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Title: Correlation between Matrix Metalloproteinase presence and caries surface appearance

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Abstract (250 words)

Introduction: Cariogenic bacterial acids dissolve the inorganic elements in dentine, leaving the dentine matrix exposed. Host-derived matrix metalloproteinases (MMPs) play an essential role in caries progression as they are significant regulators of extracellular matrix turnover and can degrade exposed collagen. This paper investigates the expression of MMP2 and MMP9 across various stages of caries in primary human teeth and relate this with a diagnosis recorded by The International Caries Detection and Assessment System (ICDAS). **Methods**: Twenty-four sections (150um in thickness) from extracted teeth, clinically diagnosed using ICDAS, were immunohistochemically treated with monoclonal anti-MMP2 and anti-MMP9 antibodies. Positive staining was visualised by immunofluorescence using a VectorFluor Duet Double Labeling Kit. Images from triplicate samples for each ICDAS score were analysed using ImageJ software. Collagen degradation in caries lesions was detected using a hydroxyproline assay. **Results**: MMPs were weakly detected in caries with ICDAS 1-2 scores, and an insignificant increase was detected in ICDAS 3. However, a significant increase in MMP expression was seen in caries with an ICDAS score of 4-6. There was a strong positive correlation between the ICDAS score and MMP2, [$r(6) = .86$, $p = .002$] and between ICDAS and MMP9, [$r(6) = .82$, $p = .004$]. Data were analysed using two-way ANOVA followed by Tukey multiple comparison test ($^*p < 0.05$). **Conclusion:** The use of ICDAS to assess the severity of caries lesions and how this correlates with the presence of MMP in these lesions validates the modern approach to caries management with a minimally invasive concept.

Introduction:

Dental caries is a common global disease that exacts a significant toll on individuals' health-related quality of life [1,2]. The aetiology of this condition is rooted in the metabolic activities of cariogenic bacteria, which ferment dietary carbohydrates to release lactic acid. In dentine this acid initiates the dissolution of tooth hydroxyapatite, leading to the gradual depletion of the collagen matrix [3,4]. Despite the prominent role played by bacterial proteases in breaking down the organic components within carious lesions, the integrity of collagen remains surprisingly intact [5-8]. In this context, matrix metalloproteinases (MMPs), a family of calcium-dependent zinc-containing endopeptidases, emerge as pivotal players in both physiological and pathological tissue remodelling (9,10).

Of particular interest, among the various MMPs implicated in dental caries, are MMP2 and MMP9, often referred to as gelatinolytic MMPs. These enzymes play a central role in the intricate cascade of events underlying the progression of carious lesions. During the early stages of caries development, the oral environment becomes acidic due to bacterial metabolism, resulting in a decrease in oral pH (10,11). This acidic milieu not only dissolves the hydroxyapatite of the tooth but also promotes the activation of endogenous host-derived enzymes, including MMPs [10]. Upon activation, these MMPs assume their enzymatic roles and initiate the degradation of extracellular matrix molecules [12]. Their ability to dismantle the collagen matrix within the dentine structure is a critical aspect of caries lesion progression. As MMP2 and MMP9 break down collagen fibrils, the structural framework of the tooth becomes compromised, setting the stage for further lesion development [13].

Dental caries is not an isolated event but rather an ongoing process, characterised by demineralisation, that commences with microscopic mineral loss and culminates in the destruction of hard tissues and dental pulp. Accurate determination of the presence and stage of caries is pivotal for effective prevention and management, as it forms the foundation for risk assessment and treatment planning [14]. The principles and techniques of minimally invasive dentistry underscore the need for precise diagnosis to guide treatment strategies [15]. Thus, the identification of a lesion's extent and activity

level is indispensable in determining whether invasive interventions or more conservative approaches are indicated.

The International Caries Detection and Assessment System (ICDAS), offers an evidence-based framework that finds utility in dental education, clinical practice, research, and epidemiological studies [15-17]. Building upon this foundation, our research endeavours to further elucidate the correlation between MMPs and dental caries. Specifically, the aim is to explore the presence of gelatinolytic MMPs (MMP2 and MMP9) in caries lesions in human primary teeth that have been labelled with a specific ICDAS score prior to extraction. In addition, we aim to explore the presence of these MMPs at different stages and depths of carious lesions in primary teeth and simultaneously investigate their correlation with collagen degradation and the recorded diagnosis and clinical appearance using ICDAS.

