Salivary IL-4: A Bleeding Predictor on Probing in Descendants of Severe Periodontitis Patients

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Objective: *Periodontitis in younger patients can cause severe periodontal destruction, and cases are usually more numerous in members of the same family due to the sharing of susceptibility factors. Thus, the use of a familial study design could improve our understanding of initial alterations in periodontal tissue. This observational study aimed to evaluate the salivary inflammatory pattern in descendants of periodontitis patients and identify any correlation with the clinical periodontal condition.* ***Study design:*** *Fifteen children of Generalized Aggressive Periodontitis (GAgP) patients and 15 children with periodontally healthy parents were evaluated for their plaque index (PI), gingival index (GI), bleeding on probing (BoP), and probing depth (PD). The concentrations of interferon (IFN)-γ, interleukin (IL)-10, IL-17, IL-1β, IL-4, and tumor necrosis factor (TNF)-α were measured in unstimulated saliva using the Luminex MAGPix platform.* ***Results:*** *Children from the GAgP group presented higher probing depth (PD) and bleeding on probing (BoP) ($p<0.05$) and lower release of IL-4 in saliva ($p<0.05$) than the periodontally healthy group. The cytokines IL-10, IFN-γ, IL-17, and IL-4 were negatively correlated with the gingival index, while IL-4 was negatively correlated with BoP. A regression analysis revealed that salivary IL-4 and plaque were predictors of BoP.* ***Conclusions:*** *Children of GAgP parents presented lower salivary IL-4 and higher BoP and PD than children from periodontally healthy families. Additionally, salivary IL-4 was a predictor of bleeding on probing in the children, suggesting that the lower presence of this anti-inflammatory cytokine is related to higher clinical inflammation.*

Keywords: *disease susceptibility; family; IL-4; inflammation; periodontal disease; saliva*

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INTRODUCTION

Periodontitis is a multifactorial disease associated with an imbalance between the microbial aggression promoted by a dysbiotic biofilm and host immune response mediated by the inflammatory process[1]. Recently, the central role of the immune system in modulating the subgingival environment was proposed[2], and host factors were suggested as the main cause of disease occurrence. Thus, understanding how they modulate the homeostatic breakdown and initiation of periodontitis is essential for identifying susceptibility factors and clinical management.

One way to understand disease susceptibility is to evaluate a risk population for its development and identify the local changes that occur before periodontal breakdown. Thus, our research group has been using the familial aggregation characteristic of the most severe and progressive cases of periodontitis in younger patients[3–5], previously classified as Generalized Aggressive Periodontitis (GAgP)[3], to assess several etiopathogenic aspects in a risk population for periodontitis development. By evaluating GAgP patients' descendants, it was demonstrated that alterations in the clinical, microbiological, and subgingival inflammatory conditions can occur during

childhood[6–9], even before the clinical diagnosis of disease. These results suggest that preliminary signs of disease or susceptibility factors could be identified from an early age, and therefore, they could be used for the identification of susceptible individuals, as well as for disease monitoring. Despite the need for periodontal examination in children, probing a child can be challenging when dealing with inflamed sites and children with reduced compliance[10]. This fact is encouraging the identification of less invasive diagnostic tools and biomarkers.

Saliva is a promising source of biomarkers for disease and for monitoring a risk population as it can represent the organism's physiological condition and give a full picture of the condition of the oral cavity[11,12]. Salivary exams have the advantages of being easy to collect, non-invasive, painless, and low-cost exams, which facilitates wider population screening. Moreover, they can be used to screen a vulnerable population, such as children, when a clinical examination could be hard to do[11,13]. Many studies have focused on saliva to search for biomarkers in oral and systemic conditions[13,14] as their identification could substantially improve the early diagnosis, risk prediction, and monitoring of many disorders[11]. However, the use of saliva in the diagnosis of periodontitis remains limited, unpredictable, and should be more explored to identify reliable markers and the individualized and precise periodontology[13,14].

