMP activity was also statistically significantly reduced in the SDF group compared to the control group (no treatment) (p < 0.001).

  The mean final value of the residual activity of the MMPs in the SDF group was lower than that of the SDF+KI experimental group, and these differences were statistically significant (p = 0.02). However, the difference in inhibition between baseline and SDF+KI was not statistically significant (p = 0.07), with a percentage of inhibition of 55.5%. (Figure 6.2)

 After four weeks of incubation, final MMP activity levels were also measured, and SDF was statistically significantly lower than baseline and control (p < 0.001). SDF significantly inhibited total MMP activity by 91% (p < 0.001), while the application of KI decreased activity to 48%. The difference in residual MMP activity between the baseline group and the SDF+KI experimental group was not statistically significant (p=0.06). (Figure 6.3)

 After 12 weeks of incubation, SDF significantly inhibited total MMP activity by 55%, while the application of KI reduced activity to 20%. The difference between the endpoint value of MMP activity at baseline was statistically significantly reduced after SDF treatment (p = 0.002), while the decrease in the SDF+KI group was not statistically significant (p = 0.31) (Figure 6.4).

**Table ‎6.2** End-point values of the residual MMP activity at baseline, following SDF, SDF+KI and control (caries without treatment).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Period of incubation** | **Sample** | **Baseline reading** | **SDF** | **SDF+KI** |
| **1 week** | **1** | 0.033 | 0.002 | 0.020 |
| **2** | 0.050 | 0.002 | 0.021 |
| **3** | 0.038 | 0.003 | 0.030 |
| **4** | 0.069 | 0.001 | 0.032 |
| **Control 1** | 0.033 | 0.044 | |
| **Control 2** | 0.036 | 0.069 | |
| **4 weeks** | **5** | 0.034 | 0.003 | 0.022 |
| **6** | 0.041 | 0.006 | 0.034 |
| **7** | 0.051 | 0.005 | 0.031 |
| **8** | 0.041 | 0.002 | 0.024 |
| **Control 3** | 0.023 | 0.062 | |
| **Control 4** | 0.036 | 0.043 | |
| **12 weeks** | **9** | 0.036 | 0.015 | 0.023 |
| **10** | 0.025 | 0.009 | 0.019 |
| **11** | 0.029 | 0.016 | 0.025 |
| **12** | 0.031 | 0.015 | 0.029 |
| **Control 5** | 0.023 | 0.032 | |
| **Control 6** | 0.033 | 0.044 | |

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**Figure 6.2** Bar graph showing total MMP residual activity in caries lesions after application of SDF and SDF+KI measured after 1 week of incubation in natural saliva. SDF significantly inhibited total MMP activity by 93%, while the application of KI reduced activity by **55.5%.** Data were analysed using ANOVA and the Tukey multiple comparison test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, \*\*\*\*p-value < 0.0001).

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**Figure 6.3** Bar graph showing total MMP residual activity in caries lesions after application of SDF and SDF+KI measured after 4 weeks incubation in natural saliva. SDF significantly inhibited total MMP activity by 91%, while the application of KI resulted in a reduction in activity to **48%.** Data were analysed using ANOVA and the Tukey multiple comparison test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, \*\*\*\*p-value < 0.0001).

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**Figure 6.4** Bar graph showing total MMP residual activity in caries lesions after application of SDF and SDF+KI measured after 12 weeks incubation in natural saliva. SDF significantly inhibited total MMP activity by 55%, while the application of KI reduced activity to **20%.** Data were analysed using ANOVA and the Tukey multiple comparison test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001).

### Long-term inhibitory effect of SDF and SDF+KI on total MMP activity

In Figure 6.5, the residual MMP activity and the percentage of MMP inhibition were plotted in a line graph to visually show the longer-term effect of SDF on MMP activity compared to SDF+KI.

 SDF showed a significant longer-term inhibitory effect (up to 12 weeks) on MMP activity compared to SDF+KI. Within the same experimental treatments, the decline in MMP activity did not change significantly between week one and week 4 (p = 0.43). However, the decline between week one and week 12 and between week four and week 12 was statistically significant (p < 0.001).

 In the SDF+KI treatment group, there was no statistically significant decline in MMP activity between all incubation times (p = 0.89).

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**Figure 6.5** Line graph showing the long-term inhibitory effect of SDF and SDF+KI on total MMP activity. Up to 12 weeks, SDF significantly inhibits MMP activity, while the inhibitory effect of SDF + KI does not reach significance.

### Quantitative measurement of the extent of collagen degradation in caries lesions after SDF and SDF+KI application

The mean concentration of HYP in the incubation media (natural saliva) was measured using a HYP assay to determine the dissolution of collagen. The value of HYP concentration is directly proportional to collagen degradation and so, the higher the HYP value, the more collagen is broken down. This analysis was performed at three-time points: 1, 4 and 12 weeks after incubation.

 After one week of incubation, the mean HYP concentration was significantly lower (p = 0.001) in the SDF-treated group (0.141 ± 0.024) than in the control group (no treatment) (0.402 ± 0.074). In the SDF+KI treated group, the mean HYP concentration (0.245 ± 0.053) was significantly higher than in the SDF group (p = 0.041). (Figure 6.6)

 A similar trend was observed after four weeks of incubation. The SDF-treated group showed a significant decrease (p < 0.001) in the concentration of HYP compared to the control group (0.198 ± 0.012 and 0.373 ± 0.035, respectively). The SDF+KI group had a statistically significant (p < 0.001) higher concentration of HYP (0.295 ± 0.015) compared to the SDF-treated group. (Figure 6.7)

 After 12 weeks of incubation, the mean HYP concentration was significantly lower in the SDF-treated group (0.252 ± 0.033) than in the control (no treatment) group (0.359 ± 0.042) (p = 0.009) and the SDF+KI-treated group (0.324 ± 0.018) (p = 0.025).

 The difference between the SDF+KI group and the control group was not statistically significant after 12 weeks (p = 0.397). (Figure 6.8)

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| **SDF SDF+KI. Control** |

**Figure 6.6** HYP assay showing the quantity of denatured collagen in caries lesions following SDF and SDF+KI application measured after 1 week incubation in natural saliva. Data were analysed using ANOVA followed by Tukey multiple comparison test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001).

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| **SDF SDF+KI. Control** |

**Figure 6.7** HYP assay showing the quantity of denatured collagen in caries lesions following SDF and SDF+KI application measured after 4 weeks incubation in natural saliva. Data were analysed using ANOVA followed by Tukey multiple comparison test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001).

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Figure 6.8 HYP assay showing the quantity of denatured collagen in caries lesions following SDF and SDF+KI application measured after 12 weeks incubation in natural saliva. Data were analysed using ANOVA followed by Tukey multiple comparison test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001).

### Percentage of inhibition of total MMP activity in caries lesions after different application times of SDF

The activity of MMPs was measured with the Generic MMP Activity Assay (section 3.8). The endpoint values (residual activity of the MMPs) at the two application times of SDF (1 and 3 minutes) and the control (no treatment) are shown in Table 6.3. A low endpoint value indicates high MMP inhibition. The percentage of inhibition was also calculated.

 ANOVA results show that both mean endpoint values following SDF application for 1-minute and 3-minute indicated significant MMP inhibition (94% and 97%, respectively) compared to baseline and control (p < 0.001). However, the difference in MMP inhibition between applications for 1-minute or 3-minute was not statistically significant (p = 0.0982). (Figure 6.9)

**Table ‎6.3** End-point values of the residual MMP activity after 1- and 3- minutes of SDF application.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Baseline reading** | **Final reading** | |
| **1 min** | **3 min** |
| **1** | 0.041 | 0.002 | 0.002 |
| **2** | 0.042 | 0.003 | 0.001 |
| **3** | 0.048 | 0.004 | 0.003 |
| **4** | 0.042 | 0.001 | 0.001 |
| **5** | 0.036 | 0.002 | 0.001 |
| **Control 1** | 0.041 | 0.041 | |
| **Control 2** | 0.048 | 0.052 | |
| **Control 3** | 0.036 | 0.038 | |

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**Figure 6.9** Bar graph showing total MMP residual activity in caries lesions after two application times (1 min and 3 mins) of SDF. Data were analysed using ANOVA followed by Tukey multiple comparison test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, \*\*\*\*p-value < 0.0001).

