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Local and Plasma Biomarker Profiles in Localized Aggressive Periodontitis

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Abstract: Localized aggressive periodontitis (LAP) patients possess a systemic hyperinflammatory response after lipopolysaccharide stimulation. However, the levels of inflammatory and bone biomarkers in plasma, as well as possible associations between local and plasma biomarkers, are unknown in LAP. This cross-sectional study aimed to characterize gingival crevicular fluid (GCF) and plasma biomarker profiles in LAP patients, their healthy siblings (HS), and healthy unrelated controls (HC). Fifty-eight LAP subjects, 33 HS, and 49 HC (African Americans, aged 5 to 25 y) were included. Following collection of clinical parameters with GCF and plasma samples, levels of 16 inflammatory and bone resorption biomarkers were determined with Milliplex. Univariate and correlation analyses were performed among all clinical and laboratorial parameters. Discriminant analyses were used to investigate groups of biomarkers discriminating LAP from HS and HC

in GCF and plasma. GCF levels of multiple cytokines and chemokines and RANKL:OPG ratio (receptor activator of nuclear factor kappa-B ligand:osteoprotegerin) were higher in LAP disease, most of which positively correlated with probing depth and attachment loss of sampled sites. A group of IL-12p40, IL-6, IL-12p70, IL-2, and MIP-1 α discriminated LAP diseased sites from their healthy sites, as well as from HS and HC healthy sites. In plasma, only RANKL levels were increased in LAP versus controls, which positively correlated with the percentage of affected sites and deep/bleeding sites. A plasma inflammatory profile including MIP-1 α , IL-8, IL-10, and INF- γ could significantly discriminate LAP patients from HS and HC. No correlations were found between GCF and plasma levels of biomarkers. In conclusion, an inflammatory profile including groups of specific biomarkers in GCF and plasma may significantly discriminate LAP from healthy individuals. The hyperinflammatory

response previously found in the peripheral blood of LAP patients is dependent on lipopolysaccharide stimulation, apparently resulting mostly in local tissue destruction and changes in biomarker profile, with a slight influence in the systemic inflammatory profile (ClinicalTrials.gov NCT01330719).

Knowledge Transfer Statement: The results of this study can be possibly used by clinicians in the future as diagnostic tools for localized aggressive periodontitis. Thus, in the future, with proper consideration of cost, patient preference, chair-side feasibility and ultimately further studies validating the role of GCF markers for disease progression and response to treatment, this information could lead to more appropriate therapeutic decisions and the development of preventive approaches for susceptible patients.

Keywords: cytokines, inflammation, bone resorption, gingival crevicular fluid, blood, periodontal diseases

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A supplemental appendix to this article is available online.

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Introduction

Periodontal disease is a multifactorial inflammatory disease initiated by virulent subgingival microorganisms in the dental biofilm. Persistent activation of the host cells by products and components of microorganisms leads to overexpression of inflammatory mediators in the periodontal tissues, including several cytokines and chemokines (Kornman 2008). Host cytokines and chemokines may directly or indirectly cause a subsequent imbalance in the osteoclastogenesis process, leading to periodontal bone resorption (Cochran 2008). Bone resorption is regulated by several mediators, including the receptor activator of nuclear factor kappa-B ligand (RANKL), which is expressed by various cell types, as well as RANKL receptor on osteoclast precursors and the soluble decoy receptor osteoprotegerin (OPG; Graves et al. 2011). A relative increase in RANKL concentrations or decrease in OPG concentrations in periodontal tissues may result in a net increase in RANKL (increased RANKL:OPG ratio) and consequent pathologic bone resorption (Graves et al. 2011).

Aggressive periodontitis is a less prevalent but often severe and rapidly progressing form of periodontal disease, associated with early onset and a tendency for familial aggregation in otherwise systemically healthy individuals (Armitage and Cullinan 2010). Localized aggressive periodontitis (LAP) results in tissue destruction around first molars and incisors and may affect primary and permanent dentitions (Albandar 2014). In aggressive periodontitis and LAP, the role of exaggerated host responses is a major factor in the rapid tissue destruction (Kulkarni and Kinane 2014). However, little is known about the local and systemic interactions between inflammatory and bone resorption molecules in the context of LAP.

Besides the well-described phagocyte abnormalities (Ryder 2010), LAP patients possess a hyperinflammatory phenotype characterized by overproduction of individual cytokines and chemokines in response to bacterial endotoxin (Shaddox

et al. 2010). High levels of bacterial lipopolysaccharide in plasma with exacerbated production of local cytokines and chemokines were also described (Shaddox et al. 2011). Whether the exacerbated local inflammatory response is enough to cause a significant impact on the systemic levels of inflammatory and bone resorption mediators in LAP patients, in the absence of any additional stimulation with bacterial endotoxin and lipopolysaccharide, remains poorly investigated.