Although the majority of the literature has investigated inflammatory alterations related to periodontitis in the gingival crevicular fluid (GCF), some studies have highlighted saliva as a potential source of periodontal biomarkers[12,15]. Specifically, the evaluation of a combination of salivary molecules, such as interleukin (IL)-1beta, IL-6, and matrix metalloproteinase-8, has demonstrated the potential for distinguishing between healthy and periodontitis patients[16], and another study has reported a positive correlation between salivary IL-1β levels, bacterial counts and gingival inflammation in orthodontic patients[17]. Furthermore, a correlation between GCF and saliva cytokine levels and clinical inflammation has been described[18], suggesting that saliva could be used as a non-invasive and quick method for monitoring periodontal disease activity.

Thus, it was hypothesized that children from GAgP parents would present alterations in the salivary levels of cytokines, as observed in the gingival crevicular fluid[7,8], that could be used as a marker for precocious periodontal alteration in children. The present study aimed to evaluate the salivary inflammatory pattern of children from GAgP parents compared to children from healthy families and identify a correlation between salivary cytokines and clinical periodontal conditions.

MATERIALS AND METHOD

Study design and patient selection

This study was an observational case-control clinical study, with a gender and aged-matched design, registered at ClinicalTrials.gov with the identifier NCT03642353, developed according to the STROBE Guideline, and following all the recommendations of the Declaration of Helsinki as revised in 1975. It was performed at Piracicaba Dental School, University of Campinas, Brazil, with the approval of the local ethics Committee, under approval number 103/2015. Informed consent was obtained from participants before inclusion in the study, and the patient assessment was performed between June 2016 and February 2017.

Patient selection for this study followed the protocol previously described in studies with the same population[6,9] Patients diagnosed with GAgP according to the 1999 International Classification of Periodontal Diseases[3] (nowadays classified as stage III and IV Grade C periodontitis[19]) with at least one child between 6–12 years old were asked about the inclusion of their children in the study. After the inclusion of children from GAgP parents (GAgP Group), periodontally healthy families (both parents with no history of periodontitis) were assessed for the inclusion of their children in the study (Periodontally Healthy Group), matching age and gender between the two groups.

When selecting a child for the GAgP or Healthy groups, the parents had to satisify the following criteria. GAgP parents: i) less than 35 years old at diagnosis; ii) clinical attachment loss in at least three teeth other than first molars and incisors; iii) at least eight teeth with probing depth (PD) and clinical attachment level (CAL) ≥ 5 mm (with at least two sites with PD ≥ 7 mm) at diagnosis; iv) at least 20 teeth; v) be in supportive therapy for at least 6 months; vi) absence of systemic disease (e.g., diabetes, hypertension). Periodontally healthy parents: i) absence of systemic disease; ii) at least 20 teeth; iii) no history of periodontitis; iv) absence of gingival sulcus with PD>3 mm; v) proximal clinical attachment level ≤ 2 mm; vi) absence of proximal bone loss. Smoking was an exclusion criterion for both groups.

The children had to satisfy the following inclusion criteria: i) having parents that satisified the inclusion criteria for the healthy or GAgP condition; ii) aged between 6 and 12 years old; iii) absence of systemic disease. Children who had used antibiotics or anti-inflammatory medication in the 6 months before the study or used an orthodontic appliance were excluded.

Thirty children, 15 in each group, were selected for inclusion in the study. Sample size calculation was done considering alpha=5%, a power level of 80%, and an effect size of 1.0 (based on previous studies published by our group with this risk population[8]), the minimum sample size required per arm was 14.

