Brazilian Dental Science
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UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
Instituto de Ciência e Tecnologia
Campus de São José dos Campos

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ORIGINAL ARTICLE

doi: 10.14295/bds.2019.v22i3.1708

Altered levels of inhibitory cytokines in patients with thalassemia major and gingival inflammation

Níveis alterados de citocinas inibitórias em pacientes com talassemia major e inflamação gengival

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ABSTRACT

Objective: To evaluate local and systemic levels of interleukin-10 (IL-10), IL-33, and tumor necrosis factor alpha (TNF-α) in Thalassemia major (TM) in the presence of gingival inflammation. **Material and Methods:** 58 patients (TM, n=29 and systemically healthy controls, n=29) were included to the study. IL-10, IL-33, and TNF-α levels were evaluated in gingival crevicular fluid (GCF), saliva and serum. Clinical periodontal measurements were recorded. **Results:** GCF IL-33 total amounts in TM and gingivitis group were elevated compared to systemically and periodontally healthy group (p=0.01). GCF IL-10, IL-33 and TNF-α concentrations were higher in TM and periodontally healthy group than the systemically healthy and gingivitis group (p=0.02, p=0.008, p=0.003). Serum IL-10 levels were elevated in TM and gingivitis compared to the systemically healthy and gingivitis (p=0.0009) and systemically and periodontally healthy (p=0.0007) groups. Serum IL-10 and TNF-α levels in TM and periodontally healthy group were higher than systemically and periodontally healthy group (p=0.01 and p=0.02). **Conclusion:** TM may potentially alter circulating levels of IL-33 and IL-10 and therefore, may affect the degree of periodontal inflammation locally or vice versa. Yet, the underlying mechanism linking the hematologic condition is not clear and deserves further investigation.

KEYWORDS

Gingivitis; Thalassemia major; Interleukin-10; Interleukin-33; Tumor Necrosis Factor-alpha.

RESUMO

Objetivo: Avaliar os níveis locais e sistêmicos de interleucina-10 (IL-10), IL-33 e fator de necrose tumoral alfa (TNF-α) na Talassemia Major (TM) na presença de inflamação gengival. **Material e Métodos:** 58 pacientes (TM, n = 29 e controles sistemicamente saudáveis, n = 29) foram incluídos no estudo. Os níveis de IL-10, IL-33 e TNF-α foram avaliados em fluido crevicular gengival (FCG), saliva e soro. As medições periodontais clínicas foram registradas. **Resultados:** As quantidades totais de IL-33 no FCG do grupo de TM e gengivite foram elevadas em comparação com o grupo sistemicamente e periodontalmente saudável (p = 0,01). As concentrações de IL-10 , IL-33 e TNF-α do FCG foram maiores no grupo TM e periodontalmente saudáveis do que no grupo sistemicamente saudável e gengivite (p = 0,02, p = 0,008, p = 0,003). Os níveis séricos de IL-10 estavam elevados na TM e gengivite em comparação com os grupos sistemicamente saudável e gengivite (p = 0,0009) e sistemicamente e periodontalmente saudáveis (p = 0,0007). Os níveis séricos de IL-10 e TNF-α no grupo TM e periodontalmente saudáveis foram maiores do que os grupos sistemicamente e periodontalmente saudáveis (p = 0,01 ep = 0,02). **Conclusão:** A TM pode alterar potencialmente os níveis circulantes de IL-33 e IL-10 e, portanto, pode afetar o grau de inflamação periodontal localmente ou vice-versa. No entanto, o mecanismo subjacente que liga a condição hematológica não é claro e merece uma investigação mais aprofundada.

PALAVRAS-CHAVE

Gengivite; Talassemia major; Interleucina-10; Interleucina-33; Fator de necrose tumoral alfa.

INTRODUCTION

Thalassemia major (TM) or Cooley anemia, is an autosomal recessive hematologic disorder and characterized by decrease or absence of β-globin chain production [1,2]. Several immunological abnormalities such as functional alterations of T-lymphocytes, polyclonal activation of B-lymphocytes and impaired activity of macrophages and neutrophils have been found in TM patients due to the secondary effects of erythrocyte transfusion and splenectomy [3].

