Association of thalassemia major and gingival inflammation: A pilot study

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ABSTRACT

Objectives: This cross-sectional study aimed to investigate the relationship between thalassemia major (TM) and gingival inflammation through the salivary, serum, and gingival crevicular fluid (GCF) levels of matrix metalloproteinase (MMP)-8, MMP-9 and tissue inhibitor of MMP (TIMP)-1.
Methods: Biofluid samples and full-mouth clinical periodontal recordings were obtained from 29 otherwise healthy patients with TM and 25 systemically healthy (SH) individuals. Biofluid samples were evaluated by immunofluorometric assay (IFMA) and enzyme-linked immunoassays (ELISAs). Data were tested statistically by Kolmogorov Simirnov, Mann–Whitney U tests, Spearman correlation analysis.
Results: Age, smoking status, bleeding on probing, plaque index were similar in the study groups, but probing depth, gender data exhibited significant differences ($p = 0.037$ for both). Salivary MMP-8, MMP-9, TIMP-1 concentrations were significantly higher in the TM than SH group ($p = 0.014$; $p < 0.001$; $p = 0.042$, respectively). Serum TIMP-1 concentrations were significantly higher; MMP-8/TIMP-1, MMP-9/TIMP-1 molar ratios were significantly lower in the TM than SH group ($p < 0.001$; $p = 0.005$; $p = 0.022$, respectively). Very few GCF samples revealed biochemical data above the detection limits. Numerous correlations were found between clinical periodontal parameters and biochemical data.
Conclusions: It may be suggested that TM may exacerbate the local inflammatory response as manifested in salivary MMP-8, MMP-9, TIMP-1 levels.

1. Introduction

Thalassemia is considered as the most common genetic disorder worldwide, presenting major public health and social problems particularly in the high incidence areas. Based on genetic heterogeneity, clinical and haematological variability, thalassemia can be homozygous, heterozygous, or compound heterozygous. The heterozygous form of the disease (beta-thalassemia) is the most common form with minimal clinical expression (Hattab, 2012). Thalassemia major (TM), which is the homozygous type of beta-thalassemia, is an autosomal recessive hereditary anaemia (Skordis & Toumba, 2011). Systemically-released inflammatory markers due to the iron overload play a key role in multi-organ complications seen in patients with TM (Aggeli et al., 2005)

Moreover, low levels of sexual hormones together with iron overload lead to decreased inhibition of osteoclast activity and bone metabolism. Therefore, skeletal complications like osteopenia/osteoporosis, pain, and fractures are quite common in TM (Aydinok, 2012).

Gram-negative, anaerobic and microaerophilic bacteria that colonize in microbial dental plaque are considered as the main etiological factor of inflammatory periodontal diseases. These bacteria are capable of stimulating host immune cells to increase their matrix metalloproteinase (MMP) release, thereby leading to the irreversible tissue destruction seen in periodontitis (Sorsa et al., 1992; Sorsa et al., 2006). MMPs can collectively degrade almost all components of extracellular matrix and basement membrane and their excess activity play a major role in periodontal tissue destruction. MMPs can also process bioactive non-matrix substrates such as cytokines, chemokines, growth factors and immune modulators, thus they mediate anti-inflammatory and pro-inflammatory processes (Kuula et al., 2009; Sorsa et al., 2006). Upon bacterial insult triggered leukocytes migrate to

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the site of inflammation and release MMP-8 and MMP-9, which are activated locally [Tissue inhibitors of MMPs (TIMPs) regulate the activities of these enzymes and TIMP-1 is more effective on interstitial collagenases (Howard, Bullen, & Banda, 1991). An imbalance between MMPs and TIMPs is a key element in the pathological tissue destruction observed in periodontitis (Aiba, Akeno, Kawane, Okamoto, & Horiuchi, 1996; Bıyıkoğlu et al., 2009; Özçaka et al., 2011).

The possible relationship between TM and gingival inflammation is unclear. Iron release from tissue stores and reactive oxygen species production are considered as adequate stimuli for the production of MMPs (Zamboni et al., 2005). It is hypothesized that the iron overload in TM patients may affect levels of MMPs in saliva, serum and/or gingival crevicular fluid (GCF) and thereby deteriorate clinical periodontal status. Therefore, the aim of the present study was to evaluate salivary, serum, and GCF levels of MMP-8, MMP-9, TIMP-1 together with clinical periodontal status in TM patients and systemically healthy counterparts.

