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Article type: Original article

Received: January 15, 2020.

Accepted: February 7, 2020.

Published online: February 11, 2020.

ISSN: 1897-9483

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Characteristics of salivary inflammation profile in obesity

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Short title: Salivary cytokines in obesity

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Conflict of interest: none declared.

No funding to declare.
What’s new?

We already know, that obesity is an inflammatory disease and that is possible to determine the levels of pro-inflammatory cytokines in saliva. We found differences between the concentration of selected salivary cytokines in individuals with obesity and with normal weight. The salivary marker sCD40L (soluble CD40 Ligand) has the best discriminatory value for obesity. Our research can help determine the correct reference values for selected inflammatory cytokines and may improve the diagnosis of inflammatory diseases.
Abstract

**Introduction:** A new direction in the study of obesity-related problems resulted from the discovery of adipose tissue secretory function. Adipokines are present not only in the blood but also in the saliva. Many scientific reports indicate that obesity affects their salivary concentration.

**Objectives:** To evaluate selected inflammatory markers in the saliva of people with obesity and determine their discriminatory values.

**Patients and methods:** The study included 125 patients (82 female and 43 male), between 20 to 65 years of age. The study group consisted of 59 patients with obesity (BMI [body mass index] >30 kg/m²), and the control group 66 patients with normal body weight (BMI <25 kg/m²). From all subjects mixed saliva samples were collected to determine the concentration of the following selected markers of inflammation: receptor 1 and receptor 2 for tumor necrosis factor-alpha (TNFα-R1 and TNFα-R2), pentraxin 3 (PTX-3), interleukin 15 (IL-15), monocyte chemoattractant protein-1 (MCP-1), soluble intercellular adhesion molecule-1 (sICAM-1) and soluble CD40 Ligand (sCD40L).

**Results:** Significantly higher levels of selected salivary markers of inflammation (excluding lower sCD40L) were found in individuals with obesity. The salivary marker sCD40L seems to be the best to discriminate obesity, regardless of patients’ sex (the highest AUC [area under the curve] values among tested markers), with the determined optimal cut-off point below 3.28 pg/ml.

**Conclusions:** Obesity can cause increased levels of selected inflammatory markers in saliva. The determined discriminatory values may assist in diagnosis of metabolic diseases.

**Keywords:** inflammatory cytokines, obesity, saliva
**Introduction**

Adipose tissue plays an important role in storage of excessive nutrients and regulating energy balance. Due to limited ability of adipocytes for fat depositing, overnutrition generates the production of oxygen free radicals resulting in oxidative stress and consequently inflammation. In obesity, low-grade inflammatory status is primarily caused by the activity of adipocytes and immune cells secreting various pro-inflammatory molecules, such as e.g. receptor 1 and receptor 2 for tumor necrosis factor-alpha, pentraxin 3, interleukin 15, monocyte chemoattractant protein-1, soluble intercellular adhesion molecule-1 or soluble CD40 Ligand. All these signaling molecules are responsible for i.a. chronic systemic inflammation, adipogenesis, atherosclerosis, insulin resistance, and their severe consequences like cardiovascular diseases, diabetes, metabolic disorders, carcinogenesis, severe asthma [1-5].

New molecular technologies within the last decade focused on analyses salivary proteome and transcriptome. Saliva has been studied as an alternative biological fluid in the non-invasive diagnostics of several systemic diseases, such as endocrine, cardiovascular, autoimmune or infectious diseases [6, 7].

Saliva is generally composed of water (ca. 99%), different proteins (e.g. enzymes, immunoglobulins) and electrolytes (e.g. sodium, calcium). All these substances are provided to saliva mainly by salivary glands, gingival crevices and upper respiratory mucosa. Majority of blood components passes to saliva using intracellular mechanisms, such as passive or active transport or ultrafiltration between cell junctions, by analogy to renal production of urine. In patients with obesity, concentrations of several markers identified in blood and saliva (like
inflammatory cytokines, adipokines or cortisol) are correlated between blood and saliva [8, 9]. Saliva collection is less stressful for the patients, also easy and requires no qualified personnel. These findings indicate that salivary biomarkers have valuable potential for developing new non-invasive diagnostic techniques. The study aims to evaluate selected inflammatory markers in the saliva of people with obesity and determine their discriminatory values (as an alternative biological fluid to blood).