Periodontal diseases are initiated by bacterial pathogens within the dental biofilm and the activated host inflammatory responses are responsible for the destructive events in the periodontium [4]. It has been reported that IL-1, IL-6 and TNF-α levels are increased over the alterations from gingival health to periodontal disease [5,6]. It was suggested that IL-2, IL-6 and TNF-α might have biological and clinical importance in TM patients [7-9].

IL-33 mainly induces T helper 2 (Th2) immune responses [10] and similar to IL-10, it enhances LPS-induced production of TNF-α via its chemoattractant action on Th2 cells [11].

Altered erythropoiesis, reduced erythrocyte survival, and bone marrow overstimulation may lead to the expansion of cranial and facial bones which is characterized by mongoloid appearance [1,12]. Reported oral health problems in TM are mostly seen as facial deformities with different forms and incorrect relations or abnormalities of the dental arches [13-16]. Moreover, higher prevalence of dental caries was reported in TM patients compared to systemically healthy controls [17-19]. At present, there is limited data on molecular determinants of clinical periodontal status as well as possible relationship between anti- and pro-inflammatory cytokine levels in biofluids and gingival inflammation in patients with TM [9, 20-22]. Recent evidence shows that the possible variation of the cytokine levels in thalassemic patients might be associated with the anemic crisis and increased TNF-α and IL-10 levels might contribute to anemia or affect erythropoiesis [23]. The authors hypothesised that presence of chronic gingival inflammation would lead to a maintained systemic inflammatory response, thereby affect the cytokine levels of patients with TM. Therefore, present study aimed to evaluate local and systemic IL-10, IL-33 and TNF-α levels in patients with TM presenting either gingival inflammation or periodontal health.

MATERIALS AND METHODS

Study protocol was approved by the local Ethics Committee (13-11/72) and conformed to STROBE guidelines [24] and followed the requirements of the World Medical Association's Declaration of Helsinki. Prior to enrolment each individual gave written informed consent. Fifty-eight patients (age 18-40 years) were included (from September 2012 to April 2014) and were classified into 4 groups; 16 patients with TM and gingivitis, 13 patients with TM and periodontally healthy, 13 systemically healthy individuals with gingivitis, and 16 systemically and periodontally healthy individuals as controls. Each individual completed comprehensive medical as well as demographic and anthropometric data questionnaires with history of all medical and dental treatments. Age 18 or above, no history of any other systemic diseases, no hepatitis B, C or HIV infection, treatment with chelation therapy, calcium and vitamin D and regular erythrocyte transfusion were the inclusion criteria for TM. Exclusion criteria were state of pregnancy or lactation, presence of systemic diseases and treatment with any other medications in the last 6 months.

Full-mouth clinical periodontal evaluations including probing depth (PD), plaque index (PI) [25], and bleeding on probing (BOP; +/-) were registered to all included patients by calibrated examiners at 6 sites on each tooth and periodontal diagnosis were assigned [26]. All biofluid samples

including GCF, saliva and serum were collected as described previously [9].

Measurement of biofluids

The pooled GCF samples from each patient were eluted into 0.5 mL phosphate-buffered saline. Specific ELISA kits for IL-10, TNF-α and IL-33 (eBioscience, San Diego, CA) were used for quantitative analyses. 50 microliter samples of GCF, saliva and serum were used for each assay and all assays were performed in duplicate and according to the recommendations of the manufacturers. The minimum detection limits were 1.0 pg/mL, 5 pg/mL, and 0.2 pg/mL for IL-10, TNF-α, and IL-33, respectively. The data obtained in GCF were presented as total amounts (picograms per 30 seconds) as well as concentrations (picograms per microliters) whereas saliva and serum samples were presented as concentrations (pg/mL).

Statistical analysis

D'Agostino-Pearson omnibus normality test was used to test the distribution of the data. Kruskal-Wallis test was used for comparisons of non-normally distributed variables (cytokine levels) between the study groups and Dunn's test was used to correct for multiple comparisons. For normally distributed variables (age, PI, BOP and PD) one-way analyses of variance (ANOVA) test with Holm-Sidak's multiple comparison test (family-wise significance and confidence level 0.05) was used. Correlations between clinical periodontal parameters and cytokine levels were analysed by Spearman's correlation test. Statistical software (GraphPad Prism version 6.00c for Mac OS X, GraphPad Software, La Jolla California) was used and statistical significance was considered at $p<0.05$.