Materials and methods:

Sample preparation

This in-vitro laboratory study used primary molar teeth, diagnosed with various proximal caries lesion levels retrieved from a biobank, which was kept frozen at -80 degrees following extraction. The teeth were clinically diagnosed by a trained and calibrated ICDAS examiner prior to extraction as part of a previous study [18]. Ethical approval for further use of these teeth 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).

In total, 32 extracted teeth were included in this study and investigated for the presence of MMPs using an immunofluorescent technique (n=24) and the status of collagen integrity within and around the proximal carious lesion with a hydroxyproline assay (n=15). The teeth were sectioned longitudinally in a mesiodistal direction. One section of each ICDAS-scored tooth (the middle section) was used; each section was approximately 500 µm thick but for our purposes the sections were further ground to 150 µm. Each section had two proximal surfaces which had been clinically examined to allocate an ICDAS

coding. The total number of surfaces used was 48. For Collagen integrity analysis 15 carious dentine blocks with dimensions of 3mm (width) x 3mm (length) x 500µm (thickness) were obtained using a diamond tapered fissure bur (Henry Schein, Gillingham, UK) with a high-speed dental handpiece (W&H, St Albans, UK) under water cooling.

Immunofluorescence

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. A VectaFluor™ Duet Double Labelling Kit (DyLight® 488 Anti-Rabbit IgG, DyLight® 594 Anti-Mouse IgG) (Vector Laboratories, Peterborough, UK) was used to achieve double-label immunofluorescence. Samples were washed in phosphate buffered saline (PBS) for five minutes in multi-well (12-well) plastic plates. Antigen retrieval was carried out by heating a citrate buffer (0.01M sodium citrate, pH6) in a microwave for eight minutes. Samples were then washed, cooled in PBS for five minutes, placed on slides and incubated for thirty minutes with 100% normal horse serum to block non-specific protein binding. Excess serum was removed, and samples were incubated with mouse MMP2 primary antibody (ab86607, Abcam) diluted in normal horse serum (1:750) at 4^{o}C overnight in a humidified chamber. A no primary antibody control was prepared by incubating tooth sections in serum without primary antibodies. 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^{o}C 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 five minutes.

Samples were mounted in a media suitable for fluorescence, VectaShield Antifade Mounting Media (Cat#: H-1200; Vector Laboratories, Peterborough, UK), 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^{o}C and analysed as soon as possible to reduce the chance of photo-bleaching. Images were captured using a

fluorescence microscope (Carl Zeiss, Vistec Inc., Germany) and analysed with ImageJ software (https://imagej.net, Maryland, USA) to quantify the mean intensity of areas with positive expression. Specific areas of the image were isolated for later analysis using the tool MACRO, which defined the area of interest (AOI) within the ImageJ software. Once created, an AOI was saved and re-applied to subsequently captured images to ensure consistency of results (blind analysis). Five Fields of interest were employed for quantitative analysis: 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. The reproducibility of field selection and image analysis for the quantification of MMPs in different fields of interest was measured 2 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.

Analysis of collagen integrity using Hydroxyproline assay

The assay was performed using a Hydroxyproline Assay Kit (Colorimetric) (ab222941, Abcam). Tissue lysate of carious lesions was prepared by adding 100µL of dH2O to each caries dentine block and homogenising using a pestle and mortar. 100µL of the 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). 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.

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 (until a crystalline

residue was formed) by heating the plate at 65°C on a microplate incubator (JENCONS PLS, Bedforshire, UK). 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°C, then 50µL of DMAB Concentrate was added and mixed thoroughly. The plate was sealed with a microplate sealer film and incubated at 65°C for 45 minutes in the microplate incubator and the absorbance measured at OD 560 nm using a spectrophotometer (Tecan, Switzerland).

Statistical analysis

All statistical tests were performed using GraphPad Prism version 8 software (GraphPad Software, San Diego, California, USA). All data was checked and passed for normality using the Shapiro-Wilk normality test. One-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test was applied to assess group differences ($p < 0.05$). Pearson's correlation coefficients were used to measure the correlation between MMP expression and caries surface appearance, as assessed by ICDAS and the correlation between MMP expression and the amount of collagen degradation in each caries lesion assessed by ICDAS.

