MMP-9 in dentinal fluid correlates with caries lesion depth

Ballal, Vasudev ; Rao, Sheetal ; Bagheri, Azadeh ; Bhat, Vinutha ; Zehnder, Matthias ; Attin, Thomas

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MMP-9 in Dentinal Fluid Correlates with Caries Lesion Depth

Vasudev Ballal[a] Sheetal Rao[a] Azadeh Bagheri[b] Vinutha Bhat[b] Thomas Attin[c] Matthias Zehnder[c]

[a]Department of Conservative Dentistry & Endodontics, Manipal College of Dental Sciences, Manipal University, Manipal, Karnataka, India

[b]Department of Biochemistry, Kasturba Medical College, Manipal University, Manipal, Karnataka, India

[c]Clinic for Preventive Dentistry, Periodontology, and Cariology, University of Zurich, Zurich, Switzerland

Short title: Dentinal fluid MMP-9 in carious teeth

Key words: dental pulp; Molecular Diagnostics; Dentinal Fluid; Matrix Metalloproteinase; Caries

Correspondence:
Dr. Matthias Zehnder
Division of Endodontics
Clinic of Preventive Dentistry, Periodontology and Cariology
University of Zurich, Center of Dental Medicine
Plattenstrasse 11, CH-8032 Zurich
Switzerland
Phone: +41 44 634 3284
FAX: +41 44 634 4308
E-mail matthias.zehnder@zzm.uzh.ch

Abstract

The analysis of molecular cues in dentinal fluid from an excavated cavity could improve diagnostics in the context of minimally-invasive caries treatment. In the current clinical trial we assessed whether the dentinal fluid levels of MMP-9 (neutrophil gelatinase) would increase with the progression of carious lesions. MMP-9 is associated with neutrophil-related tissue breakdown in the pulp. Absolute MMP-9 levels were contrasted against the levels of MMP-2, an enzyme related to normal tissue turnover. Dentinal fluid was collected below deep and shallow caries from molars and premolars within the same patients aged 16 and older (N = 30, 1 tooth per group/patient). Experimental teeth were isolated under rubber dam prior to excavation. Dentinal fluid was collected from the bottom of the cavity using a size-25 paper point. MMP levels were assessed using enzyme-linked immunosorbent assay (ELISA). Non-parametric methods were applied to test for differences between groups. Significantly more ($p < 0.05$, Wilcoxon test) MMP-9 was collected form the deep carious lesions than from the shallow counterparts. Pairwise comparison of MMP-9 values within patients revealed that there was more MMP-9 collected from deep lesions than from shallow counterparts in 27 of the 30 individuals under investigation (pairwise Wilcoxon test, $p < 0.001$). In contrast, no such difference existed for MMP-2. There was a high correlation between MMP-9 from deep and shallow lesions (Spearman's Rho = 0.72, $p < 0.001$), indicating that patients with more MMP-9 in the deep carious lesion also tended to have more MMP-9 in the shallow lesion.

The analysis of molecular cues in dentinal fluid from an excavated cavity or otherwise exposed dentin is a relatively new approach that could improve diagnostics in the context of caries treatment and restorative dentistry [Zehnder et al., 2011, Rechenberg and Zehnder, 2014]. This method appears to be especially promising to further the concept of indirect pulp capping in teeth with caries that appears close to the pulp space on the radiograph [Leksell et al., 1996, Bjorndal et al., 2010]. Ideally, markers in dentinal fluid should indicate the state of the underlying pulp. However, molecular analysis of dentinal fluid is in its infancy, and many possible impasses have yet to be overcome before any true clinical application can be considered. One core problem with this approach is that dentinal fluid yields are extremely low [Knutsson et al., 1994]. The fluid that can be collected from an exposed dentin wound is hardly enough to perform a comprehensive analysis on the molecular cues contained therein, even when an optimized collection method is used [Zehnder et al., 2014]. A second problem when dealing with caries is that the lesion itself is full of proteins formerly embedded in the mineral phase of the dentin, which may or may not be liberated by the acidic demineralization of the dentin [Tjaderhane et al., 1998, Smith et al., 2012]. Consequently, individual markers have to be identified that are indicative of pulp tissue breakdown and not present in healthy states [Vidal et al., 2014, Zehnder et al., 2014].