### Effect of SDF+/-KI application on the micro-hardness of non-cavitated caries lesions

The microhardness of non-cavitated lesions in enamel was measured with a Vickers hardness tester (section 3.9). The values are expressed as Vickers hardness number (VHN), the larger the number, the greater the hardness. In this experiment, microhardness was measured at baseline, after SDF treatment and after SDF+KI treatment. Six readings were recorded from each sample. The mean values of VHN and the percentage increase in microhardness are shown in Tables 6.4 and 6.5.

 In the SDF treatment group (n = 10), there was a statistically significant difference (p < 0.0001) in VHN between baseline (201.8 ± 26) and SDF treatment (336.6 ± 48). The mean percentage increase in microhardness after treatment was 66.8%. (Figure 6.10)

 For the SDF+KI treatment group (n = 10), there was also a statistically significant difference (p < 0.0001) in VHN between baseline (189 ± 30) and SDF+KI treatment (324.2 ± 25). The mean percentage increase in microhardness after treatment was 71.5%. (Figure 6.11)

 Although the microhardness value was higher with SDF+KI, the difference between the SDF treatment and SDF+KI was not statistically significant (p= 0.480). (Figure 6.12)

**Table ‎6.4** Vickers micro-hardness numbers (VHN) of enamel surfaces of non-cavitated caries lesion (ICDAS 2) at baseline and following treatment with SDF presented as mean ± SD and percentage of hardness increase.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Baseline reading** | **SDF treatment** | **Percentage**  **increase** |
| **1** | 158.6 ± 41 | 355.9 ± 83 | 124% |
| **2** | 216.4 ±46 | 269.4 ± 46 | 25% |
| **3** | 198.8 ±38 | 424.4 ± 80 | 113% |
| **4** | 189.1 ± 28 | 383.5 ± 47 | 102% |
| **5** | 242.6 ± 19 | 298.1 ± 45 | 22.8% |
| **6** | 232.9 ± 30 | 315.5 ±40 | 35.5% |
| **7** | 167.6 ±34 | 280.7 ±48 | 67.5% |
| **8** | 214.4 ± 21 | 365.4 ±30 | 70.4% |
| **9** | 203.2 ± 44 | 340.3 ±28 | 67.5% |
| **10** | 194.3 ± 04 | 332.5 ±32 | 71% |
| **Mean** | **201.8 ± 26** | **336.6 ± 48** | **66.8%** |

**Table ‎6.5** Vickers micro-hardness numbers (VHN) of enamel surfaces of non-cavitated caries lesion (ICDAS 2) at baseline and following treatment with SDF+KI presented as mean ± SD and percentage of hardness increase.

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **Baseline reading** | **SDF+KI treatment** | **Percentage**  **increase** |
| **1** | 146.8 ± 44 | 298 ± 27 | 102.9% |
| **2** | 138.1 ± 34 | 284± 19 | 106.3% |
| **3** | 187.7 ± 33 | 343.7 ± 32 | 83% |
| **4** | 184.2 ± 21 | 289 ± 37 | 56.8% |
| **5** | 221.6 ± 24 | 348.7 ± 26 | 57.3% |
| **6** | 198.9 ± 13 | 344.5 ± 29 | 73% |
| **7** | 224.2 ± 49 | 314 ± 24 | 40% |
| **8** | 176.5 ± 15 | 326 ± 38 | 84.7% |
| **9** | 223.9 ± 41 | 333.8 ± 45 | 50% |
| **10** | 188 ± 11 | 302.2 ± 24 | 60.7% |
| **Mean** | **189 ± 30** | **324.2 ± 25** | **71.5%** |

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**Figure 6.10** Bar graph showing Vickers micro-hardness numbers (VHN) of enamel surfaces of non-cavitated caries lesion (ICDAS 2) at baseline and following treatment with SDF. Data (n=10) were analysed using Paired t-test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, \*\*\*\*p-value < 0.0001).

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**Figure 6.11** Bar graph showing Vickers micro-hardness numbers (VHN) of enamel surfaces of non-cavitated caries lesion (ICDAS 2) at baseline and following treatment with SDF + KI. Data (n=10) were analysed using Paired t-test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, \*\*\*\*p-value < 0.0001).

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**Figure 6.12** Bar graph showing Vickers micro-hardness numbers (VHN) of enamel surfaces of non-cavitated caries lesion (ICDAS 2) following treatment with SDF and SDF + KI. Data (n=10) were analysed using Unpaired t-test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001, \*\*\*\*p-value < 0.0001).

### Differences in surface micro-hardness between sound and non-cavitated caries lesion

In this experiment, surface microhardness was compared between enamel with a non-cavitated lesion (ICDAS 2) and healthy enamel (ICDAS 0) on the same tooth (n=4). Six readings were recorded from each sample. The mean values of VHN and percentage decrease in microhardness are shown in Table 6.6. There was a statistically significant difference (p=0.014) in VHN between healthy enamel (323.5±34) and enamel of the non-cavitated lesion (190±24). The mean percentage decrease in microhardness was 41.2%. (Figure 6.13)

**Table ‎6.6** Vickers micro-hardness numbers (VHN) (mean± SD: Percentage decrease) of the enamel surfaces of sound teeth (ICDAS 0) and non-cavitated caries lesion (ICDAS 2).

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **ICDAS 0** | **ICDAS 2** | **Percentage**  **decrease** |
| **1** | 369 ± 33 | 158.6 ± 41 | 57% |
| **2** | 327 ± 81 | 216.5 ± 46 | 34% |
| **3** | 308.8 ± 16 | 198.8 ± 38 | 35.6% |
| **4** | 289.2 ± 41 | 189.1 ± 28 | 34.6% |
| **Mean** | **323.5 ± 34** | **190 ± 24** | **41.2%** |

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**Figure 6.13** Bar graph showing Vickers micro-hardness numbers (VHN) of the enamel surfaces of sound teeth (ICDAS 0) and non-cavitated caries lesion (ICDAS 2). Data (n=4) were analysed using Paired t-test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001).

Microhardness was also compared between dentine adjacent to healthy enamel (ICDAS 0) and dentin adjacent to a non-cavitated lesion (ICDAS 2) on the same tooth (n=3). Six readings were recorded from each sample. The mean values of VHN and the percentage decrease in microhardness are shown in Table 6.7.

 There was a 16.7% decrease between the microhardness of dentine adjacent to healthy enamel (72.4±14) and dentine adjacent to a non-cavitated lesion (60.3±8); however, this decrease was not statistically significant (p = 0.281). (Figure 6.14)

**Table ‎6.7** Vickers micro-hardness numbers (VHN) (mean± SD: Percentage decrease) of the dentine bordering sound enamel (ICDAS 0) and dentine bordering non-cavitated enamel surfaces (ICDAS 2).

|  |  |  |  |
| --- | --- | --- | --- |
| **Sample** | **ICDAS 0** | **ICDAS 2** | **Percentage**  **decrease** |
| **1** | 88.7 ± 11 | 60.75 ± 6 | 31.5% |
| **2** | 68.4 ± 6 | 62.4 ± 7 | 8.7% |
| **3** | 60.1 ± 7 | 51.6 ± 2 | 14% |
| **Mean** | **72 ± 14** | **60.3 ± 8** | **16.7%** |

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**Figure 6.14** Bar graph showing Vickers micro-hardness numbers (VHN) of the dentine bordering sound enamel (ICDAS 0) and dentine bordering non-cavitated enamel surfaces (ICDAS 2). Data (n=3) were analysed using Paired t-test (\*p-value < 0.05, \*\*p-value < 0.01, \*\*\*p-value < 0.001).

## Discussion

Matrix metalloproteinases have been shown to play an essential role in caries development, with each MMP taking a role in the process. Our immunofluorescence study (Chapter 5) found an increased intensity of MMP expression associated with caries lesions and also a strong positive correlation between the severity of caries and MMP intensity in dentine and odontoblast expression. Thus, inhibition of MMP activities could contribute to caries arrest. MMP inhibitors have been introduced in dentistry to control caries lesions as treatment moves towards minimally invasive dentistry with little or no carious tissue being removed (Schmoeckel et al., 2020). One such MMP inhibitor is silver diamine fluoride (SDF), which has previously been shown to have anti-MMP potential (Mei et al., 2012).