2. Materials and methods

2.1. Study population

A total number of 54 individuals were recruited for the present study between September 2012 and March 2013. Twenty-nine TM patients (aged 18–58 years) followed for at least three years by the outpatient clinic of Haematology Department, Aydın State Hospital, Aydın, Turkey were included. None of the TM patients had had splenectomy. Independent factors likely to be associated with low bone mass such as history of gonadal or pubertal dysfunction, history of iron chelating therapy, history of treatment with calcium and vitamin D, pre-transfusion haemoglobin level, serum levels of calcium, phosphorus, alkaline phosphatase, and thyroid function indices (T3, T4 and TSH) were determined and patients with any of these factors were excluded. Inclusion criteria for TM patients were age $\geq$18 years, absence of hepatitis B, C or HIV infection, and treatment with chelation therapy using deferasirox and regular erythrocyte transfusion, vitamin B and C. Patients with any other known systemic disease that can affect periodontal status and those, who received antibiotic therapy or periodontal treatment within the last three months, patients having less than 10 teeth were also excluded from the study. Smoking history was recorded, but smokers were not excluded. Twenty-five systemically healthy individuals (aged 18–40 years) seeking dental treatment in the School of Dentistry, Ege University volunteered for the control group. The study was conducted in full accordance with ethical principles, including the World Medical Association Declaration of Helsinki. The study was approved by the Ethics Committee of the Ege University with the protocol number 13-11/72. The study protocol was explained and written informed consent was received from each individual before clinical periodontal examination and biofluid sampling. Medical and dental histories were also obtained.

2.2. Saliva sampling

Whole saliva samples were obtained simply by expectorating into polypropylene tubes prior to clinical periodontal measurements or any periodontal intervention and in the morning following an overnight fast during which individuals were requested not to drink (except water) or chew gum.

2.3. Serum sampling

Five millilitres of venous blood were taken from antecubital vein by a standard venipuncture method and the plasma was separated from blood by centrifugation at $1500 \times g$ for 10 min.

2.4. GCF sampling

From each individual participating in the study, GCF samples were obtained from the buccal aspects of two interproximal sites in single-rooted teeth. Sites with obvious plaque accumulation and visible signs of inflammation, such as hyperaemia were selected for GCF sampling. Before GCF sampling, supragingival plaque was removed carefully by sterile curettes and the surfaces were air-dried and isolated by cotton rolls. Filter paper strips (PerioPaper, ProFlow, Amityville, NY, USA) were placed in the orifices of gingival sulcus/pocket for 30 s. Care was taken to avoid mechanical trauma, and strips visually contaminated with blood were discarded. The absorbed GCF volume was estimated by a precalibrated instrument (Periotron 8000, Oraflow, Plainview, NY, USA). The strips from each patient were placed into separate polypropylene tubes before freezing at $-40\,^{\circ}C$. The actual volumes of the GCF samples were calculated in μL by reference to the standard curve.

All biofluid samples were immediately frozen and stored at $-40\,^{\circ}C$ until the sample collection period was completed and thawed immediately before assays.

2.5. Clinical measurements

Subsequent to biofluid samplings, clinical periodontal recordings, plaque index (PI) (Löe, 1967), probing depth (PD), clinical attachment level (CAL), and presence of bleeding on probing (BOP; as present or absent within 15 s after periodontal probing) were performed at 6 sites (mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual and disto-lingual locations) on each tooth present, except the third molars, using a Williams periodontal probe (Hu Friedy, Chicago, IL, USA). CAL was assessed from the CEJ to the base of the probable pocket. BOP (deemed positive if it occurred within 15 s after periodontal probing) (Bıyıkoğlu et al., 2009; Nizam et al., 2014). All measurements were performed by a single calibrated examiner (ÖÖ).