Although the evidence has indicated possible disease biomarkers in LAP (Shaddox et al. 2011; Fine et al. 2014), there is an emerging need to characterize biomarker profiles that consider groups of mediators that might functionally interrelate in LAP. This would be suitable to help the development of future novel diagnostic tools, which would strongly assist current clinical and radiographic diagnostic approaches; although clinical and radiographic examinations are essential to assess tissue destruction, they may be limited or not sufficient to identify certain sites of active disease or the degree of host susceptibility to future disease (Giannobile et al. 2009).

The primary aim of this study was to determine the gingival crevicular fluid (GCF) and plasma biomarker profiles in LAP by analyzing 16 inflammatory and bone resorption mediators. Biomarker profiles were investigated to 1) identify possible associations between local and systemic biomarkers in samples and 2) characterize the groups of local and systemic biomarkers potentially discriminating LAP diseased sites versus those of healthy individuals, including siblings and unrelated controls. The hypothesis of the study is that LAP presents a differentiated local and systemic inflammatory profile when compared with that of healthy individuals.

Materials and Methods

Participants and Clinical Assessment

This study is a cross-sectional evaluation of LAP individuals, their healthy siblings (HS), and unrelated healthy controls

(HC), who were selected from a cohort recruited from December 2006 to 2014, from the following clinical centers: the Florida departments of health from Leon and Duval counties and the University of Florida, Gainesville, Florida. This study is part of a larger clinical trial (ClinicalTrials.gov NCT01330719). Inclusion criteria consisted of African Americans 5 to 25 y old who were diagnosed with LAP (Armitage 1999) or were age-, sex-, and race-matched periodontally HS or HC. LAP diagnosis was defined by the presence of at least 2 sites (incisor and/or first molar, in permanent or primary dentition), probing depth (PD) ≥ 5 mm with concomitant presence of bleeding on probing (BOP), clinical attachment level (CAL) ≥ 2 mm, and radiographically detected bone loss (Armitage 1999). HS and HC were defined as healthy by the absence of PD > 3 mm with concomitant BOP, CAL > 1 mm, and bone loss. Exclusion criteria included the following: history of systemic disease with a potential to influence periodontal diseases, having taken antibiotics or other medications that could influence the clinical characteristics or course of periodontal diseases for at least 3 mo, smoking, and pregnancy/lactation.

Medical and dental histories and clinical periodontal parameters were collected, including PD, gingival margin position (GM), CAL (calculated by PD + GM), BOP, and visible plaque. Measurements were performed by calibrated individuals, at 6 sites per tooth, with a UNC-15 periodontal probe and recorded with appropriate computer software (Florida Probe).

The study was approved by the Institutional Review Board at the University of Florida, and it followed STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines (Vandenbroucke et al. 2014). All participants or their legal representatives signed an approved informed consent to be included in the study.

GCF Sampling

Following clinical examination, periodontal sites for GCF collection were isolated with cotton rolls to prevent saliva

contamination and air-dried gently, and supragingival plaque was removed. For LAP patients, samples were collected from a diseased or affected site (LAP-D; PD ≥ 5 mm with concomitant presence of BOP, CAL ≥ 2 mm, and radiographically detected bone loss) and a healthy site (LAP-H; PD ≤ 3 mm, and absence of BOP, clinical attachment loss, and bone loss). All collected sites of HS and HC were healthy.

GCF samples were collected from each site with paper filter strips (PerioPaper) gently inserted into the sites 1 to 2 mm for ~ 10 s (Shaddox et al. 2011). Volume of GCF was calculated with a micro-moisture meter (Periotron 8000) and through calibration curves. Samples were frozen at -80°C until analyses of soluble biomarkers were performed.

Plasma Sampling

Peripheral blood samples of LAP patients, HS, and HC were collected in heparinized vacutainer tubes (13×75 mm; 2.0 to 4.0 mL). The samples were centrifuged (300 g, 15 min) to separate plasma from red blood cells. The plasma (supernatant) was stored at -80°C until further analysis.

Quantification of Soluble Biomarkers

Multiplex assays (Milliplex 14-plex cytokine/chemokine, RANKL single-plex, and bone single-plex detection kits) were used to detect and quantify 16 biomarkers (eotaxin, GM-CSF, IFN- γ , IL-10, IL-12p40, IL-12p70, IL-1 β , IL-2, IL-6, IL-8, IP-10, MCP-1, MIP-1 α , TNF- α , RANKL, OPG) in GCF and plasma according to the manufacturer's protocol. The same biomarkers were investigated in our previous studies of this LAP cohort (Shaddox et al. 2010; Shaddox et al. 2011), except the bone resorption biomarkers (RANKL and OPG), which were included here to investigate their relationship with key inflammatory mediators in the context of aggressive disease. Before the assay, GCF samples were eluted from the strip in 150 μL of phosphate buffered saline by centrifugation into an Eppendorf tube. Data were acquired on the Luminex 200 (Millipore) and analyzed with Luminex

software (Viagene Tech) and standard curves. Data were reported as pg/mL.