The efficacy of SDF in controlling caries lesions has been studied *in vivo*, where SDF was found to be an effective treatment in stopping the progression of caries lesions (Chu et al., 2002; Llodra et al., 2005; Braga et al., 2009; Dos Santos et al., 2012; Duangthip et al., 2016). One of the side effects of SDF, however, is the blackening of the caries lesion (Chu et al., 2002; Duangthip et al., 2016; Llodra et al., 2005). To overcome this side effect, the application of a salt, usually KI, after SDF application has been introduced and found to be effective in reducing discolouration (Knight et al., 2006), although the long-term effectiveness in reducing discolouration requires further study. Another clinical challenge with SDF application is that it needs to be applied for three minutes (Horst et al., 2016), which is sometimes challenging in young patients. Therefore, our study aimed to investigate SDF as an MMP inhibitor and evaluate its long-term effect on caries inhibition in relation to two important clinical parameters: the co-application of KI and the duration of application.

### Experimental approach

The main objective of this study was to investigate the activities of MMPs and the influence of cariostatic agents on their activity. Therefore, it was essential to test these agents against the native substrate of natural carious human dentine, in which complex regulation of endogenous proteases takes place. In our study, we used extracted carious human primary teeth. Previous studies have used other *in vitr*o models, such as demineralised dentine beams from the laboratory or recombinant proteins (Tezvergil-Mutluay et al., 2010, Tezvergil-Mutluay et al., 2011; Mei et al., 2012; Thompson et al., 2012; Mei et al., 2013; Altinci et al., 2019), which do not reflect the complex natural caries process or the effects of the challenging oral environment. Furthermore, multiple MMPs have been identified in caries lesions, with each MMP playing a role in the process. Beginning with the initial degradation of collagen by MMP1, MMP8 and MMP13, leading to the loss of the triple-helical conformation of collagen, further degradation is caused by MMP2 and MMP9 (Sulkala et al., 2007). All of these MMP activities reflect what would constitute a carious lesion. The use of recombinant proteins to represent matrix-bound MMPs in dentine in the study of MMP activity has limitations including measuring a specific MMP at a specific time and not the combined action of MMPs; host MMPs can cause activation and deactivation effects on other MMPs.

  In this study, we used carious dentine, with caries severity being determined by ICDAS scores. Caries with ICADS scores of 5 and 6 were included based on our immunofluorescence study' results which identified almost identical MMP immunoreactivity in the carious lesion at both of these ICDAS scores.

  To investigate the effect of SDF+/- KI and application times on overall MMP activity, the activity would ideally need to be measured before and after treatment on the same carious lesion. In this study, we were able to use the same lesion for comparison by examining carious dentine blocks obtained from three serial sections and applying each treatment to one block. It would have been better if we examined changes in activity in the same section, however, the assay used requires each section to be used once to ensure accurate measurements. In addition, the study aimed to examine the effect of SDF alone and then SDF+KI and using the same section would not allow us to achieve this goal. We conducted a preliminary experiment to measure MMP activity in the same carious lesion in three serial sections and to verify the agreement between the three measurements. Our results showed that the measurement of MMP activity in each carious lesion was almost identical, proving that this method is suitable for this study.

  SDF was the agent of choice in our study as one of its mechanisms of action is the inhibition of endogenous enzymes, (Mei et al., 2012). In the UK, the only available SDF agent for clinical use, approved for tooth sensitivity, is marketed as Riva Star. It has been used 'off-label' for caries control (Seifo et al., 2020). The Riva Star kit contains 38% SDF, a concentration that has been shown to be the most effective in controlling caries than either 30% or 12% (de Almeida et al., 2011; Savas et al., 2015; Fung et al., 2018; Tolba et al., 2019). In addition, one of our objectives was to compare the effects of post-SDF application of KI on MMP activity. KI is included in the Riva Star kit for post-SDF application to minimise the discolouration of caries lesions caused by SDF (Knight et al., 2006).

  When investigating SDF as a treatment option to control caries in children, we had to consider the use of primary teeth. Primary teeth and permanent teeth differ in their structural and micromechanical properties, with primary teeth having less minerals and fewer interdental tubules (Oliveira et al., 2010). In addition, primary teeth have been shown to be more susceptible to degradation by endogenous enzymes compared to permanent teeth (Scheffel et al., 2020), despite having the same collagen matrix composition (Torres et al., 2018). Another important aspect related to our study is whether there is a difference in the activity of endogenous enzymes between primary and permanent teeth. Gobbi et al., (2021) investigated the endogenous enzymes, MMP2 and MMP9, in primary and permanent dentine. They analysed dentine powders from intact and demineralised teeth by zymography. Their study showed that primary teeth had higher enzymatic activity compared to permanent teeth. This change was attributed to the difference in morphology and composition of the two dentitions. Furthermore, MMP9 was involved in tooth resorption in primary dentition, whereas this physiological function is not required in permanent dentition (Oshiro et al., 2001).

   In our study, the SensoLyte® Generic MMP Assay Kit (Anaspec, San Jose, CA, USA) was used to detect residual total MMP activity in carious dentine and to measure the anti-MMP potential of SDF+/- KI. The assay works by incubating an MMP source, for this, we used the natural carious lesion, with a chromogenic thiopeptide substrate, which is cleaved by the MMPs to release a sulphydryl group. This group reacts with 4,4'-dithiodipyridine or the Ellman reagent, a colour-developing thiol reagent, and was detected at 412 nm with a microplate reader. Tezvergil-Mutluay et al. (2010) investigated the effect of polyvinyl phosphonic acid on MMP activity with the same generic assay, using in vitro demineralised dentine beams as the MMP source, and found the acid to be a potent inhibitor of MMPs. Altinci et al. (2019) investigated the inhibitory effect of potassium fluoride in inhibiting endogenous enzymes with the generic assay, using a demineralised dentine beam as MMP source and found a limited inhibitory effect of potassium fluoride on MMPs.

### Percentage of inhibition of total MMP activity in caries lesions after SDF and SDF+KI application

In caries lesions, inhibition of MMPs is important to stop the progression of the lesion and thus reduce the need for restorative treatment. As mentioned previously, SDF has been shown to be effective in arresting caries lesions due to its antibacterial effect (Suzuki et al., 1976; Knight et al., 2005; de Almeida et al., 2011; Mei et al., 2013; Savas et al., 2015; Yu et al., 2018), remineralisation effect (Suzuki et al., 1976; Klein et al., 1999; Knight et al., 2005; Mei et al., 2013; Mei et al., 2014) and inhibition of endogenous enzymes (Mei et al., 2012). Our study found that SDF was a potent inhibitor of total MMP activity in caries lesions after one week of incubation (93%), which is consistent with the study of Mei et al. (2012). They first investigated the inhibitory effect of SDF on MMPs by analysing the activity of purified recombinant human MMP2, MMP8 and MMP9 separately using a fluorometric assay kit. Their study found that SDF inhibited MMP2, MMP8 and MMP9 by 79%, 94% and 82%, respectively, which is comparable to our results. However, it should be noted that their study used recombinant MMPs, which may not represent the matrix-bound MMPs in natural carious dentine. Furthermore, they tested each MMP separately, which did not consider the overall MMP activities typically found in dentine.

  The mechanism of action of MMP inhibitors and SDF in inhibiting endogenous MMPs is not fully understood. In general, MMP inhibitors act by altering the tertiary protein structure and masking or inactivating some functional MMP domains that are critical for MMP activity (Tezvergil-Mutluay et al., 2010). The inhibitory potential of SDF for MMPs could be due to the components of SDF or the synergistic effects between them. The silver ions in SDF have a greater affinity for proteins due to their low oxidation state and large ionic radius. In addition, silver has the ability to interact with the reactive side chains of enzymes, leading to the inactivation of their catalytic functions (Mei et al., 2013, Mei et al .,2018).