RESULTS

Clinical data

Demographic variables including age ($p=0.36$), gender ($p=0.61$) or rate of smokers ($p=0.06$) were similar between the study groups (Table 1). PI and BOP scores were similar in the TM and gingivitis and systemically healthy and gingivitis groups ($p>0.05$) (Table 1). Elevated PI, BOP scores were found in both TM gingivitis and systemically healthy and gingivitis groups compared to periodontally healthy groups ($p<0.001$) (Table 1). Similar PD values were found in all groups ($p=0.07$).

Table 1 - Demographics and clinical periodontal parameters

	Thalassemia major		Systemically healthy	
Periodontal status	Gingivitis (n=16)	Healthy (n=13)	Gingivitis (n=13)	Healthy (n=16)
Age (Years)	27.63 ± 9.98	24.15 ± 5.31	23.54 ± 6.42	24.00 ± 4.98
Gender (F/M)	8/8	6/7	7/6	5/11
Smokers/ non-smokers	5/11	6/7	2/11	1/15
BOP %	73.75 ± 24.39†‡	9.23 ± 8.37	76.42 ± 19.87§¶	7.81 ± 7.29
PI (Score 0-3)	2.11 ± 0.58†‡	0.63 ± 0.30	2.09 ± 0.48§¶	0.39 ± 0.26
PD (mm)	1.82 ± 0.31	1.79 ± 0.33	1.90 ± 0.39	1.58 ± 0.34

BOP = Bleeding on probing (%), PI = Plaque index, PD = Probing depth (mm). Clinical diagnosis groups: Thalassemia major and gingivitis (n=16); Thalassemia major and periodontally healthy (n=13); systemically healthy and gingivitis (n=13); systemically and periodontally healthy (n=16). Values are shown as mean ± standard deviation. † Statistically significant difference between gingivitis and periodontally healthy groups in Thalassemia major ($p<0.001$). ‡ Statistically significant difference between Thalassemia major and gingivitis group and systemically and periodontally healthy group ($p<0.0001$). § Statistically significant difference between gingivitis and periodontally healthy groups in systemic health ($p<0.0001$). ¶ Statistically significant difference between systemically healthy and gingivitis group and Thalassemia major and periodontally healthy group ($p<0.001$).

Biochemical data

All cytokine levels in biofluids are presented in Figures 1-4. GCF IL-10 and TNF-α total amounts were similar in all groups (Figures 1A, C). GCF total amounts of IL-33 were significantly elevated in TM and gingivitis group than the systemically and periodontally healthy group (p=0.01) (Figure 1B). GCF IL-10, IL-33 and TNF-α concentrations were elevated in the TM and periodontally healthy group than the systemically healthy and gingivitis group (p=0.02, p=0.008 and 0.003, respectively) and also higher in the systemically healthy and gingivitis group than the systemically and periodontally healthy group (p<0.0001, p<0.0001 and p=0.002, respectively) (Figure 2). GCF concentrations of IL-33 and TNF-α were significantly higher in the systemically and periodontally healthy group than the TM and gingivitis (p=0.04 and p=0.0012) (Figures 2B, C), whereas lower GCF TNF-α concentrations were found in TM and periodontal health compared to TM and gingivitis (p=0.04) (Figure 2C).

Serum IL-10 levels were significantly higher in the TM and gingivitis group than the systemically healthy and gingivitis (p=0.0009) and systemically and periodontally healthy groups (p=0.0007) (Figure 3A). Similarly, serum IL-10 levels in TM and periodontally healthy patients were significantly higher than those in both systemically and periodontally healthy (p=0.01) and systemically healthy and gingivitis individuals (p=0.01) (Figure 3A). Similar IL-33 levels in serum were found in all groups (Figure 3B). Elevated serum TNF-α levels were found in the TM and periodontally healthy group compared to systemically and periodontally healthy group (p=0.02) (Figure 3C). Salivary IL-10, IL-33 and TNF-α data revealed no differences between the study groups (p>0.05) (Figure 4).