Clinical and inflammatory evaluation

All fully erupted teeth were clinically evaluated at six sites per tooth. The following parameters were recorded: i) plaque index (PI)[20]; ii) gingival index (GI)[20]; iii) bleeding on probing (BoP)[21]; iv) probing depth (PD). All clinical examinations were performed by the same experienced and calibrated clinician. Calibration was performed with two patients not included in the study, who were evaluated twice in 24 hours for PPD (MFM—Intra-class correlation = 92% of PPD). The examiner was blinded regarding the group of each child. The same clinician collected the saliva before the clinical evaluation for the inflammatory cytokines analysis. Unstimulated whole saliva was collected in the early morning hours, and children were asked not to eat or perform oral hygiene 2 hours before sample collection. The saliva was collected into sterile Falcon tubes over 5 minutes,stored in an Eppendorf tube, and immediately frozen at -80 °C until laboratory evaluation.

Saliva was evaluated for the levels of interferon (IFN)-γ, interleukin (IL)-10, IL-17, IL-1β, IL-4, and tumor necrosis factor (TNF)-α using the Sensitivity Human Cytokine 06-plex (Millipore Corporation, Billerica, MA). According to the manufacturer's recommendations, assays were carried out using the MAGpix™ instrument (MiraiBio, Alameda, CA). Concentrations were estimated from the standard curve using a five-parameter polynomial

equation using Xponent® software (Millipore, Corporation, Billerica, MA). The mean concentration of each marker was calculated using the individual as a statistical unit and expressed as pg/ml.

Statistical analysis

The data were evaluated for normality by the Shapiro-Wilk test. Fisher's exact and Student's t-tests were used to analyze gender distribution and age, respectively. Due to a non-normal distribution, the differences in the clinical data and the cytokine concentration between groups were evaluated using the Mann-Whitney test. The cytokine concentrations were correlated with the clinical parameters using the Spearman correlation. After that, a Forward Multiple Linear Regression was performed using the BoP as a dependent variable and cytokine concentrations and other clinical parameters as independent variables. All tests considered alpha=5% and were done by a blinded statistician.

RESULTS

The demographic characteristics confirmed the gender and age-matching of this study (Table 1), and there was no difference in the percentage of female participants and age between groups ($p>0.05$). Both groups presented a similar number of teeth and percentage of deciduous teeth in the mouth ($p>0.05$). No children in either group presented clinical attachment loss or radiographic bone loss. Children from the GAgP group presented higher PD and BoP than the periodontally healthy group ($p<0.05$), and no difference was observed for PI and GI ($p<0.05$). Additionally, children from the GAgP group presented a lower salivary IL-4 level ($p<0.05$), while no difference was demonstrated for TNF-α, IL-10, IL-1β, IFN-ɣ, or IL-17 ($p\geq0.05$).

The correlation between the clinical parameters and the inflammatory response in saliva was assessed. The cytokines IL-10, FN-ɣ, IL-17, and IL-4 were negatively correlated with the GI, while IL-4 was negatively correlated with BoP (Table 2). No other correlation was found between clinical parameters and cytokines ($p\geq0.05$). Furthermore, a regression model was fitted using the cytokines and clinical data as independent variables and BoP as a dependent variable (Table 3). The regression analysis revealed that PI and the salivary level of IL-4 were predictors of the children's BoP. IL-10 and IFN-ɣ were kept as adjustment variables for optimal fitting of the model ($R^2= 0.47$).

DISCUSSION

Identifying biological events related to the initiation of periodontal disease is crucial for understanding susceptibility to periodontitis, especially those cases associated with precocious initiation and rapid destruction. The present study used the familial approach to evaluate a higher risk population for GAgP development and demonstrated that children from periodontitis parents presented alterations in inflammatory cytokines in the saliva even before clinical diagnosis. Additionally, salivary IL-4 concentration was a predictor of gingival inflammation even when adjusting for plaque accumulation. Thus, salivary assessment, a non-invasive and easy-to-do exam, could facilitate the examination and screening of this at-risk population, impacting preventive approaches and the clinical management of periodontal changes in childhood.

