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Analysis of gingival crevicular fluid biomarkers in patients with metabolic syndrome

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ABSTRACT

Objectives: To assess associations between gingival crevicular fluid (GCF) markers in patients with metabolic syndrome, with or without concomitant periodontitis.
Methods: A total of 95 patients with Metabolic Syndrome (MetS) had a periodontal examination and gingival crevicular fluid samples taken. Proteomic analysis of gingival crevicular fluid (GCF) was carried out by Human XL Cytokine protein arrays in 12 selected patients, followed by multiplex ELISA of 11 analytes in 95 participants.
Results: Increased levels of Aggrecan, IL-6 and IL-8 were found in patients with periodontal health compared with moderate and severe periodontitis. The inverse stepwise association between severity of periodontitis and reduced Aggrecan levels was also observed at adjusted linear regression analysis. Diagnosis of diabetes was associated with higher GCF levels of IL-8 and MMP-8.
Conclusion: Diabetes may affect GCF levels of cytokines, irrespective of periodontal status. Periodontal status may be associated with Aggrecan levels in the GCF of patients affected by metabolic syndrome.
Clinical significance: Investigation of GCF biomarkers may potentially help have diagnostic potential in patients with MetS.

1. Introduction

Obesity, insulin resistance, hypertension and dyslipidemia often cluster in the same group of individuals as part of a condition named metabolic syndrome (MetS), which is in turn associated with an increased risk of developing diabetes and cardiovascular events [1]. Associations between periodontitis and metabolic syndrome have been suggested by epidemiological investigations including large national surveys in the United States, Korea and Japan [2–4] and confirmed in systematic reviews and meta-analyses [5]. A complex network of inflammation, oxidative stress, genetic and behavioral factors probably explain the association between periodontal disease and MetS [6–8]. The local inflammation triggered by subgingival microbial biofilm accumulation may have systemic repercussions via bacterial influx in the systemic circulation, as well as by the stimulation of a systemic inflammatory response [9].

The collection and analysis of gingival crevicular fluid (GCF) is now considered a very important tool for the detection of molecular biomarkers associated with periodontitis [10–11]. The composition of GCF may at least partially reflect the systemic circulation [10]. Therefore, studies of the composition of GCF could shed light into the relationships between periodontal and systemic conditions. However, not many comprehensive analyses of GCF markers have been conducted, as most studies have focused on one or a handful of cytokines [11].

We hereby hypothesized that different inflammatory signatures might be detected in the GCF of MetS patients according to periodontal status. Therefore, the aim of this analysis was to assess associations between GCF markers in patients with metabolic syndrome, with or

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without concomitant periodontitis.

2. Material and methods

2.1. Study population

This analysis was part of a larger case-control study investigating clinical, periodontal, inflammatory, genetic and microbial factors in patients with MetS. The study population has been reported previously [12] and included 103 patients diagnosed with MetS based on the revised NCEP ATP III criteria [13]. The analysis described in this paper focused on associations between periodontitis and GCF markers. The STROBE checklist was followed during the conduct and reporting of the study. Participants were recruited among MetS patients attending the Department of Internal Medicine (University of Catania). All participants signed informed consent to take part in the study and were included in the study from July 2015 to July 2017. Ethics approval was obtained by the sponsor institution, University of Catania (reference 4242/01), and separately by the clinical center (reference 1497/Cs). The study was registered on clinicaltrials.gov (identifier NCT03297749).

2.2. Inclusion criteria

- Caucasian ethnicity;
- Age 25- 75;
- Diagnosis of metabolic syndrome as defined by the revised NCEP ATP III (e.g. the presence of at least 3 of the following factors) [13]:
- Waist circumference $>$ 102 cm for men and $>$ 88 cm for women;
- High triglycerides: $\geq$ 150 mg/dL (1.7 mmol/L), or specific treatment for this lipid abnormality;
- Low HDL cholesterol: $<$ 40 mg/dL (1.03 mmol/L) in males, $<$ 50 mg/dL (1.29 mmol/L) in females, or specific treatment for this lipid abnormality;
- High blood pressure: systolic BP $\geq$ 130 or diastolic BP $\geq$85 mm Hg, or treatment of previously diagnosed hypertension;
- High fasting plasma glucose: FPG $\geq$100 mg/dL (5.6 mmol/L), or previously diagnosed type 2 diabetes;
- Presence of at least 12 teeth;