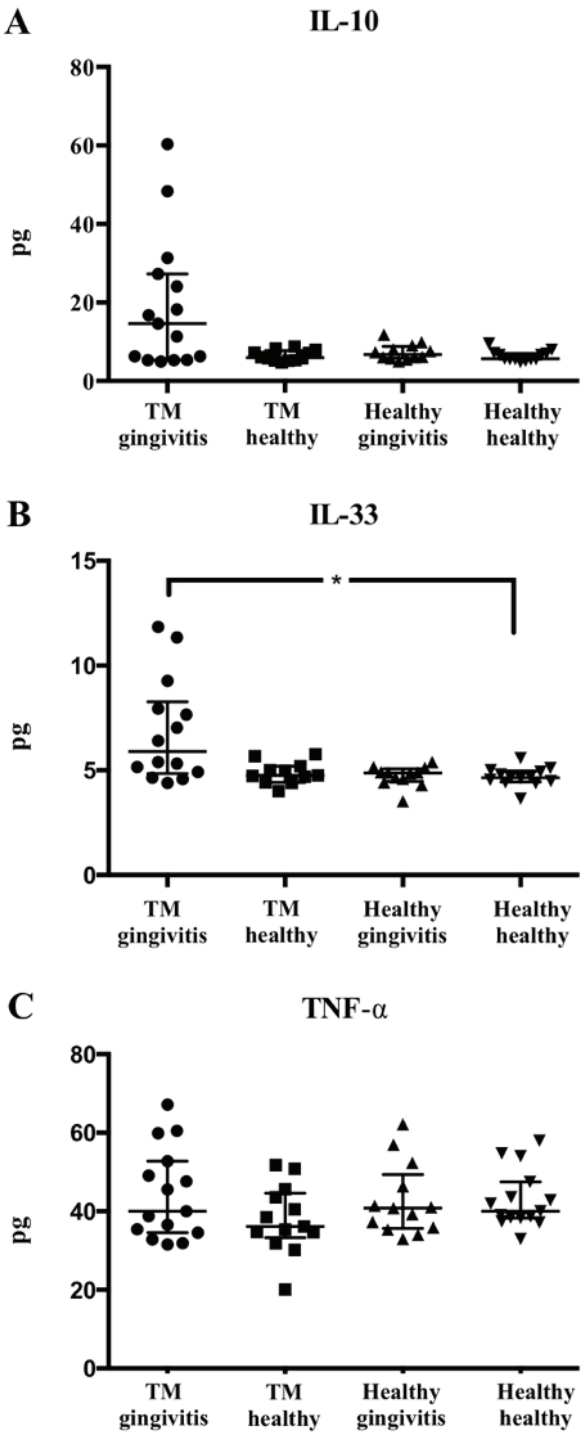

Figure 1 - GCF total amounts of the analysed cytokines. Clinical diagnosis groups: Thalassemia major and gingivitis (TM gingivitis) (n=16); Thalassemia major and periodontally healthy (TM healthy) (n=13); systemically healthy and gingivitis (Healthy gingivitis) (n=13); systemically and periodontally healthy (Healthy healthy) (n=16). The horizontal lines in the boxplots represent the median values and the whiskers represent the 5-95 percentiles. Values below and above the whiskers are drawn as individual dots. * p < 0.05.

