Published in final edited form as:

Laryngoscope. 2018 June ; 128(6): E214–E221. doi:10.1002/lary.27002.

Oral sampling methods are associated with differences in immune marker concentrations

Carole Fakhry, M.D., M.P.H., Fares Qeadan, Ph.D., Robert H. Gilman, M.D., Pablo Yori, M.P.H., Margaret Kosek, M.D., Nicole Patterson, David W. Eisele, M.D., Christine G. Gourin, M.D., Chandala Chitguppi, M.B.B.S., M.S., Morgan Marks, M.D., and Patti Gravitt, Ph.D.
Johns Hopkins School of Public Health, Baltimore, Maryland and University of New Mexico Health Sciences Center, Albuquerque, New Mexico

Abstract

Objective—To determine whether the concentration and distribution of immune markers in paired oral samples were similar.

Study type—Clinical research

Study design—Cross sectional study

Methods—Paired saliva and oral secretion samples were collected. The concentration of immune markers was estimated using Luminex multiplex assay. For each sample, the concentration of respective immune markers was normalized to total protein present and log transformed. Median concentrations of immune markers were compared between both types of samples. Inter-marker correlation in each sampling method and across sampling methods was evaluated.

Results—There were 90 study participants. Concentrations of immune markers in saliva samples were significantly different from concentrations in oral secretions samples. Oral secretions samples showed higher concentrations of immunoregulatory markers while the saliva samples contained pro-inflammatory markers in higher concentration.

Conclusions—The immune marker profile in saliva samples is distinct from the immune marker profile in paired oral secretions samples.

Keywords

Oral samples; immune markers; head and neck cancer

Corresponding author: Carole Fakhry, 601 N. Caroline St., Johns Hopkins Outpatient Center 6th floor, Department of Otolaryngology Head and Neck Surgery, Baltimore, Maryland 21287, Telephone: (443) 287- 2024, cfakhry@jhmi.edu.

Thesis number: 2016-2

Level of Evidence: Level 2b

Financial disclosure information: None

Conflict of Interest: None

Presented at: n/a

INTRODUCTION

There is a growing appreciation for the pivotal role that the immune system plays in the development and progression of malignancies, which serves as the basis for novel immunomodulatory therapies.¹ Indeed, immune checkpoint inhibitors as well as other immune-based therapies provide great promise for the successful treatment of head and neck malignancies.²⁻⁴

In head and neck cancer, the immune system plays a crucial role in pre-malignancy, malignant transformation and progression of disease.⁵ The importance of immune factors is evident in patients with cancer at the level of the tumor^{6,7} (e.g. cytokines and immune marker expression can be tested for in tumors), locally in oral rinses,^{8,9} as well as systemically in serum.^{10,11} In the context of immunotherapy clinical trials, surveillance of the local immune milieu is of interest as this may be indicative of local response to therapy, which may not be observed systematically in serum immune markers. Indeed, there is evidence that immune responses are anatomic site-dependent and may not necessarily reflect systemic immune markers.¹² Therefore, the emerging significance of immunotherapies implies a potential role for determining levels of local immune markers which relies upon accurate sampling of oral cavity immune markers.

Given the increasing use of oral sampling in study designs, it is important to understand the advantages and disadvantages of distinct oral sampling methods. It is currently unknown whether saliva provide similar information with regard to immune markers when compared with fluid absorption as has been used routinely when evaluating the genital tract immune microenvironment.¹³⁻¹⁷ Therefore, the goal of this study was to determine whether the concentrations of immune markers and cytokines were similar in paired samples using distinct sampling methods of the oral cavity. We hypothesized that the concentrations of immune markers of interest would be similar in saliva when compared with oral secretions (OS) [oral fluid absorbed onto sponge].

MATERIALS AND METHODS

Study population

A study population was sampled from a larger study of women designed to assess differences in cervical human papillomavirus prevalence in Peruvian populations, Iquitos and Lima, with and without endemic soil transmitted helminthic infections respectively.¹⁸ Women were eligible if they were between 25–45 years of age and had an intact uterus. The study was approved by the Johns Hopkins University and AB-PRIMSA (Asociación Benéfica - PRISMA) institutional review boards/ethics committee. Study participants included in this analysis were from Iquitos site in the Peruvian Amazon.