  Fluoride has also been shown to play a role in MMP inhibition, showing potent inhibition of MMP2, MMP8 and MMP9 (Mei et al., 2012). Kato et al., (2014) investigated the inhibitory effect of sodium fluoride on the activities of MMP2 and MMP9 using zymography and found that fluoride inhibited MMPs in a dose-dependent manner, with partially reversible inhibition achieved at 250-1500 ppm, while complete and irreversible inhibition was achieved at 5,000 ppm. The proposed mechanism was that the fluoride ions have a high electronegativity that allows them to bind to zinc and calcium ions, which are required for the activity and catalytic function of these endogenous enzymes.

  Another property of SDF that may contribute to MMP inhibition is the fact that it is an alkaline solution with a pH of 13 in products such as Riva star. MMPs have been shown to work effectively at a neutral pH, and a higher pH can interfere with their activity (Yan et al., 2001).

 As mentioned earlier, staining of caries lesions is one side effect of SDF treatment, and the use of KI after SDF has been introduced to reduce this staining (Knight et al., 2005). While several studies have shown the effectiveness of KI in reducing staining (Miller et al., 2016; Zhao et al., 2017; Zhao et al.,2019; Garg et al., 2019; Detsomboonrat et al., 2022), concerns have been raised about the impact of this treatment on the effectiveness of SDF. Several studies have investigated this combination and its effects on antibacterial properties (Knight et al., 2006; Hamama et al., 2015; Vinson et al., 2018; Abdullah et al., 2020; Haiat et al., 2021), and bond strength (Greenwall-Cohen et al., 2020; Knight et al., 2006; Koizumi et al., 2016) with conflicting results being reported. However, no study to date has investigated the possible influence of KI on the anti-MMP potential of SDF. Our study is, therefore, the first to investigate this and we found that the anti-MMP potential of SDF was negatively affected by the use of KI. The percentage of inhibition by SDF decreased from 93% to 55.5% after the application of KI. Although SDF + KI showed some degree of MMP inhibition, there was no significant difference compared to baseline activity.

  Potassium Iodide reduces the staining of SDF by reacting with free silver particles to form silver iodide. Therefore, the number and availability of silver ions are reduced when KI reacts with silver, reducing the caries-inhibiting effect of SDF. This mechanism of action may be the reason why KI reduced the potential of SDF to inhibit MMPs in our study, as well as for other studies that found that KI affected the efficacy of SDF in preventing the development of secondary root caries (Zhao et al., 2017) and that SDF alone showed higher efficacy in inhibiting the development of new root lesions than SDF+KI (Li et al.,2016).

  The negative effect of KI on the efficacy of SDF, calls into question the use of KI to reduce discolouration and compromises the anti-caries effect of SDF. However, further research and correctly powered *in vivo* work are needed to resolve the side effect of discolouration by SDF without compromising the anti-caries effect.

### Long-term inhibitory effect of SDF and SDF+KI on total MMP activity

Measuring the immediate effect of a caries treatment would give the first impression of effectiveness, however, it is important to consider the role of saliva and the challenging environment of the oral cavity on the long-term effectiveness of treatment. Mei et al. (2012), measured the inhibitory effect of SDF on MMPs only in the short term (60 minutes), whereas our study also measured the long-term effect by treating carious dentine blocks with SDF, with and without KI, and incubating in natural saliva for up to 12 weeks.

 In our study, residual MMP activities were measured at three incubation time points: 1, 4 and 12 weeks. The results after one week of incubation should provide initial information on the efficacy of SDF in inhibiting MMP compared to SDF+KI. In their article on the clinical use of SDF in dentistry, Seifo et al. (2020) recommended that patients be reviewed after four weeks of SDF application to assess the clinical success of the treatment and to look for signs of caries arrest. We measured the efficacy of SDF on MMPs after four weeks to get an idea of what happens to the efficacy of SDF around the 4-week review point. We assume that the clinicians will get information about what is likely to happen in the carious lesion when they combine this knowledge with their clinical examination. In our study, we found significant efficacy of SDF in inhibiting MMP, but this had not changed significantly in comparison to the 1-week analysis. After 12 weeks of incubation, the efficacy of SDF had decreased to 50% of baseline. Although the efficacy of SDF decreased significantly between week four and week 12, the efficacy of SDF was still statistically significantly higher than at baseline.

  From our study, we can conclude that SDF has a long-term effect on MMP activity that lasts up to 12 weeks. Lou et al., (2011) investigated the effect of SDF on hydroxyapatite powder using transmission electron microscopy and electron diffraction and found that metallic silver formed on the surface of hydroxyapatite, which was resistant to washing. The same results were obtained by Mei et al. (2013), who found that SDF reacted with hydroxyapatite, resulting in the formation of nanoscopic silver particles bound to hydroxyapatite crystals. Mei et al. (2014) examined arrested caries after the application of SDF using electron microscopy and found nanoscopic metallic silver particles bound to dentine collagen, however, after six months, they found that the silver particles were no longer present in the tooth samples. From these previous studies and our results, it appears that the application of SDF to caries lesions results in silver particles becoming embedded in dentine tubules and collagen, which is a potential means of sustainability, allowing a gradual release of silver and a long-lasting inhibitory effect on MMPs. All these findings further explain the results of *in vivo* studies showing that the bi-annual application of SDF provides better clinical outcomes than annual application (Zhi et al., 2012; Duangthip et al., 2015; Fung et al., 2018). However, there is no *in vivo* study investigating the efficacy of three-monthly applications of SDF on arresting caries compared to bi-annual applications.

 The long-term effect of using KI after SDF was also investigated in our study. We found that the percentage of MMP inhibition one week after SDF+KI treatment was low and insignificant compared to the baseline. The same trend was seen in the 4-week analysis and up to 12 weeks, where they showed a lower percentage of inhibition, reaching 20% after 12 weeks.

### Quantitative measurement of the extent of collagen degradation in caries lesions after SDF and SDF+KI application

To measure the degree of collagen degradation after SDF treatment, carious dentine blocks were incubated in natural saliva. As collagen degradation products seep into an incubation medium the assessment of the HYP concentration in natural saliva allowed us to directly assess collagen degradation and the effect of SDF in preventing this degradation. As explained in section 3.7.1, this assay is based on the assumption that 90% of the dry mass of dentine contains type I collagen and 10% of its mass consist of HYP (Linde et al., 1980; Goldberg et al., 2011).

  In dentine caries, collagen degradation is a crucial component of caries progression, as dentine contains 20% organic components (Nansi, 2008). The relationship between endogenous enzymes and collagen degradation has been studied previously. Chaussain-Miller et al. (2006), investigated the role of MMPs in human dentine and found that, in carious dentine lesions, the activation of MMP2, MMP8 and MMP9 played an important role in the degradation of the collagen matrix. Kato et al. (2012) investigated the effect of protease inhibitors on collagen matrix degradation using the HYP assay and found that inhibition of proteases reduced the degree of collagen degradation. Our study investigated the role of SDF as an MMP inhibitor in preventing collagen degradation of the carious lesion in an attempt to obtain useful information on the microstructural changes in the collagen of the carious dentine after SDF treatment.

  Our study showed that SDF is a potent inhibitor of collagen degradation in dentine caries lesions. The levels of HYP were significantly lower in the first week after treatment when compared to the control. Mei et al., (2013) investigated the inhibitory effect of different concentrations of SDF on collagen degradation. They treated healthy dentine blocks with SDF and exposed them to a pH cycle with demineralisation and remineralisation solutions for 8 days. The degradation of the collagen matrix was measured by analysing the HYP concentration in the remineralisation solution using HYP assay. They found that the concentration of HYP was significantly lower in the SDF group compared to the control group. Our results are in agreement with these results, as both found that SDF prevents collagen degradation. However, it should be noted that Mei et al (2013) used demineralised healthy dentine blocks and not natural carious dentine lesions.

  Another point to discuss is that there were differences in the amount of HYP. In the study by Mei et al. (2013), in which demineralised healthy dentine blocks were incubated with a high concentration of bacterial collagenase for a more extended incubation period, a higher concentration of HYP was noted. Bacterial collagenase leads to different effects on collagen molecules than mammalian collagenases as they cause multiple cleavages in collagen, which cause ¼- and ¾-cleavage. Therefore, prolonged use of bacterial collagenase is expected to result in a greater amount of degradation; hence, the assay detects a higher HYP, which does not reflect what normally occurs clinically, as less bacterial collagenase is found in the oral cavity (Kato et al. 2012). In our study, a natural demineralised caries lesion was used. During the incubation period, degradation was most likely caused by mammalian collagenases, as saliva was sterilised before incubation to exclude bacterial influence on our measurement. Therefore, the direct correlation between the inhibition of MMPs and the degree of collagen degradation could be measured.