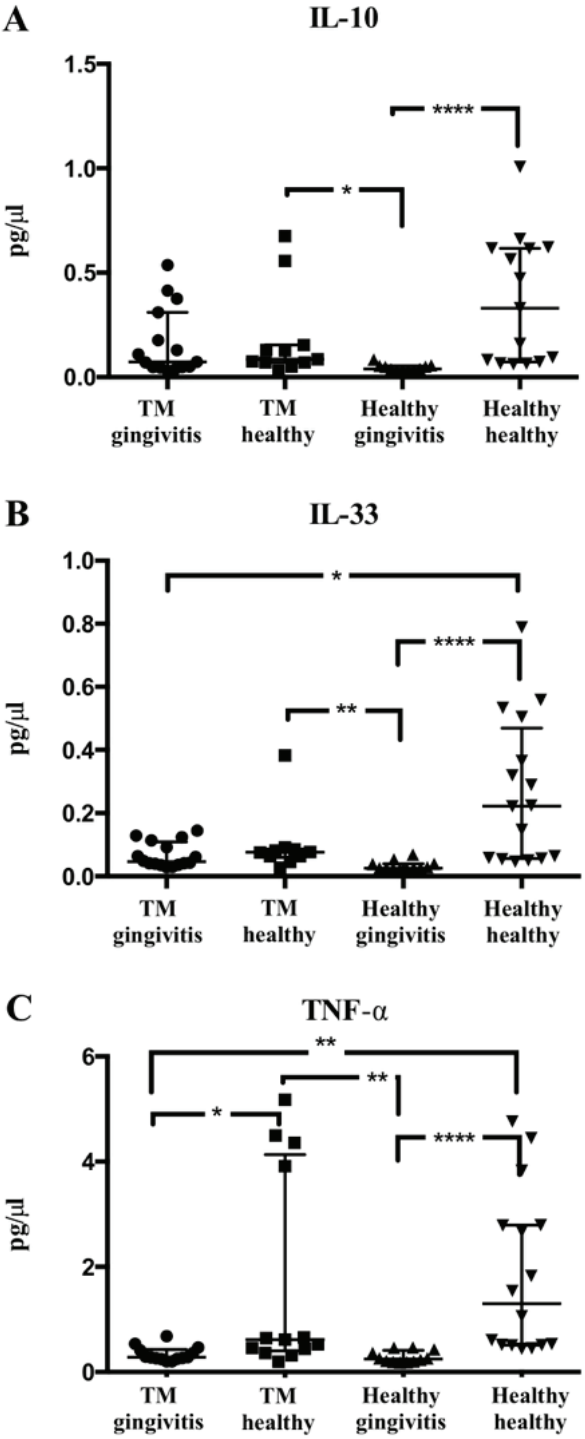

Figure 2 - GCF concentrations of the analysed cytokines. Clinical diagnosis groups: Thalassemia major and gingivitis (TM gingivitis) (n=16); Thalassemia major and periodontally healthy (TM healthy) (n=13); systemically healthy and gingivitis (Healthy gingivitis) (n=13); systemically and periodontally healthy (Healthy healthy) (n=16). The horizontal lines in the boxplots represent the median values and the whiskers represent the 5-95 percentiles. Values below and above the whiskers are drawn as individual dots. * $p < 0.05$, ** $p < 0.01$.