Statistical Analyses

From our previous study on local markers of LAP (Shaddox et al. 2011), a sample size of 10 per group was determined to be sufficient to detect differences in biomarkers among the groups, with a power $>80\%$. Means and standard deviations of parameters were calculated. One-way analysis of variance and Tukey's multiple comparisons or Kruskal-Wallis with Dunn's multiple comparisons, when appropriate, were used to compare clinical and demographic parameters and biomarker levels among groups. Spearman correlations were performed to evaluate associations between clinical parameters and biomarker levels and between GCF and plasma biomarker levels. Missing data were considered in the analysis, as we used nonparametric approaches.

Biomarker Profiles among Sites and Groups

Given the large number of biomarkers measured and how they may not function independently, we used principal component analysis (PCA) as a data reduction technique to reduce the number of variables in our statistical analysis, as well as to explore their associations (Allin et al. 2016). Our aim was to guide selection of the biomarkers that accounted for the greatest explanatory variance within our sample. The biomarkers that accounted for the greatest percentage of variance (i.e., made up the primary principal component) were entered into a discriminant function analysis (DA) to evaluate predictive ability among patient groups (LAP, HS, and HC), locally (among sites) and systemically (among patient groups). DA also was used to determine the combination of variables that best predicts the groups. We conducted subsequent analyses including age and sex as covariates in all analyses. Statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software) and SPSS 20.0 (SPSS Inc.).

Results

Participants and Clinical Parameters

Table 1 presents a summary of the demographic and clinical characteristics of the LAP patients ($n = 58$), HS ($n = 33$), and HC ($n = 49$). The LAP population comprised 12 patients with primary dentition affected and 46 patients with permanent dentition affected.

GCF and Plasma Biomarker Levels

The GCF levels of the majority of the biomarkers (except IL-8, IP-10, and OPG) and the RANKL:OPG ratio were significantly higher in LAP-D versus LAP-H subjects. For LAP-D, levels of GM-CSF, IL-10, IL-12p40, IL-1 β , IL-2, IL-6, MCP-1, MIP-1 α , and RANKL were increased when compared with all other healthy sites, even after adjusting for multiple comparisons. In HS sites, levels of IFN- γ and OPG were significantly elevated in comparison with HC sites, while eotaxin, IL-12p70, and IP-10 levels were lower than those of HC sites (Tables 2 and 3).

In plasma, only RANKL was significantly higher in LAP patients versus HS and HC, while the RANKL:OPG ratio was higher in LAP against HS only. Certain biomarkers were significantly lower in LAP versus HS (eotaxin, GM-CSF, IFN- γ , IL-6, and TNF- α ; Tables 2 and 3).

Correlations of Clinical Parameters and GCF/Plasma Biomarkers

In GCF, levels of biomarkers, except IL-8 and OPG, positively correlated with PD and/or CAL from sites sampled for GCF ($P < 0.05$). Eotaxin, GM-CSF, IL-12p40, IL-12p70, IL-1 β , IL-2, IL-6, MCP-1, MIP-1 α , and RANKL:OPG ratio significantly correlated with PD ($P < 0.05$ to $P < 0.001$). All these cytokines, as well as IFN- γ , IL-10, IP-10, TNF- α , and RANKL, positively correlated with CAL ($P < 0.05$ to $P < 0.001$; Table 4).

In plasma, IL-8 was positively correlated with CAL ($P < 0.05$), while RANKL levels positively correlated with the percentage of affected sites and the percentage of deep sites (PD > 4 mm) with concomitant BOP (i.e., deep and bleeding sites;

Table 1.

Demographic and Clinical Parameters of Patients with LAP, Their HS, and Unrelated HC.

Variable	LAP (<i>n</i> = 58)		HS (<i>n</i> = 33)	HC (<i>n</i> = 49)
	LAP-D	LAP-H		
Female:male, <i>n</i>	34:24		18:15	31:18
Age, y	13.8 ± 3.3		11.56 ± 3.5 ^a	14.7 ± 5.7
Affected sites, mm				
PD	5.7 ± 0.9		N/A	N/A
CAL	3.2 ± 1.2		N/A	N/A
% ^b				
Plaque	34.0 ± 23.3		37.3 ± 19.9 ^c	27.0 ± 19.9
BOP	19.0 ± 13.2 ^d		8.4 ± 8.0	10.5 ± 9.4
Affected sites	10.9 ± 9.6 ^d		0	0
DB sites	9.2 ± 7.8 ^d		0	0
Site, mm ^e				
PD	7.0 ± 1.9 ^d	2.6 ± 0.0	2.7 ± 0.5	2.8 ± 0.6
CAL	4.4 ± 2.0 ^d	0	0	0

Values are shown as mean ± SD.

BOP, bleeding on probing; CAL, clinical attachment level; DB, deep and bleeding; HC, healthy controls; HS, healthy siblings; LAP, localized aggressive periodontitis; LAP-D, diseased sites of LAP; LAP-H, healthy sites of LAP; N/A, not applicable; PD, pocket depth.