  The role of fluoride in maintaining the collagen matrix was highlighted in the study by Kato et al. (2012). They found that the HYP concentration in the protease inhibitor groups was significantly lower than that of the fluoride-treated group but significantly higher in the fluoride-treated group than that of the control groups, suggesting that fluoride plays a role in collagen maintenance through its remineralisation capacity; this depends on the maintenance of the collagen matrix (Ganss et al., 2010). Kato et al. (2012) concluded that products combining protease inhibitors and fluoride have a more significant potential to maintain the collagen matrix. SDF combines these two components, which may explain its strong inhibitory effect on collagen degradation.

The synergistic effect of the SDF components in maintaining the collagen matrix can be explained by the following formula, which shows the reaction that occurs when SDF is applied to a caries lesion (Lou et al., 2011):

             Ca 10 (PO4)6(OH)2 + Ag (NH3)2F     ⟶         Ag3PO4 + CaF2 + NH4

CaF2 is formed in this reaction, which serves as a fluoride reservoir. When exposed to low pH, fluoride is released to regulate pH and form a more acid-resistant fluorapatite, which is modulated by high pH for SDF (Mei et al., 2013). When the collagen matrix is exposed, it becomes more susceptible to protease activities. In addition to the remineralising effect of fluoride, which can shield the collagen matrix, fluoride has been shown to have an inhibitory effect on the activities of endogenous MMP enzymes (Kato et al., 2014), which subsequently inhibit collagen degradation.

  The incubation period in the study by Mei et al. (2013) was relatively short, at eight days, so does not reflect the long-term effect of SDF on collagen. In our study, SDF's short- and long-term effects were considered, and the analysis of collagen degradation was measured for up to 12 weeks. Although the HYP concentration was higher at week 12 than at weeks one and four, the difference from the control was still statistically significant. Therefore, the inhibitory effect of SDF on collagen degradation was effective in both the short and long term. This result also supports the relationship between MMP inhibitors and collagen degradation.

  The influence of the application of KI on the efficacy of SDF in inhibiting collagen degradation was also investigated. The concentration of HYP in the SDF+KI group was low compared to the SDF group, suggesting that KI impairs the ability of SDF to maintain the collagen matrix.

### Percentage of inhibition of total MMP activity in caries lesions after different application times of SDF

Treatment of caries lesions with SDF is a simple technique, especially in children with limited cooperation or special needs (Horst et al., 2016). It has been recommended that the application time of SDF is between 1 and 3 minutes to ensure that the SDF is sufficiently adsorbed by the lesion (Horst et al., 2016; Seifo et al., 2020). However, the real rationale for using these time points is unclear. In addition, three minutes is a very long time compared to one minute in a clinical situation, especially when dealing with young patients who cannot cope with the treatment.

  Laboratory studies have focused on the effect of application duration on one aspect of the SDF mechanism of action in caries control, namely the remineralisation effect. It was found that a 30-second (Srisomboon et al., 2021) and a 1-minute (Punhagui et al., 2021) application of SDF showed comparable results to a 3-minute application. In our study, we investigated the influence of SDF application times on the MMP-inhibitory effect of SDF by comparing 1- and 3-minute application times with a generic MMP assay. We found that SDF had a strong MMP inhibitory effect regardless of the application time. Therefore, even a 1-minute application of SDF could be clinically effective in stopping caries activity. However, since Srisomboon et al. (2021) found that a 30-second application time of SDF is as effective as one and three minutes in the mineral deposition of caries lesions, it would be of interest to investigate the effects of a shorter application time of SDF, such as 30 seconds or less, on the anti-MMP inhibitory effect.

### Effect of SDF+/-KI application on the micro-hardness of non-cavitated caries lesions

The pathogenesis of caries in enamel and dentine is different. Enamel is a hard and mineralised structure (95

% by weight), and only 5% of its weight consists of organic matrix and water (Cate, 1998). Enamel caries is basically the dissolution of minerals by bacterial acids. Therefore, enamel caries could be explained by the degree of mineral loss. One method of measuring this mineral loss in enamel is the micro-hardness test, which was first used over 50 years ago in pioneering studies measuring enamel hardness(Avery, 1962) and continues to be used today in similar studies (Topbaş et al., 2019b; Sadyrin et al., 2020; Sorkhdini et al., 2020; Punhagui et al., 2021; Sorkhdini et al., 2021). In our study, we investigated the differences in surface micro-hardness between healthy enamel and enamel from non-cavitated carious lesions using the Vickers hardness test which is a type of micro-hardness test. We then investigated the effect of SDF application on the micro-hardness of carious enamel lesions and compared it with the application of KI after SDF.

The micro-hardness results in our study replicate the results of other studies. For example, we found that the average hardness of healthy enamel in primary teeth was 323.5 VHN, comparable to a previous study in which healthy enamel had a hardness of 354.4 VHN (Punhagui et al., 2021). Our study showed a statistically significant, 41% decrease in surface micro-hardness of carious enamel compared to healthy enamel. Previous study (Punhagui et al., 2021) have found a higher decrease of 69%, however, our results examined natural carious lesions, whereas, in their study, enamel lesions were induced in vitro by pH cycling with a demineralising solution. This difference between healthy and carious enamel was also found in another study with, a reduction in mineral density of up to 50% (Huang et al., 2010), however, this study was conducted on permanent teeth and the enamel of primary teeth differs from that of permanent teeth in several aspects. Primary enamel is about 50% thinner than permanent enamel, making primary teeth more brittle and less mineralised, which makes them more susceptible to the rapid development of caries compared to permanent teeth (Koutsi et al., 1994; Oliveira et al., 2010). These differences may lead to different demineralisation and remineralisation patterns. Therefore, it is important to investigate the differences between primary healthy and carious enamel and the effect of SDF+/- KI on primary enamel.

 It was interesting to study the differences in micro-hardness of dentine at the margin of healthy enamel and dentine at the margin of enamel from non-cavitated lesions on the same tooth to investigate the changes that may occur in dentine as a result of an early carious lesion. We found that the micro-hardness of dentine-bordering enamel lesions showed a reduction (by 16%) compared to dentine-bordering healthy enamel. Sadyrin et al. (2020) found fewer changes in dentine-bordering enamel lesions in the permanent tooth compared to the healthy side. However, these differences can be attributed to the differences between primary and permanent teeth, as mentioned earlier. In early carious lesions, when the enamel barrier is disrupted, the metabolic activity of cariogenic bacteria leads to the release of bacterial components into the dentinal tubules, resulting in mineral dissolution (Love and Jenkinson, 2002). In our immunofluorescence study (Chapter 5), we noted a very early response of the dentine-pulp complex to early carious lesions, which may also be due to the permeability-promoted hardness changes in dentine adjacent to non-carious lesions of the dentine upon penetration of potential irritants into the pulp (Love and Jenkinson, 2002).

  We investigated the effect of SDF+/- KI on enamel micro-hardness in non-cavitated carious lesions (ICDAS 2). Our study found that the application of SDF resulted in a significant 66.8% increase in micro-hardness. Two studies have also investigated the effect of SDF on the mineral density of enamel lesions (Kim et al., 2020; Punhagui et al., 2021). A smaller percentage increase in microhardness (32%) was reported in the study by Punhagui et al. (2021), which investigated the effect of 38% SDF on enamel micro-hardness using the hardness test. However, they used induced enamel lesions, resulting in a much lower baseline hardness than our baseline measurements on natural enamel lesions. Kim et al. (2020) investigated the remineralisation effect of 38% SDF compared to 5% sodium fluoride varnish on artificially induced enamel caries using energy-dispersive X-ray spectroscopy and scanning electron microscopy. They found that SDF had a more substantial remineralising effect on enamel lesions than sodium fluoride. However, they used induced enamel lesions in bovine teeth, which may be different from natural caries in human teeth. In addition, other methods were used to analyse mineral density compared to our results such as energy-dispersive X-ray spectroscopy and scanning electron microscopy.