Results

The presence of MMP2 and MMP9 in the dentine of carious lesions and their expression in odontoblasts for each ICDAS score

Immunofluorescent examination of human primary teeth showed that MMP2 and MMP9 were present in both sound and carious dentine (Fig. 1). However, immunoreactivity varied significantly within the dentine region and with the presence of caries. Furthermore, although varying in intensity, immunoreactivity was predominantly observed in the odontoblasts regardless of the presence of a carious lesion. Relatively similar immunoreactivity was observed for MMP2 and MMP9 in all tooth sections; although MMP2 showed weaker staining in all sections, this difference was not statistically

significant (p= 0.998). The immunoreactivity for MMP2 and MMP9 in the tooth sections corresponding to each ICDAS score is detailed as follows.

ICDAS = 0. 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, expression was more intense in odontoblasts at the dentine-pulpal junction region (4.627±0.867, 8.588±0.277 respectively (Fig. 2).

ICDAS = 1. 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).

ICDAS = 2. The immunoreactivity of both MMPs in the dentine was similar to ICDAS 0 and 1, however, noticeable intensity of 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.

ICDAS = 3. 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).

ICDAS = 4. Significantly higher immunoreactivity of MMP2 and MMP9 (57.03±11.78, 65.81±16.94 respectively) was observed in the enamel-dentine junction and 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 an ICDAS score of 3 (P= 0.001).

ICDAS = 5. 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), but again this

reactivity becoming weaker in the inner third of dentine (22.87±2.626, 27.71±5.402 respectively).

ICDAS = 6. A similar trend was observed to that of ICDAS score 5.

The control sections showed no immunoreactivity, confirming that no cross-reactions had occurred.

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

The data (Figure 2) 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 and although it was higher in ICDAS 3, this was not statistically significant ($p=0.605$). 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.

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. However, 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 ($p= 0.01$). In the middle third of the dentine, very weak MMP immunoreactivity was observed in lesions from ICDAS 0 to 3 while In contrast, an increase in intensity was observed in ICDAS 4 to ICDAS 6. In the outer third of the dentine, MMP immunoreactivity was first detected in ICDAS 3, with a non-statistically significant ($p= 0.873$) higher intensity of MMP9 compared to MMP2. From ICDAS 4 to ICDAS 6, the immunoreactivity of both MMP2 and MMP9 was significantly increased ($p<0.001$).

Pearson correlation coefficients were calculated to understand whether the MMPs in the dental tissue correlated with the surface appearance of caries. 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, [$r (5) = 0.92$, $p=.004$] (Fig 3).

Quantification of collagen degradation

The results of the HYP assay are shown in Figure 4. A one-way ANOVA 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 significantly ($p=0.015$) larger for ICDAS 3 compared to non-cavitated lesions (ICDAS 0-2), the amount of HYP was higher in carious lesions with ICDAS 5 compared with ICDAS 3 ($p=0.004$). 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 $r(3)=0.82$, $P=0.004$

Discussion

The present study makes a unique contribution to this topic by demonstrating a positive correlation between ICDAS scores and MMP2 and MMP9 expression, and demonstrates that MMP expression in odontoblasts and their presence in dentine and caries lesions, escalates with the progression of caries stage and severity. This is clinically significant and improves our understanding of the role of these enzymes during the caries process. We have, therefore, provided further data to support the staging used by ICDAS and its associated care recommendations [17,19].

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

In healthy human dentine (ICDAS 0), results show that MMP2 expression levels are consistently low and uniform across various dentine regions, which is consistent with previous studies on human coronal dentine and dentine extracts from permanent teeth [20,21]. This is in contrast to a study conducted on rat teeth, suggesting possible

interspecies differences in MMP expression [22]. The patterns of MMP2 and MMP9 immunoreactivity in dentine and at the dentine-pulp junction in ICDAS scores 1 and 2 are similar to those observed in ICDAS 0 teeth. An increase in intensity occurred in ICDAS 2, notably in the dentine-pulp region, with MMP9 showing greater prominence than MMP2. This increased activity during early caries lesions signifies a swift response to enamel demineralisation and marks the onset of carious lesions characterised by bacterial proliferation and acid release. Understanding non-cavitated lesions is crucial as our study highlights their early effects and the subsequent increase in MMP expression in odontoblasts. Early detection of caries lesions allows non-invasive/micro-invasive interventions that can arrest lesion progression, especially considering that early dental caries is reversible and controllable if the biofilm is removed [4,23]. Non-cavitated proximal caries lesions progress more slowly than cavitated lesions, providing a window for non-invasive/micro-invasive interventions [24]. However, the use of sensitive diagnostic methods is crucial for effective monitoring of proximal caries lesion and the success of non-surgical treatments.