Data and sample collection

Using an interviewer-administered questionnaire, information pertaining to demographics, socioeconomic status, sexual behavior, reproductive history, smoking, drinking and illicit drug use was collected. An un-induced saliva sample was collected into a sterile collection vial. Oral secretions (OS) were collected by placing a Merocel ophthalmic sponge under the

tongue for 30 seconds, allowing fluid absorption, which was then placed into a sterile 5mL screw cap cryovial. Samples were stored at 4 °C for a maximum of 8 hours and then at -80 °C until protein extraction could be done. After protein extraction, samples were packed on dry ice and shipped to centralized laboratory at Johns Hopkins Bloomberg School of Public Health (Baltimore, MD) for cytokine analysis.

Processing of the sample and immune marker measurement

The sponges were thawed for ten minutes at room temperature and placed in a microcentrifuge tube with a 0.2 µm filter (SpinX, Costar), equilibrated by using 300 µl of extraction buffer (10 mg/ml aprotinin in phosphate buffered saline with 10% sodium azide). After incubating this for 30 min at 4 °C, it was centrifuged at 4 °C for 30 min at 14,000 rpm. Then, the samples were stored at -20 °C until the time of immune marker measurement.

Immune marker measurements were performed as previously described. Briefly, samples were thawed and diluted using a 1 to 3 ratio in protein extraction buffer. Magnetic bead-based multiplex assay according to the manufacturer's protocol (Bio-Rad, Hercules, CA) was utilized for the measurement. The following cytokines, chemokines and growth factors (referred to collectively as immune markers hereafter) were measured: eotaxin-1 (eotaxin), fgf-basic (fgf-B), granulocyte colony stimulating factor (GCSF), granulocyte macrophage colony stimulating factor (GMCSF), interleukin-1β (IL-1β), interleukin-1 receptor antagonist (IL-1ra), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-13 (IL-13), interleukin-15 (IL-15), interleukin-17a (IL-17a), interleukin-17f (IL-17f), interleukin-21 (IL-21), interleukin-22 (IL-22), interleukin-23 (IL-23), interleukin-25 (IL-25), interleukin-31 (IL-31), interleukin-33 (IL-33), interferon-γ (IFN-γ), inflammatory protein-10 (IP-10), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), monocyte chemotactic protein (MCP-1), platelet derived growth factor BB (PDGF-BB), regulated upon activation, normal T cell expressed and secreted (RANTES), SCD 401, tumor growth factor β1 (TGF β1), tumor growth factor β2 (TGF β2), tumor growth factor β3 (TGF β3), tumor necrosis factor α (TNF-α), and vascular endothelial growth factor (VEGF). Luminex instrument (Bio-Rad, Hercules, CA) was used to read the plates. An 8-point standard curve was generated and fit using a 5-parameter logistic model. This assay has an intra-assay coefficient of variation <20% and depending on the cytokine target the reported lower limit of detection is 1–20 pg/ml.¹⁹ Each sample pair from a given individual was tested on the same assay plate to reduce across plate heterogeneity despite prior data showing reproducibility across plates.²⁰

Normalization of total protein

As previously published, cytokine concentrations were normalized to the concentration of total protein in the extracted sample to reduce the potential for sampling bias.²⁰ Hence, bicinchoninic acid (BCA) assay was utilized per manufacturer's protocol (Pierce, Rockford IL) to calculate the total concentration of protein. Colorimetric recognition of samples was normalized to background samples. Total protein concentration was expressed as µg/ml and assessed using an 8-point standard curve. The ratio of immune marker concentration to total

protein concentration was then found and expressed as ng of immune marker per mg of total protein.²⁰

Statistical analysis

Based on their presumed primary function, immune markers were classified as either pro-inflammatory, immunoregulatory, or growth factors. Pro-inflammatory markers included IL-1 β , IL-1ra, Eotaxin, IL-6, IL-8, IP-10, MCP-1, MIP-1 α , MIP-1 β , RANTES, TNF- α and SCD 401. Immunoregulatory immune markers included IFN- γ , IL-2, IL-4, IL-5, IL-7, IL-9, IL-10, IL-13, IL-15, IL-17 α , IL-17f, IL-21, IL-22, IL-23, IL-25 and IL-31. Immune markers having both the pro-inflammatory and immunoregulatory actions included IL-33, TGF β 1, TGF β 2 and TGF β 3. Growth factors included fgf-basic, GCSF, GMCSF, PDGF-BB and VEGF.