**Patients and methods**

**Patients**

One-hundred-and-twenty-five (82 female and 43 male) subjects aged between 20 to 65 years were included in the study. The study group included 59 patients (body mass index >30 kg/m²) from the Department of Internal and Metabolic Diseases, Poznan University of Medical Sciences (PUMS), and the control group comprised 66 patients (BMI <25 kg/m²) from the Department of Conservative Dentistry and Periodontology, Poznan University of Medical Sciences. The following criteria were used to exclude subjects: diabetes, change in body weight ± 3 kg within the last three months, history of surgical treatment for obesity, pregnancy or use of contraceptive agents. This study was approved by the Bioethical Commission at PUMS (No. 189/14). Every patient was informed about the aim and type of research to be carried out and signed written consents for participation were obtained from all patients. All procedures performed in studies involving human participants were in accordance with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.
Saliva collection

Unstimulated mixed saliva was used for laboratory studies. Saliva was collected at a specific time of the day, i.e., between 10 a.m. and 1 p.m. All samples were collected at least 2 hours after a meal. For proper saliva flow into calibrated tubes on ice, patients were seated in an upright position with their trunks bent forward and were asked to hold swallowing. The tubes containing saliva were kept on ice at all times during the experiment. The unstimulated saliva was collected for a total of 20 minutes and the secreted amount was calculated in ml/min. Immediately after calculation, the saliva was centrifuged for 10 minutes with 2000 rpm using Centrifuge MPW-223e (MPW Med. Instruments, Poland), aliquoted into tubes and frozen at -80 °C until it was used for the measurement of inflammatory markers. No protease inhibitors were used.

Biochemical and immunological analyses

The CG840 pH meter (Schott, Germany) was used to measure the pH of saliva after collection. For the estimation of the salivary concentration of selected inflammatory markers, such as tumor necrosis factor-alpha receptor-1 (TNFα-R1), tumor necrosis factor-alpha receptor-2 (TNFα-R2), pentraxin 3 (PTX-3), interleukin 15 (IL-15), monocyte chemoattractant protein-1 (MCP-1), soluble intercellular adhesion molecule-1 (sICAM-1), and soluble CD40 Ligand (sCD40L), enzyme-linked immunosorbent assay, using the DuoSet Immunoassay Development Kit (R&D Systems, Minneapolis, Minnesota, USA), was performed. The spectrophotometric readout of the results was executed using the VERSA microplate reader (Molecular Devices LLC. San Jose, CA, USA).

Statistical analysis
The Shapiro-Wilk test was used to verify normal distribution of the results and non-parametric methods were used for analyses of results that were not normally distributed. The comparison between the study group and control group with respect to the previously mentioned parameters was conducted using the Mann-Whitney’s test. The Spearman’s rank test was used to measure the degree of association between measured variables. Also, ROC curves (receiver operating characteristic curves) were analyzed for tested salivary markers. The significance level was estimated at $\alpha=0.05$. The analyses were performed using the Statistica 13.1 software (StatSoft, Cracow, Poland).

Results

Patients’ characteristics, including the detailed demographic and physical parameters of the subjects, are shown in Table 1.

The amount of unstimulated saliva secreted by these patients with obesity was statistically lower when compared with the normal-weight individuals. However, salivary pH was not lower in the group with obesity.

There were statistically significant differences in the concentrations of all inflammatory markers in saliva, between patients with obesity and patients with normal weight (Figures 1a-1g). Statistical significance was observed in correlations between different inflammatory markers of both groups. The correlation coefficients are presented in Table 2.

Moreover, the analysis of ROC curves by gender was performed to assess the ability of salivary markers to discriminate obesity and determine potentially optimal cut-off points for them. The results are shown in Table 3 and Figure 2. All markers had discriminatory value for obesity but the best was sCD40L. It is worth noting that unlike other markers, lower levels of this ligand are related to higher risk of obesity.
Discussion

The saliva of individuals with obesity showed statistically higher concentrations of all studied cytokines, except sCD40L. The present study proves that significant differences exist between components and properties of saliva originating from patients with obesity and individuals with normal body weights.