ICDAS 3 represents localised enamel loss without visible dentine and is often referred to as microcavitated lesions [17]; MMP expression in the dentine of these teeth has not previously been investigated. The increased MMP expression might be attributed to increased bacterial invasion of the dentine due to enamel degradation, prompting odontoblasts to express more MMPs and attack the dentine matrix in this region. Among caries lesions, ICDAS 3 is of particular importance to dentists as while non-cavitated lesions are reversible, cavitated lesions often necessitate operative intervention. However, the optimal treatment strategy for proximal microcavitated lesions (ICDAS 3) remains controversial. Several studies have suggested that sealing can effectively stop the progression of caries without the need to remove carious tissue in both primary [25] and permanent dentition [26,27]. These studies are consistent with the principles of minimally invasive dentistry and the success of sealing in ICDAS 3 lesions may be due to the low MMP expression in carious lesions. Significantly higher immunoreactivity of MMPs was observed in lesions classified as ICDAS 4 which is consistent with previous studies whereby abundant MMP2 and MMP9 were noted in caries lesions of permanent teeth [11,13,22,28]. However, these previous studies did not consider the clinical diagnosis or stage of caries when comparing MMP expression in healthy and carious

lesions. The increased MMP immunoreactivity suggests a more advanced progression of the carious lesion and a higher degree of collagen degradation. This raises the crucial question as to whether cavity sealing alone would be sufficient to stop an ICDAS 4 carious lesion.

Caries lesions classified as ICDAS 5 and ICDAS 6 are identified as cavitated caries lesions, with ICDAS 5 indicating visible dentine and ICDAS 6 representing extensive caries lesions with visible dentine [17]. A significant increase in staining intensity was observed suggesting that caries lesions stimulate the expression of MMP2 and MMP9 which is consistent with previous findings: Tjaderhane and colleagues (1998) detected active MMP9 in dentine extracts of carious teeth using zymographic analysis [10]. In addition, studies by Toledano et al. (2010) reported increased MMP2 levels in carious lesions [29], while others found a significant increase in both MMP9 and MMP2 in carious lesions [11, 13, 22, 28]. However, it is crucial to note that these previous studies were conducted on demineralised tooth sections, rat teeth or carious lesions, without considering the stage of caries severity. Furthermore, our observations showed a decreasing distribution of MMP2 and MMP9 from the superficial to the deep dentine layer that is consistent with previous findings by Nascimento et al. (2011), who demonstrated decreased MMP activation with increasing carious depth in permanent human teeth [30]. Conversely, Nordbo et al. (2003) noted a significant increase in salivary MMPs, particularly MMP8, in individuals with carious lesions [31]. This is consistent with Shimada et al., who found increased MMP8 and MMP9 levels in the outer layer of carious lesions compared to the inner layer in human permanent teeth [32]. These studies collectively suggest a possible involvement of salivary MMPs in caries pathogenesis, particularly in severely damaged teeth that allow molecular penetration of salivary MMPs into the pulp [33].

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

In carious dentine lesions, there are two distinct layers: the inner layer (affected dentine) and the outer layer (infected dentine) [34]. Affected dentine, which is close to the normal

dentine, is partially demineralised and most of the collagen is intact, making it remineralisable and potentially suitable for adhesion of dentine restorations. However, the presence of endogenous MMPs in this layer, which has been confirmed in several studies could hinder remineralisation as the organic matrix is subject to self-degradation [11,29,32,35-37]. Interestingly, in our study, MMP immunoreactivity was significantly lower in inner dentine, representing affected dentine, than in middle and outer dentine, indicating infected dentine. This observation supports the principle of minimally invasive dentistry, which advocates selective removal of caries tissue to preserve dentine integrity while reducing the risk of pulpal complications [15, 38, 39].