  The possible influence of KI on the effect of SDF on the micro-hardness of non-cavitated lesions has not been investigated before; therefore, this study is innovative as it is the first to investigate this. According to our results, the application of KI after SDF did not affect the ability of SDF to increase the hardness of enamel lesions. In the SDF+KI group, a 71.5% increase in micro-hardness was observed, which is interestingly more than in the SDF group (66.8%). However, the differences were not statistically significant.

  Results of previous studies could explain the mechanism behind the ability of SDF to remineralise enamel lesions. Suzuki and colleagues (1974) found that silver phosphate formed when SDF was mixed with enamel powder, which contributed to the hardness observed in arrested lesions. This observation is consistent with Kim et al. (2020), who found that silver ions were deposited on enamel after SDF treatment. Fluoride ions have also been shown to be involved in this process. Lui et al. (2012) studied the effect of fluoride and silver ions on enamel remineralisation in 40 extracted healthy premolars. The enamel lesion was induced *in vitro* with a demineralising solution. They found that the application of fluoride inhibited enamel demineralisation to a greater extent compared to silver ions. Sorkhdini et al. (2021) also suggested that fluoride content plays a more significant role than silver content in preventing caries lesions in enamel. However, these results should be interpreted with caution, as they investigated the prevention of enamel lesions and not the treatment of enamel lesions.

  The present results suggest that SDF offers an alternative approach to remineralising primary tooth enamel lesions. Furthermore, the application of KI after SDF did not affect the ability of SDF to remineralise enamel lesions. However, in our study, the change in microhardness was measured over a relatively short period of time. Measuring the long-term effects of SDF+/- KI in remineralising enamel lesions with more extended incubation periods might have provided helpful information to predict the longevity of these outcomes.

## Summary

Silver diamine fluoride is used to control caries lesions, especially in young patients, because it is simple and easy to use and conforms to minimally invasive dentistry. In this study, SDF was shown to inhibit MMPs in the short and long term. Inhibition of these endogenous enzymes in dentine is one of the mechanisms of action of SDF in arresting caries. Accordingly, SDF was able to protect and preserve the organic structure of dentine. In addition, SDF increased the surface micro-hardness of enamel caries. Understanding the mechanism of action and the ability to control caries in cavitated and non-cavitated lesions, as well as the influence of KI on the efficacy of SDF, will provide clinicians with the necessary knowledge to make a clinical decision on the use of this combination, therefore,  using KI after SDF in cavitated caries lesions reduces the effectiveness of SDF in arresting caries lesions; on the other hand, using KI after SDF in non-cavitated lesions did not affect the ability of SDF to increase the surface micro-hardness of enamel lesions. In cavitated lesions, and after consulting with parents and patients and taking their views into account, if aesthetics is of low concern and arresting caries is a priority, clinicians should not use KI after SDF treatment. Investigating the influence of SDF application time on SDF efficacy will help to reduce clinicians' concerns when they have to use SDF under challenging situations such as treating young patients or patients with special needs who have limited ability to tolerate the treatment. The main findings of this study are summarised below:

* SDF has a caries inhibitory effect on dentine caries due to its potent inhibition of total endogenous MMPs.
* The caries-inhibiting effect of SDF shows long-term inhibition of total MMP activity.
* SDF is effective in protecting and preventing the degradation of the collagen matrix in dentine caries, consequently leading to the arrest of dentine caries lesions.
* The use of KI after treatment of dentine caries with SDF to reduce discolouration leads to a reduction in the inhibitory effect of SDF on MMPs. Therefore, any potential benefit of KI in reducing staining may be obviated by a reduction in the overall efficacy of SDF.
* There were no significant differences between 1- and 3-minute application times of SDF in terms of MMP inhibition; therefore, even a 1-minute application of SDF could be clinically effective in arresting caries activity.
* Fundamental changes in micro-hardness in human primary enamel and dentine in early carious non-cavitated lesions.
* SDF increases enamel micro-hardness in non-cavitated caries lesions.
* The application of KI to the enamel lesion after SDF did not affect the efficacy of SDF in remineralising the lesion. Thus, the use of KI to reduce the discolouration of the enamel lesion does not affect the remineralisation effect of SDF on the lesion.

# CHAPTER 7

GENERAL DISCUSSION, CONCLUSION & FUTURE PERSPECTIVES

# Chapter 7: General discussion, conclusion & future perspectives

## Thesis Overview

This study investigated the role of MMPs in relation to caries diagnosis and caries management. Overall, the aims and objectives of this study were achieved, and novel and clinically relevant data were produced.

  Our results require the rejection of the first null hypothesis that MMPs do not correlate with the stage of caries and do not differ with the depth of caries.  We demonstrated the presence of gelatinolytic MMPs (MMP2 and MMP9) in healthy teeth and carious lesions with increased expression in carious lesions using an alternative immunostaining technique. Furthermore, the localisation of these MMPs was different in the different depths of the lesion, with less MMP found in the inner dentine. The correlation between MMPs in dentine and the odontoblast, and the appearance of the caries surface, as assessed by ICDAS, was investigated and a high correlation was found between them. This is a novel finding that provides the clinician using ICDAS as a scoring system with detailed information to better understand the lesion and thus avoid under or over-treatment. In addition, collagen degradation was seen to be increased with increased MMP expression and caries severity. The use of ICDAS to assess the severity of caries lesions and how this correlates with the presence and expression of MMP in these lesions validates the modern approach to caries treatment with the minimally invasive concept (Banerjee, 2017).

  We found SDF to be a potent inhibitor of MMPs and that even a 1-minute application of SDF could effectively arrest caries activity. For the first time, a negative effect of KI was found on the anti-MMP potential of SDF in arresting caries lesions in dentine and inhibiting collagen degradation. These results require the rejection of the second null hypothesis that the application of KI after SDF does not affect the anti-MMP potential of SDF. On the other hand, KI did not affect the ability of SDF to improve the surface micro-hardness of non-cavitated (enamel) caries lesions. This result suggests that SDF is an effective treatment and that any potential benefit of KI in reducing staining in dentine caries is negated by a reduction in the overall efficacy of SDF. In cavitated lesions, and after consulting with parents and patients and taking their views into account, if aesthetics is of low concern and arresting caries is a priority, clinicians should not use KI after SDF treatment.

## General discussion

  Endogenous enzymes (MMPs) play a fundamental role in the development of caries. The role of MMPs in relation to caries diagnosis (see Chapter 5) and caries management (see Chapter 6) was investigated in this study. In this general discussion section, the strengths, limitations and key findings is discussed. The clinical relevance of the study is outlined and recommendations for future research are made.

### Strengths and limitations

The study had a number of strengths, including the tooth samples used in our study, which were obtained from previous work by our group and had previously been investigated, for the detection of proximal caries in primary molars (Subka et al., 2018). Their in vivo study is unique as it remains the only one to investigate the diagnosis of proximal caries in primary teeth with histological validation using ICDAS, radiographs, laser fluorescence pen and temporary tooth separation.

  To achieve the aims of our study, we had to consider that MMPs should be studied in or close to their natural environment in caries lesions using a natural substrate. Therefore, the use of human teeth and natural caries lesions was essential for the  correct interpretation of the results and added to the strength of my study in comparison to previous studies where demineralised dentine beams from the laboratory or recombinant proteins were used (Tezvergil-Mutluay et al., 2010; Tezvergil-Mutluay et al., 2011; Mei et al., 2012; Thompson et al., 2012; Mei et al., 2013; Altinci et al., 2019)  However, this demineralised healthy dentine would not mimic the complex natural caries process and the  natural progression of a carious lesion, especially the time factor and the role of host factors, such as saliva, on the action of collagenolytic enzymes in caries (Tjaderhane et al., 2015).

  When investigating the MMP-inhibitory capacity of SDF as a treatment option to control caries in children, we had to consider the use of primary teeth, which further added to the strength of our study. Primary teeth and permanent teeth differ in their structural and micromechanical properties, with primary teeth having less minerals and fewer interdental tubules (Oliveira et al., 2010). In addition, studies have shown that primary teeth have higher enzymatic activity compared to permanent teeth (Oshiro et al., 2001).