Previously published studies have shown that, in individuals with obesity, there is moderate persistent underlying inflammation in the parotid glands coupled with inflammatory mediators secreted by adipose tissue and acting along the hypothalamic-pituitary-adrenal axis, which could be contributing to the reduced activity of salivary glands [10]. Although the amount of saliva was lower, we did not observe any difference in pH between the saliva in both groups of our study.

Our results demonstrated that it is possible to estimate, not only in blood but also in saliva, the concentrations of cytokines produced in cells of adipose tissue.

A recognized marker of inflammation is MCP-1. Blood MCP-1 concentrations have been used in the diagnosis and to estimate the prognosis of several diseases, such as nephritis, rheumatoid arthritis, and cardiovascular diseases [11, 12]. Currently, an increased number of investigators are now analyzing the concentrations of cytokines in saliva, in addition to blood. A very interesting report by Khan estimated 57 cytokines, chemokines, growth factors, and acute-phase proteins of twenty healthy volunteers and then compared their concentrations in serum, urine, and saliva [13]. He reported a mean salivary MCP-1 concentration of 105.0 pg/ml. This value is two-fold higher than the mean concentration obtained in the control group of our study. More recently, Godson et al. studied 10 to 12-year-old children with obesity and unequivocally found that, especially in cases of small patients in whom blood sampling may be difficult, the analysis of saliva can be successfully used to detect metabolic abnormalities induced by excessive body weight. The salivary biomarkers of obesity and
metabolic disturbances analyzed by Godson et al. included the monocyte chemotactic factor, MCP-1 [14]. Its concentration in children with obesity was 174.1 pg/ml, similar to the level seen in our study. Currently, ongoing investigations have continued to use salivary MCP-1 in the evaluation of tissue damage and in treatment prognosis of head and neck tumors [15].

Another cytokine we studied was the CD40/CD40L complex. It participates in the inflammatory cascade in several autoimmune diseases, including systemic lupus and rheumatoid arthritis [16]. The results of several studies have shown that the CD40/CD40L complex, estimated either in blood or in serum, provides a useful biomarker for the diagnosis of Sjögren’s syndrome [17]. It was also shown that, in Sjögren’s, the CD40/CD40L complex plays a role in the process of programmed cell death or apoptosis. We found, that the best discriminatory value for obesity has sCD40L, regardless of patients’ sex (the highest area under the curve values among tested markers). As sCD40L is a destimulant, based on a determined optimal cut-off point, its salivary concentrations below 3.28 pg/ml can be considered as a potential prognostic of obesity. In contrast, all other parameters belong to stimulants and their cut-off threshold is a measurement above which the probability of obesity potentially increases.