Quantification of collagen degradation

The present study showed denaturation of collagen in carious dentine, with degradation increasing proportionally to the presence of MMP 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. An explanation for this might be that carious lesions, especially in ICDAS 3, have a combination of denatured and non-denatured collagen. Collagen consists of band patterns with bonds in between and denaturation can occur either in the band patterns, in the bonds, or in both. The significant amount of HYP recorded in ICDAS 3 may, therefore, be mainly due to denaturation in the bonds, suggesting that some degree of denaturation occurred in the collagen while the cross-links and banding pattern remained intact. In the inner dentine, which represents the affected dentine, MMP levels were observed to be low. This correlates with reduced bacterial invasion in this layer, thus indicating lesser MMP presence and consequently, less collagen degradation.

It should be noted that not all caries lesions with ICDAS scores were included in our collagen denaturation study; this was due to the limited number of samples, especially for ICDAS 4. Healthy teeth (ICDAS 0), non-cavitated (ICDAS 1-2) and microcavitated lesions (ICDAS 3) were investigated and compared to cavitated lesions (ICDAS 5). The results of our study indicated comparable immunoreactivity of MMP2 and MMP9 in ICDAS 4 and ICDAS 6, suggesting a similar extent of collagen degradation, although further investigation is required.

To ensure the robustness of our study, it was essential to study MMP expression in or near their natural environment within caries lesions, which required the use of human teeth and naturally occurring caries lesions. This approach was essential for accurate interpretation and distinguishes our study from previous research using demineralised healthy dentine beams or recombinant proteins, which cannot capture the complexity of the natural caries process, including the temporal aspects and the influence of host factors such as saliva on collagenolytic enzyme activity in caries [40]. However, a notable limitation of our study was the sample size. A convenience sample was used and a sample size calculation was not performed. We carefully selected three surfaces from each ICDAS score to maintain uniformity, and despite the limited sample size, the integrity of the study was maintained due to minimal variance among the samples, all of which were from primary molars affected with coronal caries lesions. A further limitation of this study is that a formal assessment of caries activity was not performed; however, as the sample was collected from high risk caries rate patients, lesions were likely active.

This investigation does yield several significant conclusions.

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 the opinion 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.

Statement of Ethics

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)

Consent to participate statement: The parents or legal guardians of the children involved in the study gave informed written consent to the retention and use of their children's extracted teeth and the children gave assent.

Conflict of Interest Statement

Dr Lamis El Sharkasi has no conflicts of interest to declare. She was funded by the PhD scholarship from the Libyan Government.

Dr Lynne Bingle has no conflicts of interest to declare.

Professsor Nicolas Martin has no direct conflicts of Interest to declare. He has a consultancy agreement with Oral B (Proctor & Gamble).

Dr Samiya Subka has no conflicts of interest to declare.

Professor Christopher Deery has no direct conflicts of interest to declare. He is in receipt of funding for research from the UK National Institute for Health Research on other projects. He does consultancy work for Kenvue Inc.

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Author Contributions

Dr Lamis El Sharkasi made a substantial contribution to the conception, design of the work; the acquisition of data, analysis, and interpretation of data. Drafting the work and the final approval of the version to be published. She is in agreement to be accountable for all aspects of the work in ensuring that questions related to

the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Dr Lynne Bingle made a substantial contribution to the conception, design of the work; the acquisition of data, analysis, and interpretation of data. Drafting the work and the final approval of the version to be published. She is in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Professor Nicolas Martin made a substantial contribution to the conception, design of the work; the acquisition of data, analysis, and interpretation of data. Drafting the work and the final approval of the version to be published. He is in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Dr Samiya Subka made a substantial contribution to the conception, design of the work; the acquisition of data, analysis, and interpretation of data. Drafting the work and the final approval of the version to be published. She is in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Professor Christopher Deery made a substantial contribution to the conception, design of the work; the acquisition of data, analysis, and interpretation of data. Drafting the work and the final approval of the version to be published. He is in agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data Availability Statement

All data generated or analysed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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Figure Legends

Fig 1: Photographic images of tooth sections with caries lesions recorded by ICDAS score (0-6) and the corresponding fluorescence microscopic images of MMP2 (red) and MMP9 (green) in each ICDAS score in dentine and dentine-pulp junction. Bar graphs show the mean (±SEM) expression of MMPs in each ICDAS score across caries depth. Data are from triplicate (*p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001). Note that MMP immunoreactivity was measured only on the proximal surfaces in cases where teeth had multiple lesions.

Fig 2: 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).

Fig 3: Scatter plots of Pearson’s correlation coefficient between the surface appearance of caries assessed by ICDAS and (a) MMP2 and (b) MMP9.

Fig 4: 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).