  The main limitation of this study was the sample size. Although a total of 65 teeth from a previous study were included, the number of surfaces examined varied considerably for each ICDAS score. For the study of MMPs in each ICDAS score, it was not possible to obtain a large and equal number of surfaces for all ICDAS scores. Therefore, to ensure that similar samples were used, three from each ICDAS score were included in this study. It is worth noting that despite the small sample size, the variance between samples was minimal as all samples were from primary teeth of children aged 5-10 years and all were molars with coronal proximal caries lesions.

### Key findings

Figure 1.7 summarises the main findings of this thesis and their significance. A protocol for staining MMPs in the dentine of healthy and carious lesions was established. Various staining techniques were tried (see Chapter 4), and immunocytochemistry using the immunofluorescence double staining technique proved to be the most appropriate method to double-stain and co-localise MMP2 and MMP9 without overlap.

  In Chapter 5, the double immunofluorescence technique was applied to the main samples to investigate the presence of gelatinolytic MMPs (MMP2 and MMP9) in natural carious dentine at different caries stages and depths in human primary teeth. The role of MMPs in the context of caries diagnosis was discussed. The focus was not only on their presence in healthy and carious dentine of primary human teeth but also on the correlation between the presence of MMPs and the surface appearance of caries as assessed by ICDAS, which is clinically crucial to obtain detailed information about the lesion at diagnosis and to decide on a treatment plan.

  MMPs in dentine in relation to non-cavitated (ICDAS 1 and 2) lesions have not been investigated previously. Therefore, our study is the first study to investigate and report this. Low immunoreactivity of MMP2 and MMP9 was observed in dentine. Interestingly, however, a significant increase in intensity was observed in the dentine-pulp region in ICDAS 2 compared to ICDAS 0. This suggests a very early response of the dentine-pulp complex to early caries lesions.

  In ICDAS 3 code (micro-cavities lesions), the question of the best treatment approach always arises. To date, no previous study has investigated the presence of MMPs in dentine in ICDAS 3. Our study was the first to investigate this and found that the immunoreactivity of MMP2 and MMP9 was increased throughout the outer third of the dentine, with more intense staining near the enamel-dentine junction. Their expression at the dentine-pulp junction was increased compared to ICDAS 2, but the increase was not statistically significant. It was also found that the immunoreactivity of MMP2 and MMP9 was weaker in the middle and inner third of the dentine. The low MMP expression in caries lesions with ICDAS 3 may explain the results of previous studies (Hesse et al., 2014; Monoz-Sandoval et al., 2019; Lindquist and Emilson, 2020), which showed that lesions at this particular stage of caries responded well to sealing, consistent with the concept of minimally invasive dentistry (Ericson et al., 2003).

   In ICDAS 4, significantly higher immunoreactivity of both MMPs was observed at the enamel dentine junction and in the outer and middle thirds of the dentine, while it became weaker in the inner third of the dentine. A significant increase in intensity was observed in odontoblasts compared to caries lesions with an ICDAS 3 score. These results are consistent with previous studies that found a high abundance of MMP2 and MMP9 in caries lesions in permanent teeth (Sulkala, 2002; Goldberg et al., 2003; Sulkala et al., 2007; Charadram et al., 2012). However, these studies did not take into account the clinical diagnosis or stage of caries when comparing MMP expression in healthy and carious lesions. This increase in MMP immunoreactivity compared to ICDAS 3 in our study suggests that the progression of the carious lesion and the extent of collagen degradation are likely to be greater, raising the question of whether cavity sealing alone would lead to arrest of the carious lesion with ICDAS 4 compared to an ICDAS 3 lesion where MMPs have lower immunoreactivity.

  In cavitated lesions with ICDAS scores of 5 and 6, there was a significant increase in the intensity of staining in the enamel-dentine junction, outer and middle thirds of the dentine and odontoblasts, with this reactivity becoming weaker in the inner third of the dentine. This finding suggests that the caries lesion stimulates the expression of MMP2 and MMP9, leading to increased expression by odontoblasts and their presence in carious dentine. This is consistent with other studies that have found MMP9 and MMP2 to be significantly increased in carious lesions (Sulkala, 2002; Goldberg et al., 2003; Sulkala et al., 2007; Charadram et al., 2012). However, it should be noted, once more, that these previous studies were conducted on demineralised tooth sections, not on a natural caries lesion, on rat teeth and carious lesions without measuring the stage of caries severity.

  Our results showed that the expression levels of MMP2 and MMP9 are lower in the inner third of the dentine, suggesting and supporting the selective removal of caries tissue from cavities, as the treatment of caries lesions should be based on the principle and techniques of minimally invasive dentistry (Pitts, 2004). In addition, the complete removal of caries increases the risk of exposing the pulp, leading to more pulpal complications (Ricketts et al., 2013). Although low levels of MMP immunoreactivity have been found in the inner dentine, their presence in this area has been shown to potentially compromise the integrity of the resin-dentine bond. Once activated by acid etching, they degrade the collagenous matrix of the hybrid layer (Pashley et al., 2004). Understanding the role of these endogenous enzymes in the hydrolysis of the hybrid layer has led to various approaches to maintain the integrity of this layer (Carrilho, 2012; Tjaderhane et al., 2013). Therefore, this study suggests that lower expression of MMP2 and MMP9 in carious inner (affected) dentine indicates less collagen degradation and degradation of the hybrid layer, and better survival of the restorative material can be expected.

  The vital role of endogenous enzymes in physiological and pathological processes has already been explained in detail. Although MMP localisation in healthy teeth and caries lesions has been investigated in many studies, no study to date has linked MMP expression to the stage of the caries lesion or the surface appearance of the lesion. This study represents a new addition to this field by linking caries diagnosis to MMP in the dentine substrate. My study found a significant positive correlation between the ICDAS score and MMP2 and MMP9. MMP expression in odontoblasts and their presence in the dentine and caries lesion increases with increasing caries stage and severity. This is of clinical importance as it provides more knowledge about the enzymes and their function during the caries process which improves diagnosis and helps in treatment planning. Furthermore, how caries lesion assessed by ICDAS correlates with the presence and expression of MMP in these lesions validates the modern approach to caries treatment with the minimally invasive concept.

  Chapter 6 discussed the role of MMPs in caries management using MMP inhibitors. The treatment of caries in young patients is challenging and requires new or alternative approaches to control the lesion. SDF is one of these approaches. It is simple, easy to use and in line with the concept of minimally invasive dentistry. In a previous study, SDF was shown to inhibit MMPs (Mei et al., 2012). However, it should be noted that this study used recombinant MMPs, which may not represent the matrix-bound MMPs in natural carious dentine. In addition, they tested each MMP individually, which does not consider the overall activities of the MMPs typically found in dentine. In our study, SDF inhibited total MMP activity in carious lesions after one week of incubation (93%).

  There are two critical, clinical aspects of applying SDF to carious lesions: first, the application of potassium iodide after SDF treatment, which was introduced to overcome the side effect of discolouration caused by SDF, and second, the application time, as it is clinically recommended that SDF be applied to caries for 3 minutes, which can be challenging in young patients. Previous studies have shown conflicting results regarding the impact of the application of KI after SDF, to prevent discolouration, on the caries-inhibiting effect of SDF. Furthermore, no study has yet investigated the possible influence of KI on the anti-MMP potential of SDF. Our study was the first to investigate this and found a negative effect of KI on the efficacy of SDF in inhibiting MMPs and maintaining collagen degradation.

Our study also investigated the impact of SDF application times on the MMP inhibitory effect of SDF by comparing applications for 1- and 3-minute. We found that SDF has a strong MMP inhibitory effect regardless of the application time and so suggest that even a 1-minute application of SDF could be clinically effective in stopping caries activity.

  We extended our research to investigate the effect of SDF+/- KI on enamel surface micro-hardness in non-cavitated carious lesions (ICDAS 2) and found that the application of SDF resulted in a significant increase in surface micro-hardness (66.8%). Our study showed that the application of KI after SDF did not affect the ability of SDF to increase the surface hardness of enamel lesions.