All protein adjusted values were log-transformed to maintain stability of variances and hence better comparability. All analyses in this paper were done on the log-transformed data. Immune marker with concentrations that were undetectable were assigned half the lowest measured value in our sample.²¹ In the event that an immune marker had a concentration on standard curve, but values were below the manufacturer's published limit an imputed value was assigned.

The mean values for each marker were calculated and multivariate paired T-test (Paired Hotelling's T^2 test) was applied to determine whether the two samples were systematically different. Bonferroni confidence intervals were used to determine the source of any significant difference between the paired oral and saliva samples. Spearman's rank correlation was estimated to identify patterns of co-expression. A p-value of <0.01, after Sidak's correction for multiple comparisons, was considered statistically significant.^{18,22,23,24} Bartlett's test was applied to assess the equality of variance-covariance matrices. To gain a bigger picture of the results, canonical correlation was used to calculate the multivariate association between the two sets of variables.

RESULTS

The study population consisted of 90 women between ages of 25 and 45 years. All subjects had a grossly normal oral examination at the time of sample collection.

Comparison of the concentration and distribution of immune markers in the two oral sampling methods

There was heterogeneity in the distribution of concentrations of immune markers in the paired OS and saliva samples. The distribution of the immune markers was strikingly distinct between the two (Figures 1 and 2). Not only were the mean measurements of immune markers significantly different, there was also significant variability across all measurements in OS as compared with saliva samples. These observations were confirmed using a multivariate paired T-test (Paired Hotelling's T^2 test) [$T^2= 5392$, $F= 86.11$, $df=37$, 52 , $p<0.0001$].

The mean concentration for 30 out of 37 immune markers in OS samples was significantly different from that in the saliva samples (Sidak adjusted²⁵ $p < 0.05$). The mean concentrations of IL-9, IL-33, IL-6, GCSF, TNF- α , IL-13, and SCD401 were statistically similar between the OS and saliva samples (Sidak adjusted $p > 0.05$). These findings could also be observed from Figure 3; cytokines with 95% C.I. that overlap with the zero reference line are immune markers that are statistically similar between the OS and saliva samples.

Differences in mean marker concentration level by sampling method

To understand the relative contributions of immune markers to the overall observed differences between the OS and saliva samples, the mean difference in concentration of immune markers was calculated (Figure 3). The mean difference for the immune marker SCD401 was zero which indicates that the concentration of SCD401 was similar in both OS and saliva samples. The lowest mean difference was observed for IL-1b, a pro-inflammatory immune marker and the highest for IL-31, an immunoregulatory immune marker.

Values above the zero reference line in Figure 3 indicate higher OS values in both the log-transformation scale and original standardized scale. Thus, we observe that immune markers with concentrations higher in OS samples as compared with saliva samples (i.e. positive mean difference) included IL-13, IL-33, TGF- β 3, Eotaxin, IL-17 α , PDGF-BB, IP-10, IL-22, INF γ , IL-10, IL-23, IL-5, IL-25, IL-17f, IL-21, IL-4 and IL-31. This subset mainly consisted of immunoregulatory immune markers (Figure 3). By contrast, immune markers whose concentration in OS samples was lower than in saliva samples (i.e. negative mean difference) were predominantly pro-inflammatory immune markers and included IL-1b, VEGF, IL-2, IL-8, MCP-1, GMCSF, MIP-1b, TGF β 2, RANTES, IL-1 α , MIP-1 α , FGF-B, TGF β 1, IL15, IL-9, IL-6, GCSF, TNF α and IL-17a (Figure 3).

Differences in inter-marker correlations by sampling methods

The relationship among inflammatory markers of interest within OS (Figure 4) and saliva samples (Figure 5) was evaluated to understand whether concentrations of immune markers within each type of sample collection were correlated with each other.