^a*P* < 0.05, compared with other groups.^bPlaque: percentage of full-mouth visible plaque. BOP: percentage of full-mouth BOP. Affected sites: percentage of sites with PD >4 mm, BOP, CAL ≥2 mm, and radiographic bone loss. DB sites: percentage of sites with PD >4 mm with BOP.^c*P* < 0.05, compared with HC, by analysis of variance with Tukey's multiple comparisons or Kruskal-Wallis with Dunn's multiple comparisons, when appropriate.^d*P* < 0.001, compared with other groups.^ePD site: mean PD for gingival crevicular fluid-sampled sites. CAL site: mean CAL for gingival crevicular fluid-sampled sites.

P < 0.01 and *P* < 0.05, respectively; Appendix Table).

All correlation coefficients (*r*²) from the analyses between biomarker levels and clinical parameters are presented in Table 4. No correlations were found between GCF and plasma levels for any of the biomarkers (*P* > 0.05).

Principal Component Analysis and Discriminant Function Analysis

Gingival Crevicular Fluid

The number of components derived from the PCA and the variation explained by these components in our sample are presented in Table 5. In GCF, PCA revealed 3 components, all of which were able to significantly discriminate the 4 groups (LAP-D, LAP-H, HS sites, and HC sites),

as found by DA (Wilks's lambda <0.05). However, among those 3 components, DA showed that principal component 1 better discriminated all groups in all canonical functions and that IL-12p40, IL-6, IL-12p70, IL-2, and MIP-1α cytokines were identified as having the highest discriminatory role, especially in the LAP-D group (Fig. A; Wilks's lambda <0.0001). Furthermore, IL-2 and MIP-1α were significant to separate the groups in function 1, while IL-12p70, IL-6, and IL-12p40 were significant to discriminate the groups in function 2 (Fig. A). Results were not significantly influenced by age or sex (*P* > 0.05).

Plasma

Table 5 also presents the components derived from plasma data. PCA identified

5 components, all of which were able to significantly separate the LAP individuals, HS, and HC (Wilks's lambda <0.05). A group consisting of MIP-1α, IL-8, IL-10, and IFN-γ (principal component 1) better separated the 3 groups in the DA (Wilks's lambda = 0.012, in canonical function 1), as shown in Figure B. In this group, only IL-10 was significantly responsible for separation in function 1. Similar to GCF results, these findings were not significantly influenced by age or sex (*P* > 0.05).

Discussion

To the best of our knowledge, the present study is the first to simultaneously characterize the local and systemic biomarker profiles in LAP individuals and to investigate the dynamics of bone

Table 2.

GCF Biomarker Levels for the LAP Patients, Their HS, and Unrelated HC.

Biomarker	GCF, pg/mL			
	LAP-D (<i>n</i> = 58)	LAP-H (<i>n</i> = 58)	HS (<i>n</i> = 33)	HC (<i>n</i> = 49)
Eotaxin	5.88 ± 2.16 ^{a,b}	4.19 ± 1.87 ^b	2.51 ± 1.80 ^c	5.35 ± 3.29
GM-CSF	0.72 ± 0.45 ^{a,b,c}	0.43 ± 0.18 ^b	0.30 ± 0.14	0.43 ± 0.27
IFN- γ	0.61 ± 0.40 ^{a,c}	0.40 ± 0.25	0.53 ± 0.28 ^c	0.37 ± 0.22
IL-10	1.04 ± 0.52 ^{a,b,c}	0.80 ± 0.46 ^b	0.49 ± 0.22	0.62 ± 0.31
IL-12p40	8.55 ± 6.62 ^{a,b,c}	4.47 ± 3.14	3.14 ± 3.29	5.10 ± 4.82
IL-12p70	0.83 ± 0.38 ^{a,b}	0.56 ± 0.28 ^b	0.38 ± 0.22 ^c	0.68 ± 0.38
IL-1 β	7.12 ± 6.94 ^{a,b,c}	2.36 ± 1.79	2.09 ± 1.72	1.92 ± 1.80
IL-2	1.36 ± 0.61 ^{a,b,c}	0.94 ± 0.43 ^{b,c}	0.37 ± 0.00	0.49 ± 0.18
IL-6	1.11 ± 0.59 ^{a,b,c}	0.49 ± 0.23	0.37 ± 0.13	0.56 ± 0.30
IL-8	117.63 ± 74.29	115.34 ± 83.12	146.50 ± 115.23	128.52 ± 98.37
IP-10	11.30 ± 4.47 ^b	9.13 ± 6.32	7.34 ± 5.87 ^c	13.06 ± 10.13
MCP-1	4.44 ± 2.65 ^{a,b,c}	2.82 ± 1.98	2.64 ± 2.21	1.96 ± 1.28
MIP-1 α	12.96 ± 5.97 ^{a,b,c}	8.93 ± 6.46	5.80 ± 4.40	7.69 ± 5.33
TNF- α	0.70 ± 0.34 ^{a,b}	0.57 ± 0.28	0.54 ± 0.29	0.65 ± 0.28
RANKL	9.49 ± 7.54 ^{a,b,c}	5.59 ± 4.86	4.29 ± 3.26	5.81 ± 6.35
OPG	12.56 ± 10.28	12.06 ± 7.88 ^c	22.83 ± 22.89 ^c	7.31 ± 6.15
RANKL:OPG	1.14 ± 1.12 ^{a,b}	0.81 ± 1.35	0.77 ± 1.51	1.31 ± 2.12

Values are shown as mean ± SD.