The concentration of sICAM-1 in saliva is statistically higher in individuals with obesity when compared with individuals without obesity (609.12 pg/ml vs 395.89 pg/ml). This difference may point to an increased risk for leukocyte (particularly monocytes) adhesion to the vascular endothelium, which, consequently, leads to the formation of foam cells in arteriosclerotic plaque [18]. According to previous studies, increased blood concentrations of sICAM-1 may be observed in asthma, allergic or autoimmune inflammation of the skin, and in tumors. Numerous other studies have reported high levels of sICAM-1 in the saliva of individuals diagnosed with periodontal disease [19].
In our study, a statistically significant difference was detected in the salivary TNFα-R1 concentration of individuals with obesity (237.38 pg/ml) when compared with healthy individuals (113.11 pg/ml). This may indicate an increase in TNFα concentration and could explain the persistent moderate inflammation and tendency for cell apoptosis in individuals with obesity. The level of salivary TNFα-R2 of individuals with obesity (67.87 pg/ml) was statistically higher than that of the control group (47.91 pg/ml), as well. The majority of studies quantifying TNFα-R2 in saliva pertain to the diagnosis and prognosis of Lichen planus invasions [20]. Ghallab et al., examining patients with Lichen planus invasions, analyzed the alterations in salivary TNFα-R2 concentrations after treatment with prednisone. Before the application of prednisone, the average concentration of salivary TNFα-R2 was 350 pg/ml and this level went down to 14.0 pg/ml following treatment. The salivary TNFα-R2 concentration was only 45.0 pg/ml in the group of healthy individuals [21]. We also assessed changes in PTX-3, a known serum biomarker used for the evaluation of complications of cardiovascular events and for the assessment of arteriosclerotic process advancement [22]. Recent studies have shown that, in addition to the known uses of PTX-3, its estimation in the saliva is helpful in early diagnosis and estimation of prognosis related to periodontal diseases [23]. Our study showed a higher concentration of PTX-3 in individuals with obesity (420.28 pg/ml) when compared with normal-weight individuals (272.33 pg/ml). IL-15, another important inflammatory cytokine, also showed a statistically significant difference between the two groups in our study. In the group with obesity, a median concentration of 11.71 pg/ml was measured while a concentration of 8.60 pg/ml was measured in the control group. The study by Phalane et al. showed that the level of IL-15 in the saliva of patients with tuberculosis was 0.6 pg/ml. IL-15 was undetected in the serum [24]. In addition, the study by Khan discussed earlier in this paper, stated that the concentration of IL-15 could not be estimated in blood, urine, or saliva [13].
Conclusion
The results presented here will shed more light on understanding the link between inflammation and obesity. This will allow for better monitoring of general health conditions in patients with obesity and will also aid in formulating more accurate prognoses. Our study has revealed several positive correlations between inflammatory markers and excessive body weight. Hopefully, this will aid the implementation of appropriate steps to screen individuals at risk for obesity and, thus, implement proper preventative guidelines.
The saliva of patients in the study group contained higher concentrations of all the measured inflammatory markers. Salivary biomarkers have the potential to aid in the diagnosis of other systemic diseases, including many of those which are a consequence of obesity.
Our study could help to understand the pathomechanism of obesity. Establishing typical values for the pro-inflammatory cytokines in saliva would allow better monitoring of general health in patients with obesity. Further research is necessary to implement systemic solutions for patients with obesity.

Acknowledgements
We would like to thank Editage (www.editage.com) for English language editing.

Contribution statement
AL and AS conceived the concept of the study and contributed to the design of the research. All authors were involved in data collection. AL and KN analyzed the data. All authors edited and approved the final version of the manuscript. AL and KN declared shared co-first authorship.
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### Tables

Table 1. Baseline characteristics of all patients: comparison between the study and the control groups.

| Variables          | Study group | Control group | All patients |
|-------------------|-------------|---------------|--------------|
| n                 | 59          | 66            | 125          |
| Sex (Female : Male), n (%) | 46:13 (78.0:22.0) | 36:30 (54.5:45.5) | 82:43 (65.6:34.4) |
| Age               | 34 (27 – 40) | 30 (24 – 36)  | 32 (25 – 37) |
| BMI, kg/m²        | 37.1 (32.7 – 41.4) | 21.8 (19.4 – 23.9) | 28.0 (21.7 – 36.0) |
| WHR, m            | 0.92 (0.85 – 0.96) | 0.80 (0.75 – 0.89) | 0.86 (0.77 – 0.92) |
| Unstimulated saliva, ml/min | 0.30 (0.23 – 0.38) | 0.38 (0.25 – 0.63) | 0.33 (0.25 – 0.5) |
| Salivary pH       | 7.0 (6.8 – 7.2) | 7.0 (6.7 – 7.3) | 7.0 (6.7 – 7.3) |

Abbreviations: BMI, body mass index; WHR, waist-hip ratio.

Quantitative variables are expressed as median (Q25 – Q75) and categorical variables as n (%).
Table 2. Spearman’s correlation coefficients between inflammatory markers in the saliva of patients.

|         | TNFα-R1 | TNFα-R2 | IL-15 | sCD40L | sICAM-1 | PTX-3 |
|---------|---------|---------|-------|--------|---------|-------|
| MCP-1   | 0.327\(^a\) | 0.504\(^a\) | 0.112 | -0.021 | 0.512\(^a\) | 0.337\(^a\) |
| TNFα-R1 | 0.547\(^a\) | -0.011  | -0.006 | 0.457\(^a\) | 0.391\(^a\) |
| TNFα-R2 | 0.048   | 0.199\(^a\) | 0.679\(^a\) | 0.634\(^a\) |
| IL-15   | -0.243\(^a\) | -0.008  | 0.076 |
| sCD40L  |         | 0.031   | 0.019 |
| sICAM-1 |         |         | 0.437\(^a\) |

Abbreviations: IL-15, Interleukin 15; MCP-1, Monocyte Chemoattractant Protein-1; PTX-3, Pentraxin 3; sCD40L, soluble CD40 Ligand; sICAM-1, soluble Intercellular Adhesion Molecule-1; TNFα-R1, Tumor Necrosis Factor Alpha Receptor-1; TNFα-R2, Tumor Necrosis Factor Alpha Receptor-2.