In teeth affected by caries, it appears to be most promising to assess molecules associated with the innate immune system, i.e. the polymorphonuclear neutrophil (PMN) response against the invading microorganisms [Rechenberg and Zehnder, 2014]. This is because PMN infiltration and related soft tissue breakdown is the key factor to histologically discern between reversible and irreversible pulpitis [Wahlgren et al., 2002, Ricucci et al., 2014]. Some recent approaches in that direction have thus targeted PMN-related enzymes in the dentinal fluid. It was shown that MMP-9 is present in amounts sufficient for quantification in the dentinal fluid of teeth with irreversible pulpitis, while it is absent in the dentinal fluid of healthy teeth [Zehnder et al., 2011]. However, the teeth in these studies were either severely inflamed with symptomatic pulpitis or clinically healthy and caries-free. Information from such studies may be useful for a first assessment of a marker, yet do not allow any conclusions regarding its expression in the course of the disease process.

Therefore, in the current clinical trial we assessed whether the dentinal fluid levels of MMP-9, also known as neutrophil gelatinase, would increase with the progression of

carious lesions. To this end, dentinal fluid was collected below deep and shallow caries from molars and premolars within the same patients aged 16 and older. Absolute MMP-9 levels were contrasted against the levels of MMP-2, an enzyme related to normal tissue turnover, which is consistently present in pulp tissue [Gusman et al., 2002, Accorsi-Mendonca et al., 2013], embedded in healthy dentin [Vidal et al., 2014], and can be found in the dentinal fluid of healthy teeth [Zehnder et al., 2014].

Materials and Methods

Ethics

The protocol of the current study did in no way influence the treatment of the patients under investigation and was approved by the institutional review board (IEC 149/2016). This research was performed according to the Declaration of Helsinki. Each participant joined this study at free will after reading, discussing, and signing an informed consent form.

Inclusion criteria

Clinically healthy, patients aged 16 and older presenting with at least one shallow (just reaching the dentin) and one deep carious lesion reaching in the inner 1/3 of the dentin were asked to participate. The caries lesions had to be in molars or premolars, and proper rubber dam isolation of the affected teeth had to be possible. Diagnoses were reached based on clinical inspection, cold test, and bite-wing radiographs. Included teeth had to be pain-free. Possible participants were informed that they would not suffer any disadvantage, nor gain any profit from this study. The treatment protocol followed the normal clinical procedure with the exception that some dentinal fluid was collected from the cavity prior to restoration (duration: 60 s).

Exclusion criteria

Patients presenting with spontaneous pain emanating from a tooth with deep caries were not included. Patients who take anti-inflammatory drugs (NSAIDs or steroids) because of toothache or any other reason were excluded also, as were teeth that did not respond to the cold test and/or show an apical radiolucency.

Group size

To test for the goodness of fit of the data to the normal distribution, test and control group comprised 30 teeth each ["Student" (Gosset), 1906]. To avoid clustering effects, only 1 tooth per group was included in each patient.

Clinical procedures

Teeth were isolated using rubber dam (Hygenic Dental Dam, Coltène Whaledent, Altstätten, Switzerland). The rubber dam, clamp, and tooth were decontaminated using a cotton swab drenched in a 1% NaOCl solution [Ng et al., 2003]. Cavities were then cut using a diamond-coated bur in a counter-angle hand piece under water-cooling. Subsequently, the caries was excavated using a sterile rose head round bur. In the test group, some softened dentin towards the pulp in the deepest portion of the cavity was left intact, whilst in the control group with the shallow caries, excavation was performed until the hard dentin was reached (test: “cri dentaire” using a dental explorer). The cavity was dried using a cotton pellet. Dentinal fluid was collected from the bottom of the cavity by pressing the blunt end of a sterile size-25 paper point (Sirona Dentsply Endodontics, Ballaigues, Switzerland) against it for 60 s. Care was exercised that the cavity was completely dry before this procedure; no saliva and gingival crevicular fluid contamination was to occur. The paper point was transferred into a sterile micro-centrifugation tube be (kept on ice) and immediately after frozen at -80°C until further processing. This sampling procedure was developed in a previous study on maximizing the protein yields from dentinal fluid samples [Zehnder et al., 2014].