GCF, gingival crevicular fluid; GM-CSF, granulocyte-macrophage colony-stimulating factor; HC, unrelated healthy controls; HS, healthy siblings; IFN- γ , interferon gamma; IL-10, interleukin 10; IL-12p40, interleukin 12p40; IL-12p70, interleukin 12p70; IL-1 β , interleukin 1 beta; IL-2, interleukin 2; IL-6, interleukin 6; IL-8, interleukin 8; IP-10, interferon gamma-induced protein 10; LAP, localized aggressive periodontitis; LAP-D, diseased sites of LAP; LAP-H, healthy sites of LAP; MCP-1, monocyte chemoattractant protein 1; MIP-1 α , macrophage inflammatory protein 1 alpha; OPG, osteoprotegerin; RANKL, receptor activator of nuclear kappa-B ligand; TNF- α , tumor necrosis factor alpha.

^a*P* < 0.05 versus LAP-H sites.^b*P* < 0.05 versus HS sites.^c*P* < 0.05 versus HC sites.

resorption mediators in these patients. In addition to increased levels of several inflammatory cytokines and chemokines previously found to play a role in LAP disease (Shaddox et al. 2011), we detected increased RANKL levels and RANKL:OPG ratio in LAP-D. The lack of association between GCF and plasma cytokines, with distinct patterns of differentiation observed, provides a better understanding of a collective role of biomarkers in LAP pathogenesis. Jointly, our findings may contribute to the development of

noninvasive diagnostic methods, which would be valuable for LAP patients, in whom initial clinical parameters of disease are frequently overlooked.

Locally in the gingiva (GCF), a combination of IL-12p40, IL-6, IL-12p70, IL-2, and MIP-1 α better discriminated LAP-D from healthy sites of LAP patients, their HS, and HC. In accordance with our previous report (Shaddox et al. 2011), the present findings suggest a significant role of IL-12 in LAP, contrasting previous findings (Johnson and Serio

2005; Thunell et al. 2010). Nevertheless, longitudinal studies are required to confirm this assumption. IL-12 production by dendritic cells (DCs) is essential to connect innate to adaptive immune responses. Specifically, the biologically active heterodimer IL-12p70 (composed of p35 and p40 homodimers) is crucial to direct proliferation of activated T lymphocytes toward a Th1 phenotype, with high IFN- γ secretion. The IL-12p40 homodimer can also bind to the IL-12 receptor, acting as an antagonist to IL-12p70 while

Table 3.

Plasma Biomarker Levels for the LAP Patients, Their HS, and Unrelated HC.

Biomarker	Plasma, pg/mL		
	LAP (<i>n</i> = 49)	HS (<i>n</i> = 33)	HC (<i>n</i> = 48)
Eotaxin	27.74 ± 14.21 ^a	38.97 ± 15.54	42.20 ± 31.21
GM-CSF	1.37 ± 2.23 ^a	3.12 ± 2.12	N/D
IFN- γ	4.03 ± 5.30 ^{a,b}	10.80 ± 10.42	9.76 ± 11.10
IL-10	0.92 ± 1.50	0.77 ± 0.60	0.96 ± 1.05
IL-12p40	1.81 ± 2.02 ^a	0.54 ± 0.44 ^b	4.45 ± 6.53
IL-12p70	0.73 ± 0.54	1.46 ± 1.48	0.72 ± 0.49
IL-1 β	0.43 ± 0.13	0.45 ± 0.18	0.49 ± 0.22
IL-2	N/D	N/D	N/D
IL-6	0.28 ± 0.05 ^{a,b}	0.82 ± 0.96 ^b	0.74 ± 0.79
IL-8	6.17 ± 5.59	4.59 ± 4.02 ^b	8.07 ± 6.58
IP-10	222.80 ± 83.79	218.36 ± 126.89	215.33 ± 105.79
MCP-1	181.42 ± 58.10	186.64 ± 75.65	194.03 ± 66.45
MIP-1 α	8.21 ± 10.38	8.36 ± 10.91 ^b	13.16 ± 13.62
TNF- α	4.38 ± 2.74 ^a	6.88 ± 3.60 ^b	3.72 ± 2.36
RANKL	166.45 ± 87.65 ^{a,b}	111.20 ± 57.37	134.48 ± 89.12
OPG	177.99 ± 74.79	176.00 ± 74.79	171.16 ± 104.90
RANKL:OPG	1.21 ± 0.61 ^a	0.67 ± 0.46	1.39 ± 1.76

Values are shown as mean ± SD.

HC, unrelated healthy controls; HS, healthy siblings; LAP, localized aggressive periodontitis; N/D, not detected. See Table 2 for all other abbreviations.