The familial approach in this study design was based on the familial aggregation of AgP cases[4,5]. This model was used in

Table 1. Demographical, clinical, and salivary cytokine concentration data for both groups

	GAgP (n=15)	Periodontally Health (n=15)	p-value
Gender (%F)	40	40	1.0
Age (years)	9.90±2.10	9.90±1.90	0.98
PD (mm)	2.44±0.23	2.30±0.24	0.03
PI (%)	57.0±13.0	50.0±22.0	0.28
GI (%)	27.0±14.0	19.0±12.0	0.09
BoP (%)	40.0±15.0	26.0±12.0	0.002
Number of teeth	23.58±2.78	23.67±1.23	0.92
Deciduous teeth (%)	34.43±27.16	39.52±25.37	0.55
TNF-α (pg/mL)	6.7±2.8	8.4±2.9	0.30
IL-10 (pg/mL)	3.1±1.2	8.9±8.2	0.15
IL-1β (pg/mL)	1585.2±900.9	1633.0±1117.8	0.94
IFN-ɣ (pg/mL)	69.3±26.0	121.4±64.1	0.09
IL-4 (pg/mL)	15.3±4.8	26.4±9.8	0.02
IL-17 (pg/mL)	12.4±4.5	20.0±9.0	0.06

Data presented as mean ± standard deviation. *GAgP – Generalized Aggressive Periodontitis; F – female; PD – probing depth; PI – plaque index; GI – gingival index; BoP – Bleeding on probing; TNF–tumor necrosis factor; IL – interleukin; IFN – interferon.*

Table 2. Spearman's correlation (ρ (p-value)) between clinical data and salivary cytokine concentration, considering both groups

	PI	GI	PD	BoP
TNF-α	-0.14(0.49)	-0.41(0.06)	0.16(0.46)	-0.21(0.31)
IL-10	-0.28(0.18)	-0.61(0.001) *	-0.16(0.43)	-0.29(0.16)
IL-1β	0.19(0.36)	-0.11(0.60)	0.15(0.48)	-0.01(0.95)
IFN-ɣ	-0.12(0.57)	-0.59(0.002) *	0.01(0.96)	-0.39(0.06)
IL-4	-0.20(0.35)	-0.60(0.002) *	-0.003 (0.98)	-0.42 (0.04) *
IL-17	-0.14(0.52)	-0.57(0.004) *	0.05(0.81)	-0.39(0.06)

* Significant Spearman correlation ($p<0.05$). *PI – plaque index; GI – gingival index; PD – probing depth; BoP – Bleeding on probing; TNF–tumor necrosis factor; IL – interleukin; IFN – interferon.*

Table 3. Forward multiple linear regression analysis considering BoP as a dependent variable.

	Coefficient	p-value	Adjusted R^2
Intercept	0.058	<0.001	0.47
PI	0.176	0.004	
IL-4	-2.00E-04	0.018	
IL-10	-	0.335	
IFN-ɣ	-	0.403	

IL-10 and IFN-ɣ was the adjusted variable for the regression model. PI – plaque index; IL – interleukin; IFN – interferon.

previous studies and allowed the identification of clinical, microbiological, and inflammatory changes from early ages in descendants of AgP patients[6–8,22]. These characteristics could be involved in susceptibility to the disease and could favor the imbalance in the subgingival environment, leading to periodontal destruction.

Clinically, none of the included children presented clinical attachment loss, and plaque accumulation was similar between groups. Despite these similarities, children of GAgP parents showed higher PD and BoP, corroborating other studies by our research group[6,7,9] and others[23], which found that parents' oral disease can negatively impact the oral health of their descendants. Higher PD and BoP alone are not sufficient to diagnose periodontitis; however, they highlight that gingival inflammation, a precursor for periodontal destruction, may already be present.

The use of clinical periodontal parameters is fundamental for the diagnosis of periodontal destruction. However, they are limited in identifying precocious changes happening subgingivally and susceptible individuals[13]. Additionally, performing the clinical periodontal examination and the subgingival collection of samples in children can produce some discomfort and be hard to execute. Thus, less invasive diagnostic tools, such as saliva collection, and the characterization of biomarkers such as cytokines for disease onset are desirable and could favor long-term monitoring and precision periodontology[13,14].