2.3. Exclusion criteria

- Pregnancy;
- Presence of infectious diseases such as hepatitis and HIV;
- Antibiotic pre-medication required for the performance of periodontal examination;
- Previous periodontal therapy within 6 months of the study visit;

2.4. Medical assessment and sampling

Medical and smoking histories were recorded, body mass index (BMI) and waist circumference were taken [12], Patients' dental history was investigated, including family history of periodontal disease, frequency of dental appointments, date of last appointment and previous treatment, reasons for tooth loss and frequency and type of tooth brushing. A six sites/tooth periodontal examination was carried out by a single calibrated examiner including full mouth plaque scores (FMPS) [14], full mouth probing pocket depth (PPD), clinical attachment level (CAL) bleeding on probing (FMBS)[13], tooth mobility and furcation involvement. According to the study protocol, patients were classified as having periodontitis according to the criteria below [15]:

- healthy/mild periodontitis: $<$ 2 sites on different teeth with CAL $\geq$ 4 mm or no sites with PPD $\geq$ 4 mm;
- moderate periodontitis: $\geq$ 2 sites on different teeth with CAL $\geq$ 4 mm or one site with PPD $\geq$ 4 mm;
- severe periodontitis: $\geq$ 2 sites on different teeth with CAL $\geq$ 6 mm and $\geq$ 1 site with PPD $\geq$ 4 mm;

2.5. Gingival crevicular fluid collection and analysis

Gingival crevicular fluid (GCF) samples were taken prior to periodontal probing to avoid contamination by blood. Four samples were taken from the mesio-buccal surfaces of first molars. In the absence of these teeth, neighboring teeth were chosen (second premolars, second molars, first premolars, canines in this order). The selected sites were isolated with cotton roll and supragingival plaque, if present, was removed using a curette to prevent saliva and/or plaque contamination. GCF was collected for 60 seconds using PerioPaper strips (OraFlow, Inc.) placed gently until slight resistance was felt. The four samples were pooled into Eppendorf tubes and then placed in the laboratory freezer at –80 °C for storage.

2.6. Gingival crevicular fluid analysis

Samples were then thawed and eluted in PBS (pH 7.2±0.2) supplemented with Easypack Protease Inhibitor Cocktail (Roche) as described in Curtis et al. 1988 [16]. Briefly, the periopaper strips were placed in a perforated 0.5 ml microcentrifuge tube which was held inside a 1.5 ml tube. The elution buffer (50μL) was added on to the strips and placed in a vortex shaker for 2 minutes before centrifugation for 15 minutes at 11, 000 rpm and 4℃. The centrifugation was repeated with a further 50μL of the elution buffer added on to the strips, to yield ~90μL of total eluted GCF. Protein concentration was quantified using an LVis plate (BMG Labtech CLARIOstar) calibrated with Bovine Serum Albumin (Sigma). 50 μg of protein from 12 patient samples each were analysed using the Human XL Cytokine protein array (R&D Systems) which consisted of a panel of 105 cytokines. These 12 patients were consecutively selected based on diagnosis of severe periodontitis (n=6) and periodontal health (n=6). Densitometric dot blot analysis of the x-ray films exposed to array membranes were performed using the ImageJ software [17]. The normalized pixel density values were used in multivariate data and statistical analysis. Based on these analyses, 11 analytes were chosen for quantification in all samples using Luminex assays. These were Aggrecan, CCL-2, Complement factor D, IL-11, IL-6, IL-8, IL-17, MMP-8, Resistin, TFF-3, DPP IV. Assays were conducted according to the manufacturer's instructions with a 4-fold sample dilution for all analytes except TFF-3 and DPPIV, which were quantified in a 10-fold diluted sample.