2.6. Measurement of MMP-8, MMP-9 and TIMP-1 in saliva and serum samples

2.6.1. MMP-8 analysis by immunofluorometric assay (IFMA)
MMP-8 levels in the salivary and serum samples were determined by a time-resolved immunofluorescence assay (IFMA) as described previously by (Gürsoy et al., 2010). The monoclonal MMP-8 specific antibodies 8708 and 8706 (Medix Biochemica Oy Ab, Kauniainen, Finland) were used as a catching and tracer antibody respectively. The tracer antibody was labelled using europium-chelate. The assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM $CaCl_2$, 50 μM $ZnCl_2$, 0.5% bovine serum albumin, 0.05% sodium azide, and 20 mg/l dietyhlenetriaminepentaacetic acid (DTPA). Samples were diluted in assay buffer and incubated for 1 h, followed by incubation for 1 h with the tracer antibody. Enhancement solution was added, and after 5 min fluorescence was measured using a fluorometer (1234 Delfia Research Fluorometer, Wallac, Turku, Finland). The specificity of the monoclonal antibodies (Gürsoy et al., 2010) against MMP-8 was the same as that of polyclonal MMP-8 antibodies (Sorsa et al., 1999). The salivary and serum concentrations of MMP-8 were expressed as ng/ml and the detection limit for MMP-8 was 0.8 ng/ml.

2.7. MMP-9 and TIMP-1 analysis by enzyme-linked immunosorbent assays (ELISAs)

MMP-9 and TIMP-1 levels in the salivary and serum samples were determined by enzyme-linked immunosorbent assay (ELISA) as described earlier (Rautelin et al., 2009). MMP-9 and TIMP-1 concentrations were determined using commercially available ELISA kits (Quantikine ELISA, human MMP-9, R&D Systems, Minneapolis, MN, USA, Amersham TIMP-1, Human, Biotrak, ELISA System, GE Healthcare, Buckinghamshire, UK, respectively) and the assays were performed according to the manufacturers' recommendations. The salivary and serum concentrations of the proteins were expressed as ng/ml and the detection limit for MMP-9 was 0.156 ng/ml and for TIMP-1 1.25 ng/ml, respectively. For the calculation of MMPs/TIMP-1 molar ratios the levels were converted to mol/l.

2.8. Statistical analysis

A pilot experiment, in which MMP-8 levels were measured and a 20% difference was obtained, was used for statistical power calculations. With a power of 80% and $a = 0.05$, the minimum number of patients required for the comparisons was 20 for each group.

A statistical software program (GraphPad Prism version 6.00c for Mac OS X, GraphPad Software, California, USA) was used for the statistical analyses. The distribution of the variables was validated by D'Agostino–Pearson omnibus normality test, the differences between groups were evaluated using Kruskal–Wallis test, and Dunn's test was used to correct for multiple comparisons. Correlations between clinical and biochemical data were assessed by Spearman rho rank correlation analysis. All the tests were performed at $\alpha = 0.05$ significance level.

3. Results

3.1. Clinical analyses

Clinical periodontal measurements and demographic variables recorded in the study groups are outlined in Table 1. The rate of males to females was higher in the systemically healthy group ($p = 0.037$). There were no significant differences between the study groups in BOP, PI, CAL, age or smoking status. PD values were significantly higher in the TM group ($p = 0.037$).

3.2. MMP-8, MMP-9, TIMP-1 levels in biofluids

The medians, interquartile ranges and min–max values of salivary and serum MMP-8, MMP-9, TIMP-1 levels are presented in Tables 2 and 3, respectively. Saliva MMP-8, MMP-9 and TIMP-1 levels were significantly higher in the TM group than

Table 2
Saliva concentrations of MMP-8, MMP-9, TIMP-1 in the study groups.

Saliva	Thalassemia major (n = 29)	Systemically healthy (n = 24)	P value
MMP-8 (ng/ml)	672.7(961.5)[a] [31.7–1496.2]	298.3(344.2) [88.1–596.7]	0.014
MMP-9 (ng/ml)	294.2(231.5)[a] [1.0–760.1]	142.9(119.1) [0–305]	<0.001
TIMP-1 (ng/ml)	732.2(448.5)[a] [91.7–1315.3]	507.9(337.9) [62.8–1286.3]	0.042
MMP-8/TIMP-1	0.29(0.51) [0–6.8]	0.22(0.27) [0.04–1.9]	0.509
MMP-9/TIMP-1	0.13(0.1) [0–0.8]	0.07(0.11) [0–0.4]	0.086

[a] Significantly higher in the TM group. All data are presented as Median (IQR) [min–max].

Table 3
Serum concentrations of MMP-8, MMP-9, TIMP-1 in the study groups.