    The study provides clinicians with the necessary evidence to make a clinical decision on the combination of SDF and KI in treating caries lesions in enamel (non-cavitated) and dentine (cavitated) and suggests that using KI after SDF in cavitated caries lesions reduces the effectiveness of SDF in arresting caries lesions, however, using KI after SDF in non-cavitated lesions did not affect the ability of SDF to increase the surface micro-hardness of enamel lesions.

## Conclusion

Several conclusions can be made as a result of this investigation as highlighted below:

**1)** The use of ICDAS to assess the severity of caries lesions and how this correlates with the presence and expression of MMP in these lesions validates the modern approach to caries treatment with the minimally invasive concept.

**2)** The low expression of MMP2 and MMP9 and the low degree of collagen degradation in caries lesions with ICDAS 1, 2 and 3 support that a restorative approach is not indicated. Furthermore, low MMP presence in the inner dentine supports the minimally invasive approach in treating cavitated caries lesions.

**3)** The early response of odontoblasts to an early caries lesion (ICDAS 2) and the decrease in the micro-hardness of dentine bordering these lesions demonstrate the importance of early detection of caries lesions and the implementation of non/micro-invasive measures that limit the progression of the lesion to an irreversible lesion.

**4)** The caries-inhibiting effects of SDF include short- and long-term inhibition of total MMP activity and effective protection and prevention of collagen matrix degradation in dentine caries.

**5)** SDF increases the surface micro-hardness of the enamel surface in non-cavitated caries lesions.

**6)** The use of KI after treatment of dentine caries with SDF to reduce discolouration, results in a reduction in the inhibitory effect of SDF on MMPs. Therefore, any potential benefit of KI in reducing discolouration may be obviated by a reduction in the overall efficacy of SDF. The application of KI to the enamel lesion after SDF had no effect on the efficacy of SDF in remineralising the lesion.

## Clinical significance

The study of MMPs and their role in dental caries at different stages of disease progression has provided a more detailed picture of the pathogenesis of caries. *In Vitro* studies and basic science research, as in this study, improve our understanding of these endogenous enzymes and helps to identify specific MMPs in carious lesions that could allow novel therapeutic options for caries prevention and treatment to be identified; for example, MMP inhibitors may be employed to inhibit the progression of dental caries. These *in vitro* studies are needed before any commercial interest and clinical trials are undertaken. Improving knowledge of the role of MMPs in the affected dentine layer will support the selective removal of carious tissue. This may help develop clinical strategies for fabricating durable restorations in which the affected dentine layer is a suitable substrate for dentine restoration adhesion. The correlation between the presence of MMPs and surface appearance gives dentists using ICDAS as a visual diagnostic method a more detailed picture to avoid over- or underestimating carious primary teeth based on the surface appearance of the carious lesion. This also validates the modern approach to caries treatment with the minimally invasive concept.

  SDF is used to control caries lesions, especially in young patients, because it is simple and easy to use and is consistent with minimally invasive dentistry. In this study, SDF inhibited MMPs in the short- and long-term. Inhibition of these endogenous enzymes in dentine is one of the mechanisms of action of SDF in preventing caries. Accordingly, SDF was able to protect and preserve the organic structure of dentine. In addition, SDF increased the surface microhardness of enamel caries. Understanding the mechanism of action and the ability to control caries in cavitated and non-cavitated lesions, as well as the influence of KI on the efficacy of SDF, will provide clinicians with the necessary knowledge to make a clinical decision on the use of this combination. Investigating the impact of SDF application time on SDF efficacy and our finding that a 1- minute application is effective in arresting caries lesions, will help alleviate clinicians' concerns when they need to use SDF in challenging situations, such as treating young patients or patients with special needs who have limited ability to tolerate the treatment. Furthermore, SDF has not been widely used in the UK. Basic research such as this study will help to support its clinical application in primary care.

**Figure 7.1** Summary of main thesis findings and their significance. Annotation: Matrix metalloproteinases (MMP), International Caries and Assessment System (ICDAS), Silver diamine fluoride (SDF), Potassium iodide (KI).

## Future perspectives

Our results answered several research questions regarding the role of MMPs in the diagnosis of caries lesions by investigating the localisation of MMPs in caries lesions and their correlation with clinical diagnosis by ICDAS. We also investigated the role of MMPs in caries management by examining SDF for anti-MMP activities in relation to two important clinical variables: application of KI and application time. Future studies should be conducted to further our understanding of the role of MMPs in caries lesions.

**1) Further immunohistochemistry**

Conduct experiment on a larger sample size to insure reliability and generalizability of the results. In our study, a protocol was optimised for staining MMP2 and MMP9. It would be useful to apply this protocol to other MMPs that have been shown to play a role in caries progressions, such as MMP8 and cysteine cathepsin, and to correlate their expression with collagen degradation and caries surface appearance.

**2) Further collagen characterisation techniques**

The degree of collagen degradation in caries lesions of varying severity assessed by ICDAS was performed in our study using the HYP assay. Other characterisation techniques, such as scanning electron microscopy (SEM), could be used to further investigate collagen. SEM is generally considered a more sensitive and accurate method for characterizing collagen degradation than hydroxyproline assay because it can directly visualize the structural changes in collagen fibres during degradation. SEM can provide information about the size and shape of collagen fibres, as well as any changes in their organization or structure. Hydroxyproline assay, on the other hand, is a chemical method that measures the amount of hydroxyproline present in a sample. While it can provide information about the overall collagen content of a sample, it cannot provide detailed information about the structure or organization of collagen fibres.

**3) Salivary transcriptomics and proteomics study**

Saliva has been proposed as a diagnostic tool and thus the identification of salivary biomarkers could be used to detect caries at an earlier stage or for the evaluation, diagnosis, treatment and prognosis of dental caries. Saliva samples could be collected from patients with and without caries to perform in-depth proteomic and transcriptomic analyses to identify disease-specific biomarkers that could form the basis for a novel, easy-to-use point-of-care test.

**4) HYP assay**

Our study used the HYP assay to quantify the degree of collagen degradation in carious dentine lesions. This assay could also measure collagen degradation after treatment with other MMP inhibitors to investigate whether these treatments help maintain collagen integrity and compare their efficacy with SDF treatment.

**5) SDF application time**

Our study found that a 1-minute application of SDF could be clinically effective in arresting caries activity. However, since another study found that a 30-second application time of SDF was as effective as one and three minutes in the mineral deposition of caries lesions, it would be of interest to investigate the effects of a shorter application time of SDF, such as 30 seconds or less, on the anti-MMP inhibitory effect.

**6) Synergistic effect of SDF's anti-MMP potential with other treatments**

Our study showed that SDF is a potent MMP inhibitor. Future studies evaluating the synergistic effect of SDF with fluoride varnish or chlorhexidine in terms of the inhibitory effect of SDF on MMPs would be beneficial.

**7) Qualitative studies**

Parents' views and opinions on SDF discolouration have already been studied in America and China. However, it would be beneficial to study this in the UK and compare the views of parents in different countries. In addition, children's views on SDF staining have not been studied. It is important to involve children in the research and consider their opinions.

**8) SDF discolouration**

Our study found KI to negatively affect the anti-MMP potential of SDF. However, further research and correctly powered in vivo studies are needed to resolve the side effect of discolouration by SDF without compromising the anti-caries effect of SDF.

**9) The effects of SDF on the microhardness of caries lesions**

Our study investigated the effect of SDF on the inhibition of MMP activity and collagen degradation. It would be beneficial to measure the mineral content in the incubation medium to evaluate the effect of SDF on mineral loss in carious dentine lesions.

**10) Long-term effect of SDF on the surface micro-hardness of the non-cavitated lesion**

Our study measured the change in surface micro-hardness of non-cavitated lesions over a relatively short period. Measuring the long-term effects of SDF+/- KI in remineralising enamel lesions with longer incubation periods would provide useful information to predict the longevity of these outcomes.

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# Appendices

**Appendix 1:** The original ethical approval (Authorisation of the project, 14/09/2012).

**Appendix 2:** List of samples retrieved from Biobank and used in the current study.

**Appendix 3:** Substantial amendments (30/06/2016).

**Appendix 4:** Approval from the University Research Ethics Committee (01/10/2020).