2.7. Statistical analysis/power calculation

The sample size calculation was based on the pulse-wave velocity outcome (primary outcome in the original study protocol), resulting in a required sample size of 102 patients. No sample size estimation was conducted for the GCF analysis.

Data were entered in a computer as an Excel file and proofed for entry errors. The resulting database was locked and loaded in SPSS Version 26.0. Continuous, normally distributed variables are reported as means ± standard deviations (SD). Comparisons of continuous and categorical data between groups were analysed with ANOVA and Chi-square test, respectively. Values of analytes were checked for normal distribution and were log-transformed for analysis if not normally distributed. Periodontal categories of healthy-mild, moderate and severe as stated above were used for analysis. Linear regression analysis was performed to test associations between periodontitis and GCF analytes, adjusted for age, gender, smoking, diabetes and BMI.

3. Results

Out of a total of 103 patients included in the study, 95 had GCF samples which were analysed. Others were either not taken or discarded

as contaminated with blood. Demographic and clinical characteristics of the 95 included subjects are reported in table 1. Patients were on average 58 years old, with a majority of males (62.1%), and had an average BMI of nearly 32. Ten subjects were classified as no-mild periodontitis, 34 as moderate periodontitis and 51 as severe periodontitis[14]. Sixty-nine patients (72.6%) were diagnosed with diabetes. Thirty-eight patients (40%) had Hb1Ac levels $\geq$ 7.0%.

3.1. Results of preliminary proteome profiler human XL cytokine array

Results of Human XL Cytokine array on 105 soluble analytes in 12 selected patients (6 periodontally healthy and 6 with severe periodontitis) are reported in Fig. 1. A total of 14 analytes were not detected in at least half of the samples. Log-transformed values of the remaining 95 analytes were analysed by ANOVA to explore potential associations with periodontal status. The biggest differences in absolute median values between periodontally healthy and severe periodontitis were registered for IL-6 (increased in healthy) and TFF-3 (increased in periodontitis). The following analytes showed some tendency to be associated with presence of moderate to severe periodontitis (at p value threshold < 0.15): IL-17, relaxin-2, cystatin C, IL-3, VEGF complement factor D, M-CSF, IL-6, FGF-19, Serpin E1 and Aggrecan. Based on these differences, on overall levels and on previous literature, the following eleven analytes were selected for multiplex ELISA: Aggrecan, CCL-2, Complement factor D, IL-11, IL-6, IL-8, IL-17, MMP-8, Resistin, TFF-3, DPP IV.

3.2. Multiplex ELISA

Results of Multiplex ELISA on the 11 selected analytes on the 95 included patients, grouped by periodontal diagnosis, are reported in table 2. Increased levels of Aggrecan, IL-6 and IL-8 were found in patients with periodontal health compared with moderate and severe periodontitis (unadjusted ANOVA p=0.034, 0.048 and p=0.038 respectively). Linear regression analysis showed that only Aggrecan was significantly associated with periodontal status (p=0.026). Cytokine interrelationships also changed between mild to moderate and severe periodontitis groups. Complement factor D, Resistin, IL-6, IL-11, IL-8 and MMP-8 showed negative associations with other cytokines in the mild group, but this relationship was observed to shift to a positive association with other cytokines in the moderate and severe groups.

Among covariates, presence of diabetes (p=0.002) and female gender (p=0.006) were associated with increased IL-8 levels. Diagnosis of diabetes was also associated with increased MMP-8 levels, irrespective of periodontal diagnosis (p< 0.001).

Fig. 2 shows correlation matrices (Pearson) of the studied analytes for patients divided by periodontal diagnosis (no-mild vs. moderate vs. severe). Some differences in correlation patterns by periodontal status were detected, especially for complement factor D, MMP8 & IL-6. Significant positive correlations were detected between DPP IV and IL-8, with negative associations between DPP IV and IL-6, Resistin and IL-8 in the mild periodontitis group (p<0.05). These associations were observed to weaken or reverse in the moderate and severe groups compared to the mild periodontitis group. Complement factor D was negatively correlated with IL-6, IL-8, MMP-8 and Resistin in the moderate periodontitis group (p<0.005), whereas no significant associations between these cytokines could be detected in the mild periodontitis group.