Serum	Thalassemia major (n = 27)	Systemically healthy (n = 25)	P value
MMP-8 (ng/ml)	24.7(39.5) [3.5–270]	36.1(38.9) [7.7–175.6]	0.602
MMP-9 (ng/ml)	551.3(609.6) [306.9–2141.7]	584.6(717.5) [160.9–1657.2]	0.640
TIMP-1 (ng/ml)	174.3(135.6)[a] [91.3–386.4]	93.8(62.4) [34.7–162.0]	<0.001
MMP-8/TIMP-1	0.06(0.14)[b] [0.01–1.1]	0.15(0.29) [0.03–8.0]	0.005
MMP-9/TIMP-1	1.04(1.51)[b] [0.3–6.2]	1.90(2.29) [0.5–10.9]	0.022

All data are presented as Median (IQR) [min–max].
[a] Significantly higher in the TM group.
[b] Significantly lower in the TM group.

the systemically healthy group ($p = 0.014$; $p < 0.001$; $p = 0.042$, respectively). Serum TIMP-1 levels were significantly higher whereas, MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios were significantly lower in the TM group than the control group ($p < 0.001$; $p = 0.005$; $p = 0.022$, respectively). Only few GCF samples revealed biochemical data above the detection limits, therefore, GCF data were excluded from the statistical analysis.

Saliva MMP-9/TIMP-1 molar ratio positively correlated with PD (rho = 0.545, $p = 0.002$), with BOP (rho = 0.476, $p = 0.009$) and with PI (rho = 0.0480, $p = 0.008$). Saliva MMP-9 levels correlated with PD (rho = 0.413, $p = 0.026$). Saliva MMP-8 levels correlated with BOP (rho = 0.370, $p = 0.048$) and with PI (rho = 0.414, $p = 0.026$).

4. Discussion

The present cross-sectional study was undertaken to comparatively evaluate the salivary, GCF and serum levels of MMP-8,

Table 1
Clinical periodontal measurements and demographic variables in the study groups.

Clinical variable	Thalassemia major n = 29	Systemically healthy n = 25	P value
Age (years)	25 (9) [18–58]	23 (14) [18–40]	0.508
Gender (F/M)	14/15[a]	19/6	0.037
Smoking (nonsmoker/smoker)	20/9	22/3	0.093
Periodontal health (healthy/gingivitis)	14/15	15/10	0.389
PD (mm)	2.7 (2)[a] [1–3.5]	1.04 (0.99) [0.9–2.3]	0.037
CAL (mm)	1.5 (1.1) [1–2.5]	1.04 (0.99) [0.9–2.3]	0.623
BOP (%)	60 (78) [0–100]	14 (68) [0–100]	0.613
PI (Score 0–3)	1.6 (2.2) [0–2.9]	0.8 (1.6) [0–2.9]	0.895
GCF sample volume (μl)	0.53 (0.44) [0.045–0.887]	0.43 (0.51) [0.05–0.995]	0.62

[a] Significantly different in the thalassemia major group ($p < 0.05$). Age and clinical periodontal parameters are presented as median (IQR) [min–max].

MMP-9, and TIMP-1 in TM patients and systemically healthy controls. To the best of our knowledge, this is the first study comparatively evaluating these parameters in TM patients in relation with gingival inflammation. According to the present findings, TM patients revealed significantly higher salivary MMP-8, MMP-9, TIMP-1 and serum TIMP-1 levels whereas; serum MMP-8/TIMP-1 and MMP-9/TIMP-1 levels were significantly lower than the systemically healthy individuals.

Studies examining the levels of proinflammatory and regulatory biomarkers in GCF and whole saliva of patients with various systemic diseases have provided insight to the potential diagnostic and prognostic utilization of these oral fluids (Edgar, 1992; Giannobile et al., 2009; Kinney, Ramseier, & Giannobile, 2007; Mandel, 1990; Sorsa et al., 2011). Levels of cytokines and MMPs in whole saliva have been shown to correlate with actively progressing periodontal diseases (Kinney et al., 2007). Whole saliva contains GCF, immune cells and tissue metabolites (Leppilahti et al., 2011; Sorsa et al., 2011) and most closely reflects the predominant intraoral condition (Leppilahti et al., 2011). Stimulation may increase the flow of GCF and this may result in false decreases in the concentration of various contents in the saliva (Kinney et al., 2007). Therefore, we collected expectorated whole saliva, where the degree of stimulation was minimal relative to that obtained using gum, citric acid, or paraffin wax.