As soon as the sample was collected, the tooth was restored using composite resin. In the test group, calcium hydroxide paste (Dycal, Dentsply, India) was placed in the deepest portion of the cavity using Dycal placing instrument and a layer of resin modified glass ionomer cement (Fuji Plus, GC Corporation, Tokyo, Japan) was placed. Then cavity was etched with 37% phosphoric acid (Eco Etch, Ivoclar Vivadent, Schaan, Liechtenstein) for 15 sec, followed by application of two layers of bonding agent, (Adper Single Bond 2, 3M ESPE, St. Paul, MN, USA) all over the cavity using a micro brush and light cured for 15 s using LED light curing unit (Shengua Ind Co. Ltd, China). Subsequently, posterior composite resin (Filtek P60, 3M ESPE) was placed incrementally into the cavity and light cured. In the control group, after caries excavation and sample collection, the cavity was etched with 37% phosphoric acid for 15 sec, followed by application of two layers of bonding agent and restored with

composite resin similar to the test group. Then, the occlusion of the patient was evaluated and corrected in both the test and control groups if there was a high point.

Laboratory procedures

On the day of analysis, the samples were eluted in 2 ml of sterile phosphate buffered saline (PBS, pH 7.2) by centrifuging at 2000 x g for 30 min at 4˚C. The supernatant was collected and used for analysis. The levels of MMP-9 and MMP-2 in these samples were measured using commercially available specific enzyme-linked immunosorbent assay (ELISA) kits (Duoset, R&D Systems, Abingdon, UK). The human MMP-9 assay measured the 92 kDa Pro-MMP-9 and the 82 kDa active MMP-9. It did not recognize the 65 kDa form. The detection range of the assay was 31-2000 pg/ml. The MMP-2 counterpart recognized active and Pro recombinant human (rh) MMP-2 but did not assess rhMMP-2 complexed to rhTIMP-2. Its detection range was 0.625-20 ng/ml. Duplicate experiments were performed per MMP and sample, and mean values were used for further calculations. All ELISA assays were run under Good laboratory practice conditions, and performance specification of each ELISA were validated for their intended purpose, as per established guidelines and read at 450 nm using a microplate reader (Lisa Plus, Rapid Diagnostics, Delhi, India). All assays were performed in duplicate and the co-efficient of variation was <10%. A standard curve was generated using a four-parameter logistic curve fit for each set of samples assayed. 30 blank paper points were used as internal controls. These were processed as described above, yet not applied to any tooth. Mean values from these blank samples were subtracted from the respective MMP-9 and MMP-2 values of the clinical samples. MMP-9 values are presented as pg/60 s of collection (full numbers), MMP-2 values as ng/60 s of collection (two digits).

Data analysis and presentation

Values related to MMP-9 and MMP-2 levels in the dentinal fluid samples were skewed (Shapiro Wilk test), and hence, non-parametric methods were applied to test for differences between shallow and deep lesions. The alpha-type error was set at 5% ($p < 0.05$). Data are presented as medians and interquartile ranges (IQRs).

Results

Thirty patients participated in this study, 13 females and 17 males, age 17 to 47 years (mean= 25 years, median = 27 years). They had a median DMFT of 8 (IQR = 6), D3MFT of 5 (IQR = 5), and 2 deep carious lesions (IQR = 2).

Significantly more ($p < 0.05$, Wilcoxon test) MMP-9 was collected form the deep carious lesions than from the shallow counterparts. The median values were 329 pg (IQR = 365 ng) and 207 pg (IQR = 184) for deep and shallow lesions, respectively. In contrast, the difference in MMP-2 that was collected from deep lesions (median = 3.71 ng, IQR = 5.83) did not differ at the 5% probability level from the MMP-2 that was collected from shallow caries (median = 2.21 ng, IQR = 5.37).

Pairwise comparison of MMP-9 values within patients revealed that there was more MMP-9 collected from deep lesions than from shallow counterparts in 27 of the 30 individuals under investigation (pairwise Wilcoxon test, $p < 0.001$). In contrast, no such difference existed for MMP-2 (Fig. 1). There was a high correlation between MMP-9 from deep and shallow lesions (Spearman's Rho = 0.72, $p < 0.001$), indicating that patients with more MMP-9 in the deep carious lesion also tended to have more MMP-9 in the shallow lesion. This was not the case with MMP-2 (Spearman's Rho = -0.23, $p = 0.21$).

Discussion

The current study showed that MMP-9 levels in dentinal fluid samples differ between deep and shallow caries lesions, especially within individuals. In contrast, the MMP-2 that was collected had similar variance as the MMP-9, yet did not differ at all between deep and shallow caries.