Table 1
Demographics and dental history of 95 cases included in GCF analysis. BMI= body mass index. DM= Diabetes Mellitus.

		Average
Age		58.45 ± 10.01
BMI		32.08 ± 4.31
		Frequency
Gender	Male	59 (62.1%)
	Female	36 (37.9%)
Diagnosis of DM		69 (72.6%)
Smoking status	Non smoker	63 (66.3%)
	Current smoker	25 (26.3%)
	Former smoker	7 (7.4%)
Tooth brushing frequency	<1/day	6 (5.9%)
	1/day	34 (33.0%)
	At least 2/day	63 (61.1%)

4. Discussion

This study consisted of a comprehensive proteomic GCF analysis in patients with the metabolic syndrome, subdivided by periodontal status. A total of 105 analytes were assessed in a subset of the study population (N=12), and then ten analytes were selected for multiplex immunoassay analysis in the larger population of 95 MetS patients. Some interesting and novel findings emerged.

Among analytes initially selected based on exploratory analysis with the Human XL Cytokine array, Aggrecan is a proteoglycan part of the extracellular matrix in cartilaginous tissue, with a potential role in cartilage deterioration during joint injury, disease and aging. It has been shown that the inflammatory cascade can decrease aggrecan synthesis and increase its catabolism through up-regulation of matrix degrading enzymes, such as MMPs and aggrecanases [18]. The loss of aggrecan molecules is thought to be an early event preceding the breakdown of cartilage tissue [19]. Although we are not aware, to the best of our knowledge, of any reports regarding the detection of aggrecan in GCF, another chondroitin sulfate proteoglycan, versican, has been suspected to be involved in epithelial differentiation and downgrowth in porcine gingiva [20]. Interestingly, total aggrecan levels have been reported to decrease in patients affected by RA compared with healthy controls, suggesting a decreased aggrecan turnover in RA patients [21]. Conversely, other studies detected higher levels of aggrecan in the circulation in patients with RA vs. healthy subjects [22–23] and in patients with osteoarthritis compared with psoriatic arthritis [24]. Furthermore, aggrecans may have a role in vascular plasticity and remodeling [25]. It has also recently emerged that T cells that recognize citrullinated aggrecan are present in patients with RA and that aggrecan-specific T cells and antibodies are potentially markers to monitor patients with RA or at-risk subjects [26]. The detection of aggrecan in the GCF probably reflects exudation of this proteoglycan or of its fragments from the blood through the gingival crevice. The decrease in aggrecan levels in patients with periodontitis observed here may reflect mechanisms of associations between periodontitis and RA [27], further compounded by a potential association between RA and MetS [28].

A recent systematic review by our group showed that MMP-8 had good sensitivity and specificity in GCF for diagnosis of periodontitis, making it a very promising biomarker [29]. In the present study, no statistically significant differences in MMP-8 levels were observed for periodontal status. However, a strong association was detected between GCF levels of MMP-8 and diagnosis of diabetes. This is in agreement with previous studies [30] and may reflect the role of MMPs in multiple pathways leading to diabetic microvascular complications [31].

Another cytokine correlated with diagnosis of diabetes in this study was IL-8, which is prevalently released from endothelial cells, gingival fibroblasts, neutrophils, monocytes, and phagocytes, and is involved in neutrophil activation [32]. The results presented in this study are in agreement with a recent systematic review and meta-analysis showing significantly lower IL-8 levels in GCF of CP patients in comparison with periodontally healthy subjects [33]. Among other cytokines studied, despite promising results in the preliminary analysis of 12 samples, no statistically significant associations with periodontal status or with diagnosis of diabetes were detected in the larger sample for Resistin, CCL-2, Complement Factor D, IL-6, IL-11, IL-17, DPP IV and TFF3.