^a*P* < 0.05 versus HS.^b*P* < 0.05 versus HC.

being involved in IL-23/IL-17 signaling (Khader et al. 2005), recently reported in generalized aggressive periodontitis (GAP; Cifcibasi et al. 2015). While IL-12p70 and IL-12p40 were highly correlated in local inflammatory sites in the present study, as well as with several other markers, including IFN- γ , IL-6, IL-2 and MIP-1 α , it is difficult to ascertain whether both contribute equally or differentially in diseased versus healthy sites. Interestingly, IL-2 has been shown to also control inflammation by inhibiting Th17 differentiation by interfering with IL-6-dependent signaling events (Laurence et al. 2007). Thus, IL-2 and IL-6 may discriminate disease via cross-regulatory roles.

MIP-1 α was another marker of the highly predictive component in GCF. Produced by epithelial cells, monocytes/macrophages, neutrophils, and DCs, this chemokine induces chemotaxis of monocytes and Th1 lymphocytes, also acting by stimulating monocytes and osteoclast progenitor cells to become active osteoclasts (Silva et al. 2007). MIP-1 α -positive cells are associated with expanded proportions of lymphocytes in tissues with increasing inflammation (Gemmell et al. 2001). The importance of MIP-1 α in the local sites of LAP patients and its association with clinical parameters of disease severity was emphasized in recent studies on other

cohorts (Fine et al. 2009; Fine et al. 2014). Here, MIP-1 α , as well as IL-12p40, IL-6, IL-12p70, and IL-2, not only pertained to the same group of discriminatory role for diseased sites but was also all significantly correlated with PD and CAL of the sampled sites. However, unlike other studies (Fine et al. 2014), the present investigation showed these values at only 1 time point and did not assess the predictive value of these markers in disease initiation or bone loss, which is a limitation.

Given the associations among GCF biomarkers found, we speculate that high activation of innate immune cells and high interactions between antigen-

Table 4.

Correlations between GCF Biomarkers and Clinical Parameters in LAP Patients.

GCF Biomarkers	Clinical Parameters ^a							
	PD All	PD Sites	CAL All	CAL Sites	Affected Sites, %	DB Sites, %	BOP, %	Plaque, %
Eotaxin	-0.240	0.228 ^b	-0.302 ^b	0.283 ^c	-0.143	-0.281	-0.277 ^b	-0.184
GM-CSF	-0.177	0.253 ^b	-0.254	0.258 ^c	0.025	-0.052	-0.035	0.004
IFN- γ	-0.286 ^b	0.141	-0.267	0.202 ^b	0.014	-0.144	-0.235	0.167
IL-10	-0.048	0.190	-0.090	0.210 ^b	0.024	0.006	0.051	0.039
IL-12p40	-0.193	0.254 ^c	-0.298 ^b	0.273 ^c	0.053	-0.033	-0.104	0.125
IL-12p70	-0.153	0.265 ^c	-0.293 ^b	0.288 ^c	0.004	-0.044	-0.176	0.121
IL-1 β	-0.058	0.378 ^d	-0.128	0.384 ^d	0.197	0.149	0.109	0.173
IL-2	-0.218	0.269 ^c	0.006	0.306 ^c	0.146	0.038	0.038	0.050
IL-6	-0.093	0.480 ^d	-0.354 ^c	0.483 ^d	0.039	-0.032	-0.075	0.036
IL-8	0.068	0.016	-0.144	0.007	-0.063	-0.226	-0.176	-0.314 ^b
IP-10	-0.179	0.192	-0.290 ^b	0.206 ^b	0.183	-0.011	-0.164	0.080
MCP-1	-0.101	0.286 ^c	-0.314 ^b	0.286 ^c	-0.151	-0.271	-0.283 ^b	-0.149
MIP-1 α	-0.017	0.282 ^c	-0.169	0.246 ^c	-0.149	-0.173	-0.063	0.084
TNF- α	0.053	0.202	0.010	0.225 ^b	0.137	0.009	0.003	-0.137
RANKL	-0.103	0.193	-0.014	0.257 ^b	0.055	-0.091	-0.072	-0.067
OPG	0.160	-0.085	0.018	-0.125	-0.178	-0.225	-0.079	-0.222
RANKL:OPG	-0.122	0.233 ^b	0.184	0.333 ^d	0.129	0.105	0.064	0.222

Values are correlation coefficients (r^2) after Spearman's correlation test. GCF correlations were made with LAP-specific diseased sites and whole-mouth parameters. BOP, bleeding on probing; CAL, clinical attachment level; DB, deep and bleeding; LAP, localized aggressive periodontitis; PD, pocket depth. See Table 2 for all other abbreviations.

^aPD all: mean pocket depth of all affected sites. PD sites: mean PD for diseased sites sampled for GCF. CAL all: mean clinical attachment level of all affected sites. CAL sites: mean CAL for diseased sites sampled for GCF. Affected sites, %: percentage of all sites with PD >4 mm, CAL $\geq$ 2 mm, radiographic bone loss, and BOP. DB sites, %: percentage of all deep pockets (PD >4 mm) with BOP. BOP, %: percentage of full-mouth sites with BOP. Plaque, %: percentage of full-mouth sites with visible plaque.