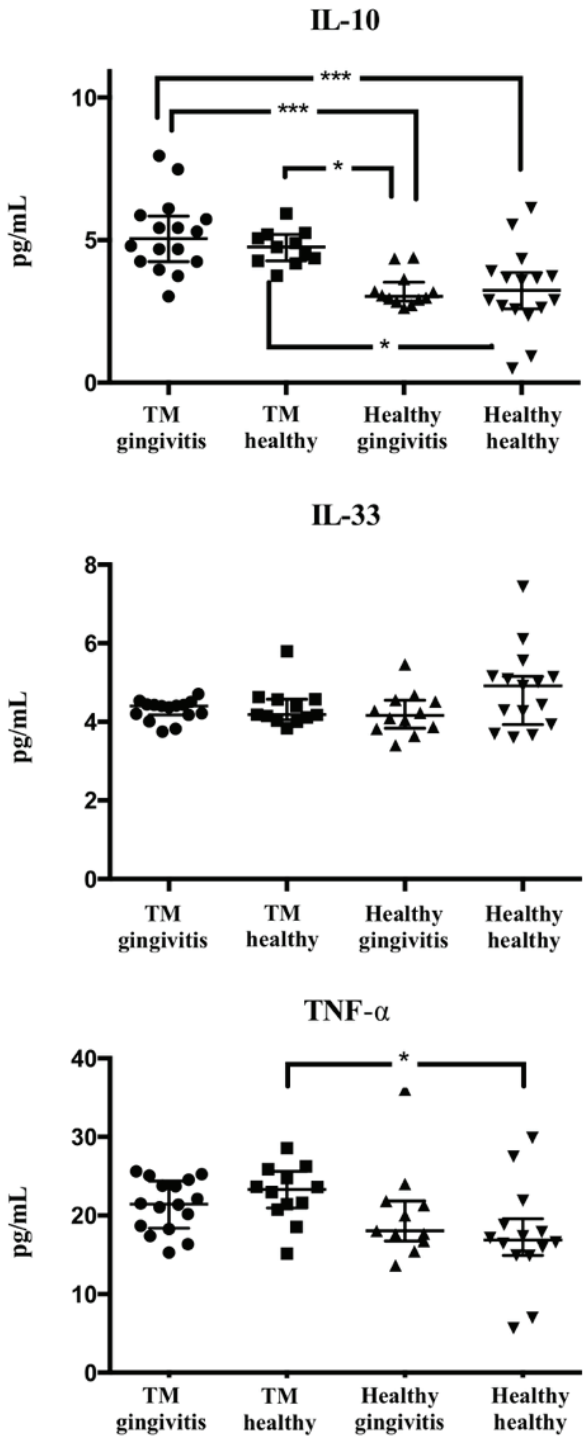

Figure 3 - Serum concentrations of the analysed cytokines. Clinical diagnosis groups: Thalassemia major and gingivitis (TM gingivitis) (n=16); Thalassemia major and periodontally healthy (TM healthy) (n=13); systemically healthy and gingivitis (Healthy gingivitis) (n=13); systemically and periodontally healthy (Healthy healthy) (n=16). The horizontal lines in the boxplots represent the median values and the whiskers represent the 5-95 percentiles. Values below and above the whiskers are drawn as individual dots. * $p < 0.05$, *** $p < 0.001$.