In OS samples a strong correlation was observed. The majority of immune markers in OS samples (55.7%, 371 of 666) were strongly and significantly correlated with one another ($p < 0.01$; Figure 4). In contrast, only 130 of 666 (19.5%) immune markers in the saliva samples were strongly correlated with one another ($p < 0.01$; Figure 5). Significant inter-marker correlations were observed in the saliva samples for growth factors (GMCSF) and immunoregulatory factors (IL-2 and IL-15). Overall, inter-marker correlation in the OS samples was significantly different from that in saliva samples according to Bartlett's test for the equality of variance-covariance matrices ($\chi^2 = 2913.4$, $df = 703$, $p < 0.0001$).

Lastly, we sought to understand whether the concentrations of immune markers in the OS samples correlated with the concentrations in the saliva samples despite the observed differences overall (Figure 6). The concentrations of immune markers in saliva samples were found to be associated with the concentrations of immune markers in OS samples; the first two canonical correlations were significant with 47.9% overlapping variance ($p < 0.0001$ and $p = 0.0131$ respectively with canonical correlations of 0.9895 and 0.9840). The plot of

canonical variables (Figure 7) shows that, of the two canonical variables, the first one has the most discriminatory power. Further, when studying the loadings of the first canonical variate (positive versus negative), the pattern of clustering was the same as that observed when differences in mean marker concentration level were evaluated by sampling method (Supplemental Table 1).

DISCUSSION

This analysis demonstrates substantial differences in concentrations of immune markers depending on the sampling method used. While we hypothesized that similar concentrations would be observed for immune markers whether measured in OS or saliva samples, the differences identified in the analysis may be explained by the complex anatomy of the oral cavity. With the rising role of immunotherapy, oral immune markers may be a potential marker of response to therapy. Therefore, understanding the bias introduced by oral sampling methods is important.

Immune marker concentrations in OS and saliva samples are significantly different, although the two are correlated with one another (Figure 6). This may reflect sampling methodology and the anatomic subsites sampled. OS sample likely only represents the focal anatomic subsite that the merocel sponge is exposed to when placed under the ventral tongue or in the floor of mouth admixed with pooled saliva, whereas saliva samples may represent multiple subsites of the oral cavity and oropharynx. This is highlighted by the high inter-immune marker correlation in OS (Figure 4) and low inter-immune marker correlation in the saliva sample (Figure 5).

These results are a reminder that the immune system of the oral mucosa is subsite specific. Each anatomic site of the upper aerodigestive tract contains variable lymphoid tissue with the highest concentration typically being in the oropharynx and variable amounts anteriorly in the oral cavity. Additionally, the distribution of dendritic cells across subsites is variable.^{26,27} The heterogeneous keratinization of the epithelial surfaces in the oral cavity²⁸ may affect the potential impact of pathogens and ensuing antigen presentation. For example, the microbial flora of the oral cavity is heterogeneous across oral subsites, which may also affect the concentration of immune markers.^{29,30}

OS samples contained higher concentration of the immunoregulatory immune markers (INF- γ , IL-4, IL-5, IL-7, IL-9, IL-10, IL-13, IL-17 and IL-23) and lower concentration of pro-inflammatory markers. The immune markers measured in the OS sample may reflect local immune factors from the oral mucosa, salivary glands and also from the adjacent lymphoid organs. The palatine and lingual tonsils which are within close proximity to the oral cavity contain a large number of antigen presenting cells (e.g. monocytes, dendritic cells, and antigen-specific T- and B-cells).³¹ As the oral cavity is exposed to a large number of antigens through food, infectious pathogens etc. there is a need for constant immunoregulation resulting in oral tolerance to avoid excessive immune response.³² The increased concentration of immune markers produced by T-cells and dendritic cells (e.g. IFN- γ , IL-7 and IL-10) observed in OS samples may be indicative of oral tolerance.^{33,34} Additionally, since saliva samples are a mixture of oral mucosal transudate and saliva, the

concentration of the immunoregulatory immune markers may have been diluted. By contrast, paired saliva samples mainly contained pro-inflammatory cytokines and epithelial cell-derived markers (e.g. IL-1b, IL-8). This may be ascribed to inflammatory milieu of the oral cavity^{35–37} and reflect the sampling directly from the epithelium of the oral cavity and oropharynx.