^b $P < 0.05$.

^c $P < 0.01$.

^d $P < 0.001$.

presenting cells and T lymphocytes are related to a subsequent bone resorption process in LAP, reflecting the comprehensive biological responses in periodontal disease. Higher RANKL levels and RANKL:OPG ratios in our LAP-D sampled for GCF compared with LAP-H and their association with clinical measurements of disease severity may contribute to confirm this hypothesis. No previous studies have evaluated

bone resorption biomarkers in the LAP model, and few studies have determined their concentrations in GAP patients, in which local RANKL or RANKL:OPG ratio was usually higher versus HC (Cochran 2008; Baltacioglu et al. 2014). RANKL is mainly produced by lymphocytes in the inflammatory tissues, and RANKL production by T lymphocytes has led these cells to be considered key regulatory cells in periodontal bone

resorption (Han et al. 2007). Lymphocyte activities remain scarcely investigated in LAP, with conflicting results (Seymour et al. 1997; Sigusch et al. 2006). Interestingly, T-cell activation is highly induced by IL-12 production via DCs, the most important antigen-presenting cells, and such IL-12 production was found to be highly stimulated in the presence of *Aggregatibacter actinomycetemcomitans* (Kikuchi et al. 2004), which plays a major

Table 5.

PCs and Their Respective Percentages of Variance Derived from the PCA of GCF and Plasma Biomarker Levels in LAP Patients, Their HS, and Unrelated HC.

PC1	PC2	PC3	PC4	PC5
GCF: LAP-D, LAP-H, HS, HC				
IL-12p40 (0.907)	TNF- α (0.810)	OPG (0.877)		
IL-6 (0.902)	IL-8 (0.755)			
IL12p70 (0.888)	IP-10 (0.600)			
IL-2 (0.838)	MCP-1 (0.504)			
MIP-1 α (0.803)				
GM-CSF (0.764)				
IFN- γ (0.748)				
IL-1 β (0.748)				
Eotaxin (0.611)				
IL-10 (0.511)				
48.93% ^a	10.24% ^a	7.41% ^a		
Plasma: LAP, HS, HC				
MIP-1 α (0.829)	IL-12p40 (0.873)	IL-12p70 (0.818)	IP-10 (0.854)	RANKL (–0.700)
IL-8 (0.714)	Eotaxin (0.850)	IL-6 (0.575)	MCP-1 (0.674)	OPG (0.606)
IL-10 (0.684)		IL-1 β (0.468)	TNF- α (0.634)	
IFN- γ (0.622)				
22.34% ^a	16.21% ^a	12.59% ^a	10.94% ^a	7.82% ^a

Biomarkers in each PC are shown with factor loads (in parentheses) after varimax rotation with Kaiser normalization. Factor loads indicate the correlation of individual markers on each component.

GCF, gingival crevicular fluid; HC, unrelated healthy controls; HS, healthy siblings; LAP, localized aggressive periodontitis; LAP-D, diseased sites of LAP; LAP-H, healthy sites of LAP; PC, principal component; PCA, principal component analysis. See Table 2 for all other abbreviations.

^aPercentage of variance.

role in LAP pathogenesis. Although more investigations are necessary, both cell types may play an important role in the development of the adaptive response in LAP.

In plasma, the combination of MIP-1 α , IL-8, IL-10, and IFN- γ better separated the 3 patient groups. As a chemoattractant of Th1-type lymphocytes, MIP-1 α stimulates the production of IFN- γ by T cells; IFN- γ , in turn, stimulates osteoclast formation and bone loss in vivo via antigen-driven T-cell activation or through the chemoattraction of RANKL-positive cells (Garlet 2010). Although RANKL was not

part of this component, it was elevated in the plasma of LAP patients. The effects of IFN- γ are directly regulated by the anti-inflammatory IL-10 produced by DCs, macrophages, granulocytes, and most lymphocyte populations, which reduces the proinflammatory cytokine or chemokine production, as well as major histocompatibility complex and costimulatory molecules produced by monocytes, DCs, and/or macrophages (Banchereau et al. 2012). Finally, IL-10 also inhibits the production of IL-8 (Wang et al. 1994), a neutrophil chemoattractant produced by epithelial cells, fibroblasts,

monocytes, and polymorphonuclear leukocytes. With MIP-1 α , IL-8 is known to recruit and activate immune cells and to induce osteoclast differentiation (Silva et al. 2007). Notably, plasma levels of IL-10 in LAP were comparable to those of HS and HC (in contrast with our GCF results), but IL-10 was the main plasma cytokine separating patients in function 1 of the DA. Individually, the plasma cytokines could not decimate LAP from healthy groups, and some proinflammatory markers were even lower in LAP than healthy groups individually; however, when examined in conjunction, some