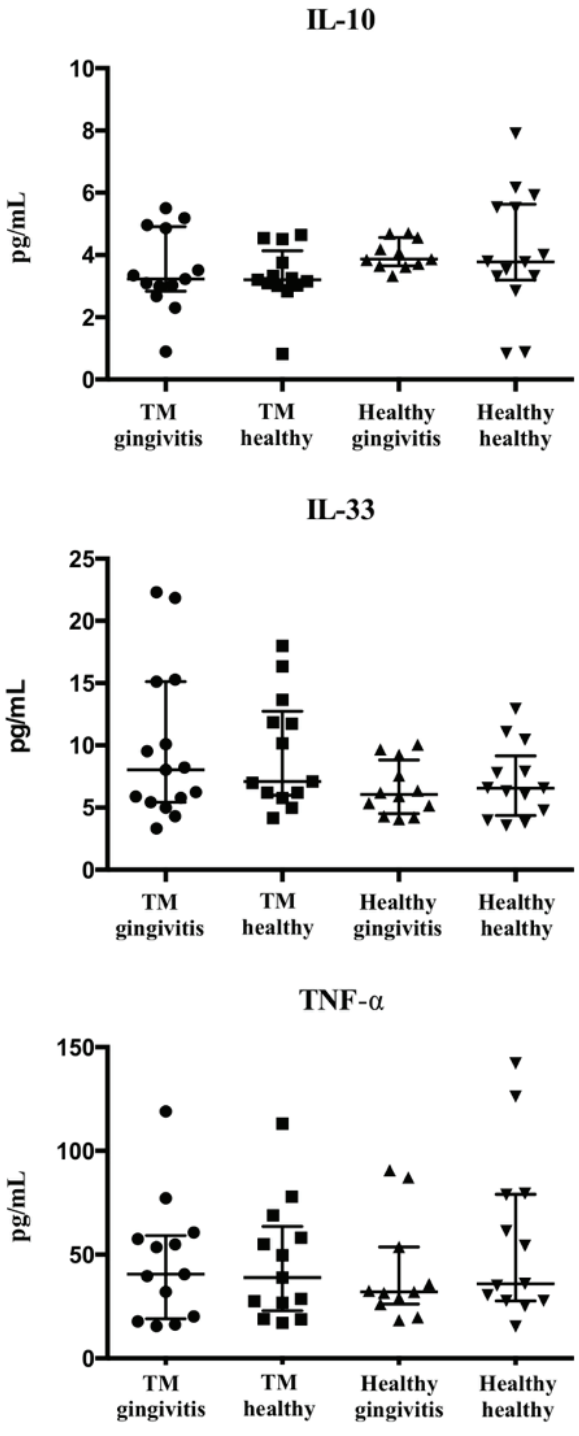

Figure 4 - Salivary concentrations of the analysed cytokines. Clinical diagnosis groups: Thalassemia major and gingivitis (TM gingivitis) (n=16); Thalassemia major and periodontally healthy (TM healthy) (n=13); systemically healthy and gingivitis (Healthy gingivitis) (n=13); systemically and periodontally healthy (Healthy healthy) (n=16). The horizontal lines in the boxplots represent the median values and the whiskers represent the 5-95 percentiles. Values below and above the whiskers are drawn as individual dots. * $p < 0.05$.

Correlations

In the TM group, a significant negative correlation was observed between GCF total amounts and serum levels of IL-10 ($p=0.026$), whereas correlation was positive between salivary IL-10 and IL-33 levels ($p=0.018$) (Appendix S1). Significant positive correlations were reported between GCF IL-10 levels and both IL-33 and TNF-α levels (for GCF total amounts $p=0.048$ and $p=0.024$, respectively and for GCF concentrations $p=0.003$ and $p=0.009$, respectively). GCF IL-33 total amounts and TNF-α were also positively correlated ($p<0.0001$). Similarly, in systemic health, numerous positive or negative correlations were found between the biochemical parameters in GCF, saliva, and serum samples (Appendix S1).

Significant but weak to moderate positive correlation was found between PD and salivary IL-10 in the TM group ($p=0.038$) (Table 2).

Table 2 - Correlation between clinical periodontal and biochemical parameters

Thalassemia Major		GCF			Serum			Saliva		
		IL-10	IL-33	TNF-α	IL-10	IL-33	TNF-α	IL-10	IL-33	TNF-α
BOP	r	.194	.286	.061	-.039	-.040	-.186	-.004	-.011	.171
	p	.352	.157	.756	.847	.846	.343	.985	.957	.404
PI	r	.261	.257	.085	-.073	-.004	-.078	.144	-.087	.140
	p	.208	.205	.667	.718	.984	.692	.482	.659	.496
PD	r	-.004	-.116	-.104	-.104	.332	.163	.409	.181	.300
	p	.985	.574	.597	.605	.098	.408	**.038**	.356	.137
Systemically Healthy		**GCF**			**Serum**			**Saliva**		
		IL-10	**IL-33**	**TNF-α**	**IL-10**	**IL-33**	**TNF-α**	**IL-10**	**IL-33**	**TNF-α**
BOP	r	-.222	-.109	.026	.021	.289	-.297	-.073	-.155	.021
	p	.286	.603	.896	.915	.152	.149	.723	.459	.919
PI	r	-.283	-.140	-.129	.050	.375	-.163	-.020	-.087	-008
	p	.170	.505	.514	.796	.059	.436	.922	.678	.971
PD	r	-.048	-.123	-.014	.103	.309	.187	-.331	.068	.246
	p	.820	.559	.943	.595	.125	.372	.099	.746	.236

Significant correlations are demonstrated in bold face.

DISCUSSION

Elevated GCF IL-33 total amounts and concentrations, serum IL-10 and TNF-α levels were found in patients with TM compared to their healthy counterparts.

TM patients are not considered as immune compromised per se, particularly if the disease is well compensated by treatment. On the other hand, many functional changes especially impaired production, chemotaxis and phagocytosis of the immune cells including CD8 suppressor cells, macrophages, and interferon gamma have been described in TM [27]. Clarification of the nature of link between diseases like periodontal disease and TM may help to understand immune-inflammatory responses of the patients and develop novel therapeutic approaches to improve the life quality in such diseases. Regarding the association between TM and gingival inflammation, it was recently demonstrated that TM might potentially affect B/T-lymphocyte stimulatory cytokines with elevated BAFF levels in serum and saliva in TM compared to controls [9]. Likewise, higher serum TNF-α levels have been reported in TM patients in comparison with systemically healthy individuals [28,29]. The present data are in line with the previous reports of higher circulating TNF-α levels in TM and healthy periodontium compared to their systemically and periodontally healthy counterparts [23,28,29]. Furthermore, the present findings suggest that local gingival inflammation does not have a major influence on circulating levels of TNF-α in patients with TM. This could be explained by the present findings on elevated levels of pro-inflammatory cytokine (TNF-α) that was only observed in clinically healthy periodontal condition.