\(^a\) p < 0.05
Table 3. Analysis of ROC curves for tested salivary markers.

|        | Female |          |          | Male |          |          | Overall |          |          | Optimal cut-off point, pg/ml |
|--------|--------|----------|----------|------|----------|----------|---------|----------|----------|-----------------------------|
|        | AUC    | p-value  | AUC      | p-value | AUC     | p-value  |         | AUC      | p-value  |                            |
| MCP-1  | 0.658  | 0.009<sup>a</sup> | 0.800  | <0.001<sup>a</sup> | 0.696  | <0.001<sup>a</sup> | 56.12  |          |          |                            |
| sCD40L | 0.801  | <0.001<sup>a</sup> | 0.800  | 0.004<sup>a</sup> | 0.800  | <0.001<sup>a</sup> | 3.28   |          |          |                            |
| sICAM-1| 0.702  | <0.001<sup>a</sup> | 0.687  | 0.03<sup>a</sup> | 0.685  | <0.001<sup>a</sup> | 606.09 |          |          |                            |
| TNFα-R1| 0.778  | <0.001<sup>a</sup> | 0.697  | 0.02<sup>a</sup> | 0.747  | <0.001<sup>a</sup> | 182.80 |          |          |                            |
| TNFα-R2| 0.624  | 0.047<sup>a</sup> | 0.595  | 0.28  | 0.620  | 0.02<sup>a</sup> | 56.39  |          |          |                            |
| PTX-3  | 0.628  | 0.049<sup>a</sup> | 0.641  | 0.11  | 0.637  | 0.006<sup>a</sup> | 319.44 |          |          |                            |
| IL-15  | 0.623  | 0.047<sup>a</sup> | 0.831  | <0.001<sup>a</sup> | 0.721  | <0.001<sup>a</sup> | 13.03  |          |          |                            |

Abbreviations: AUC, area under the curve; IL-15, Interleukin 15; MCP-1, Monocyte Chemoattractant Protein-1; PTX-3, Pentraxin 3; ROC curve, receiver operating characteristic curve; sCD40L, soluble CD40 Ligand; sICAM-1, soluble Intercellular Adhesion Molecule-1; TNFα-R1, Tumor Necrosis Factor Alpha Receptor-1; TNFα-R2, Tumor Necrosis Factor Alpha Receptor-2.

<sup>a</sup> p < 0.05
Figure 1a. MCP-1
Figure 1b. sCD40
Figure 1c. sICAM
Figure 1d. TNFα-R1
Figure 1e. TNFα-R2
Figure 1f. PTX-3
Figures 1a-1g. Concentrations of selected inflammatory markers in the saliva of patients in control (1 - normal weight) and study (2 - obesity) groups.

Abbreviations: IL-15, Interleukin 15; MCP-1, Monocyte Chemoattractant Protein-1; PTX-3, Pentraxin 3; sCD40L, soluble CD40 Ligand; sICAM-1, soluble Intercellular Adhesion Molecule-1; TNFα-R1, Tumor Necrosis Factor Alpha Receptor-1; TNFα-R2, Tumor Necrosis Factor Alpha Receptor-2.

p < 0.05, according to the Mann-Whitney’s test
Figure 2. Comparison of ROC curves for tested salivary markers.

Abbreviations: IL-15, Interleukin 15; MCP-1, Monocyte Chemoattractant Protein-1; PTX-3, Pentraxin 3; ROC curve, receiver operating characteristic curve; sCD40L, soluble CD40 Ligand; sICAM-1, soluble Intercellular Adhesion Molecule-1; TNFα-R1, Tumor Necrosis Factor Alpha Receptor-1; TNFα-R2, Tumor Necrosis Factor Alpha Receptor-2.