Besides the clinical difference, the salivary level of IL-4 was lower in children from GAgP families and predictive for gingival bleeding. IL-4 is considered a critical anti-inflammatory mediator that modulates osteoclastogenesis[24], suppresses macrophage activation, and downregulates the synthesis of pro-inflammatory mediators[25]. Its reduction has been demonstrated to be important in disease pathogenesis by hampering the proper control of the inflammatory process and exacerbating the local inflammation that causes bone loss[26]. IL-4 can modulate periodontal destruction by orchestrating the migration of Treg cells to the sites of osteolytic lesions, including by inhibiting inflammatory migration and producing pro-inflammatory and osteoclastogenic mediators associated with periodontitis[27]. Clinically, lower levels of IL-4 were observed in the serum of diseased sites of GAgP patients[28] and GCF of diseased and healthy areas of GAgP patients[29]. Additionally, periodontal treatment has been associated with increasing IL-4 levels in GCF and serum, highlighting their importance in maintaining better clinical conditions [30]. This result is also consistent with previous investigation of this risk population describing decreased cytokine levels subgingivally in descendants of GAgP individuals[7,8]. Thus, this study demonstrated that lower levels of IL-4, a periodontitis-associated condition, are observed in GAgP descendants' saliva, suggesting its importance in GAgP initiation. This observation, associated with some other findings describing the reduction of the anti-inflammatory response in GAgP patients [24-25], supports the notion that susceptibility to periodontitis may be related to a decrease in anti-inflammatory pathways, instead of just an increase in pro-inflammatory mediators, and an imbalance in the local inflammatory process.

Interestingly, this study demonstrated a connection between the saliva's inflammatory pattern and the children's clinical condition. Lower salivary IL-4 was associated with higher levels of BoP and GI, and IL-4 was a predictor of BoP, even though the regression model included an adjustment for the biofilm accumulation index. The negative correlation between the GCF concentration of IL-4 and BoP, PD, and CAL was also demonstrated in GAgP patients[29]. These associations reinforce the notion that saliva could be used to evaluate what is happening subgingivally and that such inflammatory changes impair the clinical condition, a suggestion also supported by a previous study that correlated salivary and GCF cytokines and clinical inflammatory parameters[18]. So, even though the saliva might not reveal the clinical situation precisely, it could offer essential information about risk, precocious diagnostics, and monitoring.

Although there was no clinical diagnosis of attachment loss in our sample, long-term bleeding is described as a risk for future attachment loss[31,32], so this population should be monitored. It is possible that the manifestation of inflammation could be a precocious change before the clinical diagnosis of active bone loss, which, in the long term, could negatively impact periodontal health. In the interval between immunological alterations and clinically detectable changes in disease status, there is an opportunity for prevention in the risk populations. Therefore, precocious signs of higher clinical inflammation and lower level of IL-4 in saliva should be investigated in the long-term as signs of susceptibility for periodontitis or as early signs of disease.

Meanwhile, longitudinal studies with a larger sample size should be performed to investigate the causal relationship between the disruption in salivary levels of IL-4 and attachment loss. As periodontitis is a multifactorial disease, the use of comprehensive analysis in subsequent studies is also desirable as a combination of markers could more precisely describe risk[13]. Moreover, it is also crucial to understand the cause of these changes. Intrinsic factors such as host response and microbial factors can disrupt the inflammatory response, and understanding such traits is crucial for the correct clinical management of the risk population and the proposal of preventive approaches.

In conclusion, children of GAgP parents presented lower salivary IL-4 and higher BoP and PD than children from periodontally healthy families. Additionally, salivary IL-4 was a predictive marker of BoP estimation in children, suggesting that the lower presence of this anti-inflammatory cytokine is related to higher clinical inflammation.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgment

The authors acknowledge The São Paulo Research Foundation (FAPESP) for financial support for this study (process 2018/12335-0 and 2015/50264-0).

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