Regulatory T-cell mediated cytokine IL-10 is important through its suppressive and modulatory function on inflammatory response [30]. Higher serum IL-10 levels in TM patients than healthy controls have been reported [31] and the present study revealed parallel data. Although the exact role of elevated cytokine levels still remains obscure, high circulating levels of IL-10 might imply beneficial role of this cytokine in controlling the inflammatory response in patients with TM.

IL-33 drives Th2-mediated host defence depending on the type of activation of immune response and its primary role is more likely to be antimicrobial [10]. The present findings showed that GCF total amounts of IL-33 is elevated in TM compared to controls when gingival inflammation is present. In spite of a lack of similar trend in GCF concentrations, it may be implied that local inflammation may have an influence on the impaired inflammatory response of TM patients.

Salivary levels of inhibitory cytokines IL-10 and IL-33 negatively correlated with pro-inflammatory cytokine TNF-α in systemically healthy controls, whereas positive correlation was found between GCF TNF-α and IL-l0 levels in TM patients. In healthy state, IL-10 provides a feedback control for the increased production of TNF-α. Thus, this contrary observation between systemic health and TM can be explained by impaired regulatory role of IL-10 in TM. GCF is an inflammatory transudate, primarily originated from serum and its flow is influenced by the degree of inflammation as well as capillary permeability [32]. Besides, GCF contains proteins not only derived from serum but also from the local inflammatory reactions [33,34]. In the present TM study population, a significant inverse correlation was found between GCF and serum IL-10 levels and this finding might be interpreted as the effect of local gingival inflammation.

The inclusion of the smokers to the study population might be regarded as a limitation due to possible impact of smoking on biochemical data. Although the number of smokers in each group differs, they were distributed statistically similar to the study groups, therefore, we believe smoking had a similar effect in the study groups, if any. TM is

a rare disorder and considering the strict oral and systemic criteria for inclusion, smokers were not excluded. Unequal distribution of the study groups is possibly a further limitation of this study. However, the inclusion of such subgroups based on the clinical periodontal status (periodontally healthy versus gingivitis) in the analysis provide better estimation of the influence of periodontal disease on the outcome parameters. Hence, further clinical research with a larger sample size, controlled confounding factors and an adequate power is recommended to make more homogenous group comparison which will further validate the present findings.

Based on the present findings, it might be speculated that presence of gingival inflammation has a more pronounced effect on type 2 immunity (i.e. inhibitory cytokines) rather than production of pro-inflammatory cytokines via TNF-α. The present study allows only association-assessment (presence of gingival inflammation-clinical periodontal and biochemical findings) and cannot extrapolate potential causal relationship, it may emphasise the possible role of IL-l0 and IL-33 in regulating macrophage activity in TM. IL-33 and IL-10 appear to be necessary cytokines for Th2-mediated host defence and play a central role in immune control. Therefore, it is likely that activation of cells of both innate and adaptive immunity can either promote the resolution of inflammation or drive disease pathology, which is possibly associated with severity and complications of TM.

CONCLUSION

Management of TM is complex and requires a multidisciplinary approach to provide precision dental medicine approach to care. Evaluation of pro-inflammatory and inhibitory cytokine levels in local biofluids may specify additional information on detection of gingival inflammation as well as immune-inflammatory response of patients with TM. Further studies are needed to understand the potential role of these cytokines in the pathogenesis of TM itself and its undesired complications such as altered immune responses that might in turn influence infectious episodes of these special group of patients.

ACKNOWLEDGEMENTS

This study was supported by a grant from the Research Foundation of Ege University, Izmir, Turkey (project no. 2013/DIS/030).

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Date submitted: 2018 Dec 14

Accept submission: 2019 May 13