Strengths of this study are the ethnic homogeneity of the included subjects and the comprehensive GCF analysis. The explorative approach of the first phase involving 105 cytokines is balanced by the more

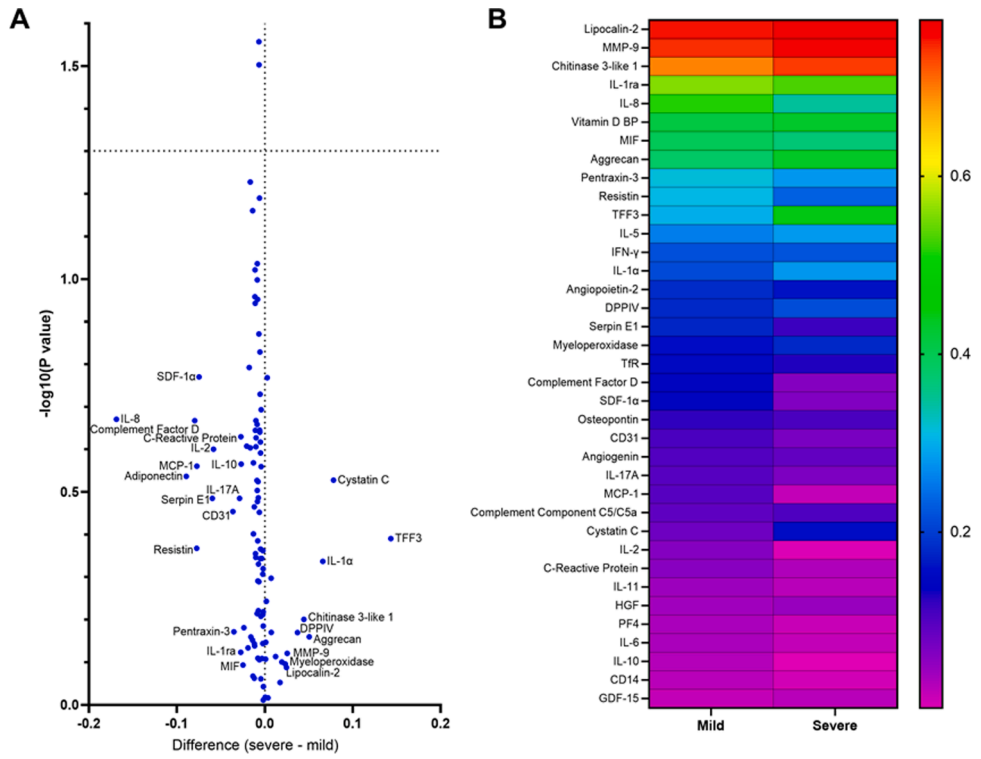

Fig. 1. Volcano plot (A) with labels highlighting some proteins that showed differences. The heatmap (B) to help show the scale of changes in the proteins that showed the most differences.

Table 2
Log-transformed mean values of analytes (± standard deviations), expressed in pog/ml, for patients divided by periodontal status.

Analyte(pg/ml log-transformed)	Healthy-mild periodontitis (n=10)	Moderate periodontitis (n=34)	Severe periodontitis (n=51)	Unadjusted p value (ANOVA)	Adjusted p value (linear regression analysis)
Aggrecan	6.63 ± 0.18	6.48 ± 0.25	6.37 ± 0.36	0.034	0.026
CCL-2	3.59 ± 0.64	3.70 ± 0.56	3.71 ± 0.36	0.836	0.722
Complement factor D	9.67 ± 0.37	9.30 ± 0.62	9.47 ± 0.75	0.267	0.490
IL-6	2.83 ± 0.38	2.24 ± 0.46	2.30 ± 0.82	0.048	0.206
IL-8	8.36 ± 0.37	7.55 ± 0.85	7.63 ± 0.96	0.038	0.265
IL-11	6.21 ± 1.42	5.59 ± 1.38	5.50 ± 1.44	0.355	0.152
IL-17	2.08 ± 1.04	2.16 ± 0.82	2.39 ± 0.53	0.218	0.172
MMP-8	13.05 ± 0.37	12.52 ± 0.85	12.83 ± 0.77	0.085	0.316
Resistin	10.33 ± 1.82	9.73 ± 1.25	10.07 ± 1.53	0.415	0.391
DPP IV	1.60 ± 4.03	3.08 ± 4.14	3.47 ± 4.26	0.436	0.262
TFF3	9.12 ± 1.22	9.41 ± 1.37	9.12 ± 1.26	0.597	0.958