This study is limited by the fact that we did not know the true histologic state of the pulp. Nevertheless, if we accept the concept that deeper caries lesions cause more pulpal inflammation than shallow counterparts [Reeves and Stanley, 1966, McLachlan et al., 2004], then the current results can be considered important. However, this being an early study on largely uncharted territory, the information to be gained is limited. We chose to assess the dentinal fluid levels of 2 individual proteins well known to be either involved with the innate immune response to bacterial invasion (MMP-9), or normal tissue turnover (MMP-2) [Birkedal-Hansen et al., 1993]. The dentinal fluid levels of MMP-2 collected from carious teeth in this investigation were in the same range as those collected from healthy teeth in a former study [Zehnder et al., 2014]. Unfortunately, it

is not possible to normalize the levels of these proteins to total protein in the sample for two reasons: first the paper points themselves contain varying amounts of protein [Zehnder et al., 2014], and second, caries itself contains solubilized proteins, which would then obscure the findings. Furthermore, tissue inhibitor of metalloproteinase (TIMP) levels were not regarded in this study, as these correlate with the degree of pulpal inflammation [Accorsi-Mendonca et al., 2013, Mente et al., 2016].

MMPs have been discussed in relation to caries largely based on their presence in saliva, and the potential that they could be involved in the progression of certain types of lesions [Tjaderhane et al., 1998, Chaussain-Miller et al., 2006]. Furthermore, it has been realized that MMPs are present in the dentin proper, but it has not necessarily been evaluated to which extent or proportion MMPs are immobilized in the hard tissue phase, attached to the collagen, or are dissolved in the dentinal fluid [Vidal et al., 2014]. This could be done in future studies by centrifuging dentin samples to collect the fluid [Geraldeli et al., 2012], followed by deproteinization/demineralization to assess the fraction that is embedded in the mineral phase [Zeng et al., 2016]. The current results strongly suggest that both, MMP-9 and MMP-2 are present in the dentinal fluid, while former studies showed that they can also be attached to the dentinal collagen [Vidal et al., 2014].

The ultimate and most straightforward goal of molecular diagnostics as described here should be to develop a chair-side assay that targets an individual molecule that, if present above a certain threshold level, indicates disease. MMP-9 may be such a molecular cue. However, further studies are necessary to investigate how MMP-9 levels in dentinal fluid correlate with e.g. the pulp survival in teeth with deep caries. We will re-assess all the patients from this study 1 year after treatment. However, because the deep cavities assessed in this study were from teeth without spontaneous pain, it is unlikely that any of these had irreversible pulpits [Ricucci et al., 2014]. Nevertheless, a high proportion of teeth with deep caries lesions are not painful [Bjorndal et al., 2010]. It would appear that pulpal breakdown does not necessarily hurt, yet it invariably results in the infection of the pulp space, with untoward consequences for the patient [Michaelson and Holland, 2002]. This is why tests that go beyond the current concept of merely triggering a nerve response in a tooth to assess its pulpal status should be developed.

One most intriguing finding made here was the fact that MMP-9 levels between deep and shallow lesions correlated so significantly within patients. We could guess that the bacterial species contained in these lesions from the same patient caused a similar host response [Hahn and Liewehr, 2007]. Conversely, the correlation of MMP-9 levels between deep and shallow lesion within patients could have to do with similarities in individual immune responses regardless of the aggressiveness of the microbiota involved in the caries. However, when MMP-9 values were cumulated for each patient and these values were compared to the amount of deep caries lesions per patient (which could be indicative of the aggressiveness of the disease), no correlation was found (Spearman's Rho = -0.27). Furthermore, there was no correlation between these cumulative MMP-9 levels and the patient's age (Spearman's Rho = -0.06). The reason why MMP-9 values vary more from patient to patient than they do within patients between deep and shallow caries should be investigated in future studies, e.g. by correlating the type of infection with markers in dentinal fluid. Specifically, it should be investigated whether and how the microbiota in caries differs within patients between deep and shallow lesions. This is a biologically important issue, yet it is strangely underinvestigated.

It may be concluded from the current study that MMP-9 can be collected consistently from the dentinal fluid of carious teeth, and that the total amount that is found is higher in deep carious lesions than in shallow counterparts. The current results can be used as a basis for further clinical investigations.