Figure. Discriminatory potential of gingival crevicular fluid (GCF) and plasma biomarkers in localized aggressive periodontitis (LAP) subjects, their healthy siblings (HS), and unrelated healthy controls (HC). Principal component analysis identified subsets of highly correlated biomarkers produced in the GCF and plasma of patients (principal components, as shown in Table 5), which were further evaluated regarding their discriminatory potential by discriminant function analysis. Panels represent plots of significant separation in discriminant function analysis according to specific biomarker subsets among groups: **(A)** GCF: Note clear separation among LAP diseased sites (LAP-D) and LAP healthy sites (LAP-H) and then a more closely related profile (although still statistically different) for HS sites and HC sites by a biomarker group of IL-12p40, IL-6, IL-12p70, IL-2, and MIP-1 α (Wilks's lambda <0.001 in canonical functions 1 and 2). **(B)** Plasma: Note less separation among the groups for the plasma inflammatory profile as compared with that of GCF, although there was a significant separation among LAP patients, their HS, and unrelated HC by a group of biomarkers consisting of MIP-1 α , IL-8, IL-10, and IFN- γ (Wilks's lambda = 0.012 in canonical function 1. *Significant biomarker in function 1. †Significant biomarker in function 2).

of them may play a significant role in this complex disease activity, even in an unstimulated environment.

Regarding the bone biomarkers in plasma, in addition to increased RANKL levels in LAP patients versus healthy groups, we observed increased RANKL:OPG ratios in LAP versus their HS (not HC), while OPG concentrations were similar among groups. Elevated systemic RANKL and RANKL:OPG values were observed in GAP and chronic periodontitis patients as compared with controls (Baltacioglu et al. 2014), while no studies have investigated systemic levels of these biomarkers in LAP. Intriguingly, here, plasma RANKL was associated with the percentage of affected sites and deep and bleeding sites in LAP patients. Moreover, PC5 (formed by RANKL and OPG) was able to discriminate

the 3 groups in this study, and RANKL significantly separated LAP, HS, and HC in function 1 (data not shown). Thus, we could speculate that since RANKL, IFN- γ , and IL-10 are highly produced by T cells and because these 3 mediators participated in the discriminatory groups from plasma data, maybe differential profiles of T-cell populations in peripheral blood might characterize the 3 groups of individuals, which may also explain the differences in plasma RANKL production among groups.

Our findings in unstimulated plasma suggest that we may not consider plasma biomarker levels in isolation to diagnose LAP. Similarly, Havemose-Poulsen et al. (2005) did not observe significant changes in cytokine levels in unstimulated blood of 18 LAP individuals versus controls. The role of the systemic

markers investigated in the prediction of the local destruction remains uncertain, as does whether LAP may represent a risk factor for systemic diseases as suggested for other periodontal diseases (Seymour et al. 2007). However, although individual proinflammatory markers here did not show higher levels in LAP plasma, our results indicate that LAP individuals, HS, and HC exhibited systemically distinct biomarker profiles of inflammatory and bone mediators.

Regarding the HS, we analyzed unaffected LAP siblings owing to the tendency for familial involvement in aggressive periodontitis and LAP (Albandar 2014), as well as their exacerbated inflammatory response, previously found to be attenuated in relation to LAP patients but increased against that of controls (Shaddox et al.

2010; Shaddox et al. 2011). The distinct profiles of HS, locally and systemically, could be the consequence of genetic susceptibility and/or the host's specific bacterial or inflammatory environment, which ideally will be elucidated by future genetic and epigenetic studies related to immune inflammatory-associated gene expressions. One of the limitations of the present investigation is the lack of longitudinal evaluation of these markers to be able to correlate these with disease progression, initiation, and resolution and thus be able to identify in future studies some predictive value to the inflammatory profiles found here.

In summary, our results demonstrated how inflammatory and bone resorption biomarker profiles are differentiated within the local and systemic environments in our LAP cohort of African Americans. The inflammatory profiles, including groups of specific biomarkers in GCF and plasma, significantly discriminated LAP versus healthy individuals, although no correlations were found between markers in the GCF and plasma of these individuals. The present findings may contribute to our understanding of the activity profile of the disease or its susceptibility, thereby stimulating the development of future diagnostic methods based on an immunologic profile, which currently do not exist, especially in the local environment. Additionally, we hypothesize that the hyperinflammatory response previously found in the peripheral blood of LAP patients is dependent on lipopolysaccharide stimulation, resulting in local tissue damage, with minimal influence in the systemic inflammatory profile.

Author Contributions

L.S. Branco-de-Almeida, Y. Cruz-Almeida, contributed to conception, design, data analysis, and interpretation, drafted and critically revised manuscript; Y. Gonzalez-Marrero, contributed to design, data analysis, and interpretation, drafted and critically revised manuscript; H. Huang, contributed to design,

data acquisition, and interpretation, critically revised manuscript; I. Aukhil, contributed to conception, design, data acquisition, and interpretation, critically revised manuscript; P. Harrison, contributed to conception, design, data analysis, and interpretation, critically revised manuscript; S.M. Wallet, contributed to conception, design, and data interpretation, critically revised manuscript; L.M. Shaddox, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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