On the other hand, serum provides information about the inflammatory stimulus and response generated in circulation towards the periodontal pathogens. The present findings indicated that serum TIMP-1 levels were significantly higher whereas, MMP-8/TIMP-1 and MMP-9/TIMP-1 molar ratios were significantly lower in the TM group than the systemically healthy group. Only few of the GCF samples revealed MMP levels above the detection limits and therefore, GCF data was excluded from the statistical analyses. This finding may be explained by the rather high variation of gingival inflammation degree between the participants.

The immune and inflammatory responses are critical in the pathogenesis of periodontal diseases. Inflammatory response activated by periodontal disease may cause exacerbation of systemic complications in TM and maintenance of oral health may help to improve the quality of life in TM patients. TM patients involved in the present study, revealed significantly higher PD values despite the similar BOP and PI scores with the systemically healthy individuals. Salivary MMP-9/TIMP-1 positively correlated with all clinical periodontal parameters, whereas, salivary MMP-8 correlated with BOP and PI, salivary MMP-9 correlated with PD in the TM group. In a recent study by our group, GCF, serum and salivary levels of B cell activating factor (BAFF), interleukin (IL)-6 and IL-8 in TM patients with gingivitis than the systemically healthy patients with gingivitis (Akcali et al., 2015). In the same study, higher levels of a proliferation-inducing ligand (APRIL), receptor activator of nuclear factor kappa B ligand (RANKL), IL-6, IL-8 were found in the TM group than the systemically healthy counterparts. The present findings suggesting an exacerbation in local inflammatory response in the TM patients are in line with our previous study pointing that TM may have a role in the underlying systemic hematologic condition and potentially affect gingival inflammation via dysregulation of lymphocytes and increased activation of osteoclasts (Akcali et al., 2015).

MMP-8 is considered to be a key mediator of the irreversible tissue destruction associated with periodontitis (Sorsa et al., 2006). Neutrophils are major cellular sources of MMP-8, and a large and persistent neutrophil influx is a hallmark of inflammatory periodontal diseases. Additionally, MMP-8 expression by several non-PMN-lineage cells found in the periodontium can be induced by pro-inflammatory cytokines such as interleukin-1beta and tumour necrosis factor-alpha (Zamboni et al., 2005). Furthermore, specific bacterial proteinases present in microbial dental plaque can activate the PMN-type MMP-8. Gingival inflammation seems to have an enhancing effect on the levels of these cytokines, which in turn are capable of up-regulating the chemotactic, secretory and phagocytic functions of macrophages (Flesch & Kaufmann, 1990) and neutrophils (Nibbering, Pos, Stevenhagen, & Van Furth, 1993).

The present study has some limitations. Apart from the relatively small number of smokers included in the present study, lack of cotinine analysis may be regarded as a limitation, since self-reports on smoking status can sometimes be unreliable (Ah, Johnson, Kaldahl, Patil, & Kalkwarf, 1994) However, the participants were not grouped according to their smoking status and the distribution of smokers and non-smokers were similar in the TM and systemically healthy study groups. Therefore, smoking is not likely to have a major effect on the present biochemical findings. On the other hand, the difference in the male/female ratios in our study groups may have a role in the present biochemical findings and imperfectly matched study groups in regards with gender may be considered as a limitation.

In conclusion, within the limits of the present study, it may be suggested that TM may exacerbate the local inflammatory response in saliva. Larger scale studies and particularly intervention studies with detection of different biochemical parameters in biofluids before and after initial periodontal treatment are required to better address this issue.

Author contributions

Author Pınar Gümüş contributed biofluid sampling, drafted the first version of the manuscript. Author Özgün Özçaka did clinical periodontal measurements and contributed study design and edited the manuscript draft. Author Aliye Akcalı contributed biofluid sampling. Author Selda Kahraman-Çeneli referred the TM patients and provided their medical records. Authors Timo Sorsa and Taina Tervahartiala did the laboratory analyses of the biofluid samples and contributed the manuscript preparation. Author Nurcan Buduneli contributed study design, critically read and edited the manuscript draft.

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