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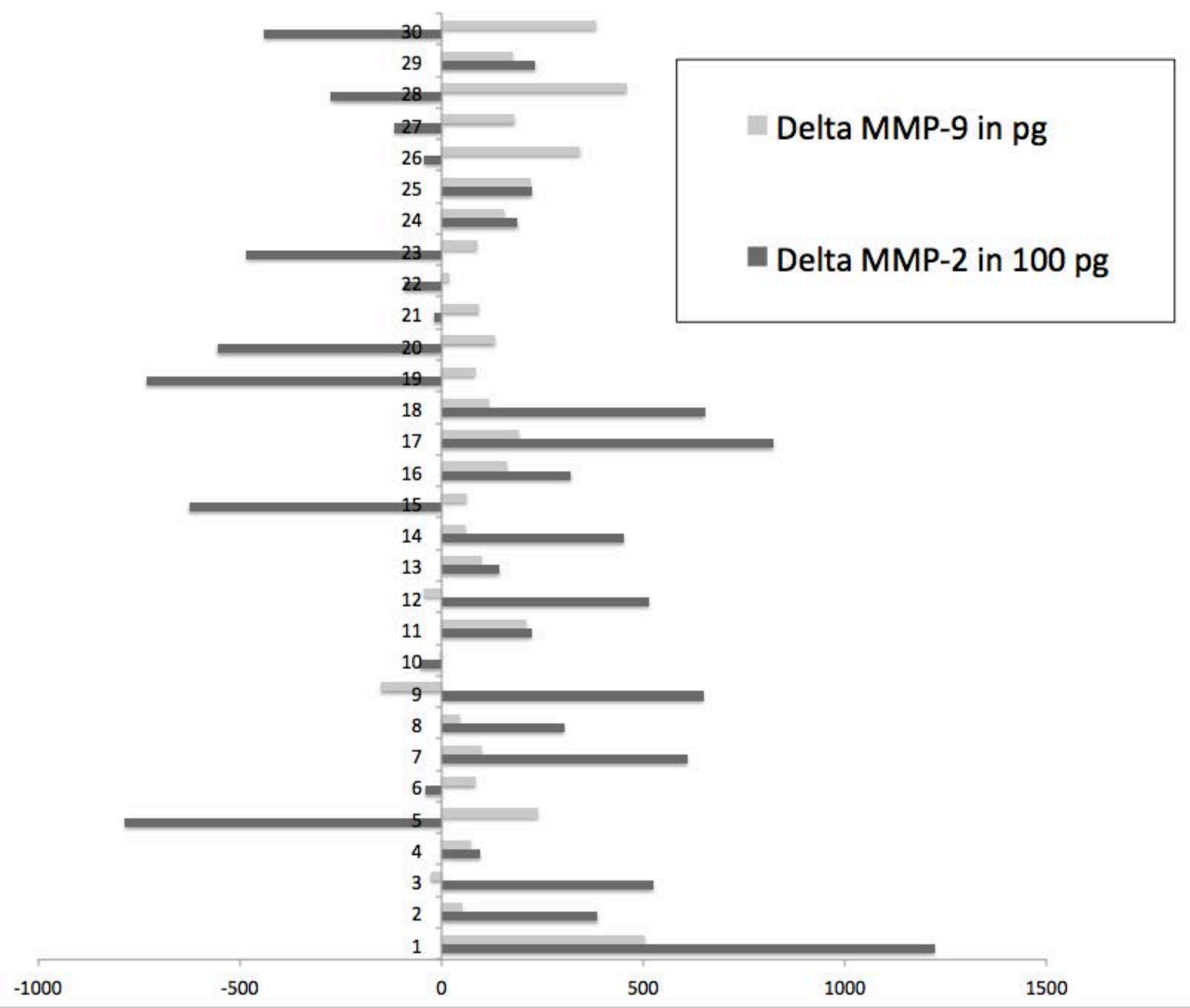
Caption:

Fig. 1 Bar diagram showing the differences in MMP-9 and MMP-2 amounts that were collected from deep and shallow caries for each individual patient (1- 30). Positive values (to the right) indicate that there was more of the respective MMP collected from the deep compared to the shallow caries lesion in that patient. Regardless of the absolute amount of MMP-9 that was collected, there was almost consistently more MMP-9 obtained from the deep compared to the shallow lesions. This was not the case with MMP-2; 13 patients had more MMP-2 in the dentinal fluid of the tooth with the shallow compared to that with the deep caries.


Delta MMP-9 in pg
Delta MMP-2 in 100 pg
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