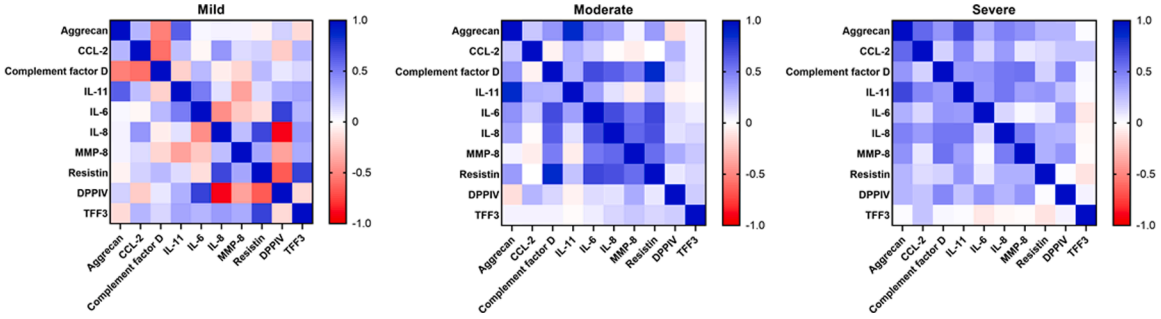

Fig. 2. Correlation matrices (Pearson) of the studied analytes for patients divided by periodontal diagnosis (no-mild vs. moderate vs. severe periodontitis).

focused approach of the second phase limited to 11 cytokines. A limitation of this study is the absence of controls without the metabolic syndrome. However, internal controls (patients with MetS with no periodontitis) were present. Results have been presented in levels per 60 seconds rather than in concentrations, as GCF volume measurement was not carried out. Although this can be considered a limitation, total cytokine amount or levels per 60 seconds have previously been suggested as more representative of the disease status than the evaluation of protein concentration [34–35].

Overall, this study suggests that presence of diabetes influences the

level of cytokines in the GCF. Furthermore, this study shows for the first time that aggrecan is present at relatively high levels in the GCF and that it may be inversely correlated with the severity of periodontitis. Future studies about this and other proteoglycans may help understanding the association between periodontal disease and metabolic syndrome. Prospective studies investigating biomarkers and treatment response in patients with MetS and periodontitis are also recommended.

5. Compliance with ethical standards

Funding: The study was funded by the 2016–2018 Research Plan of the University of Catania, Catania, Italy, Department of Clinical and Experimental Medicine (project # A).

Ethical approval: Ethics approval was obtained by the sponsor institution, University College London (reference 4242/01), and separately by the clinical center (reference 1497/Cs). The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent: All participants provided written informed consent to take part in the study.

6. Author contributions

Nibali: conceptualization, data curation and analysis, methodology, investigation, paper writing

Stephen: data curation and analysis, methodology, investigation, paper reviewing and editing

Hagi-Pavli: data curation and analysis, methodology, investigation, paper reviewing and editing

Allaker: investigation, paper reviewing and editing

Di Pino: data curation and analysis, investigation, paper reviewing and editing

Terranova: data curation and analysis, investigation, paper reviewing and editing

Pisano: data curation and analysis, investigation, paper reviewing and editing

Di Marca: data curation and analysis, investigation, paper reviewing and editing

Ferrara: data curation and analysis, investigation, paper reviewing and editing

Scicali: data curation and analysis, investigation, paper reviewing and editing

Giordano: data curation and analysis, investigation, paper reviewing and editing

Purrello: data curation and analysis, investigation, paper reviewing and editing

Donos: conceptualization, resources, methodology, investigation, paper reviewing and editing

Malatino: data curation and analysis, investigation, paper reviewing and editing

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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