Therefore, in the context of the results of this study, it is not surprising that the diversity of immune cells in the mucosal subsites would be differentially sampled depending on the method used. The OS sample which is limited to pooled saliva from the mucosa of the floor of mouth and ventral tongue as compared to the saliva samples represent immune marker production throughout the oral cavity and oropharynx. Therefore, the saliva sample may be more broadly representative of the diverse immune markers at play in the oral cavity and oropharynx.

Other reasons to explain the variability in immune markers is that each method may over- or under- sample oral mucosal transudates, the filtrate of plasma present orally or the fluid created by salivary glands.³⁸ Given the sampling methods in this study, it is possible that the OS samples may have had a predominance of oral mucosal transudate, while the saliva samples may have been limited to saliva.

This study has limitations which warrant discussion. Firstly, it does not involve males and therefore, the effect of gender was not accounted for. Secondly, individuals with malignancy were not included. Additionally, the order of sampling was not accounted for in analysis which may contribute to the lower concentration of immune markers in the second sample. Furthermore, since these are paired samples, the correlation is not surprising as one would expect a strong overlap. Additional factors which could affect the sample composition including local and systemic pathological conditions, and physiologic states like phase of a menstrual cycle,³⁹ hydration status and general health factors^{40,41} have been unaccounted for. Furthermore, no control was used to determine whether the act of sample collection stimulated saliva which could inherently affect salivary proteins concentrations and secondarily affect immune marker concentrations. One or more of these factors may also have contributed to the apparent difference in the immune marker levels between the two samples. Therefore, further studies should take these factors into account.

CONCLUSION

The oral cavity is comprised of distinct sub-sites, histologically and immunologically. Using two sampling methods of oral fluids, OS and saliva samples had distinct profiles of concentration of measured immune markers. While they were distinct, they did remain correlated with one another. Therefore, future studies will need to determine the optimal sampling method as this may affect interpretation of results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Study participants, Maribel Paredes Olortegui, Alberto Mejia Medrano, Dixner R. Trigs, Sarah Qureshi, Christine Huang for their invaluable contributions. Work was supported by the National Cancer Institute (grant R21 CA156537 to PEG) and the Johns Hopkins Center for Global Health (GHEFP Program, Qureshi). We acknowledge the University of New Mexico Clinical & Translational Science Center (CTSC) (grant UL1TR001449) and the Mountain West Clinical Translational Research Infrastructure Network (grant 1U54GM104944). University of New Mexico Health Sciences Center (Albuquerque, New Mexico).

Source of financial support or funding: National Cancer Institute (grant R21 CA156537 to PEG) and the Johns Hopkins Center for Global Health (GHEFP Program, Qureshi).

References

1. Li Z, Chen L, Rubinstein MP. Cancer immunotherapy: are we there yet? *Exp Hematol Oncol*. 2013; 2(1):33. [PubMed: 24326015]
2. Varilla V, Atienza J, Dasanu CA. Immune alterations and immunotherapy prospects in head and neck cancer. *Expert Opin Biol Ther*. 2013; 13(9):1241–1256. [PubMed: 23789839]
3. Ferris RL, Blumenschein GR, Fayette J, et al. Further evaluations of nivolumab (nivo) versus investigators choice (IC) chemotherapy for recurrent or metastatic (R/M) squamous cell carcinoma of the head and neck (SCCHN): CheckMate 141. *ASCO Meet Abstr*. 2016; 34(15_suppl):6009.
4. Seiwert TY, Burtneiss B, Mehra R, et al. Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial. *Lancet Oncol*. 2016; 17(7):956–965. [PubMed: 27247226]
5. Ferris RL. Immunology and Immunotherapy of Head and Neck Cancer. *J Clin Oncol Off J Am Soc Clin Oncol*. 2015; 33(29):3293–3304.
6. Suresh A, Vannan M, Kumaran D, et al. Resistance/response molecular signature for oral tongue squamous cell carcinoma. *Dis Markers*. 2012; 32(1):51–64. [PubMed: 22297602]
7. Kwon M, Kim JW, Roh J-L, et al. Recurrence and cancer-specific survival according to the expression of IL-4R α and IL-13R α 1 in patients with oral cavity cancer. *Eur J Cancer Oxf Engl* 1990. 2015; 51(2):177–185.
8. Prasad G, McCullough M. Chemokines and cytokines as salivary biomarkers for the early diagnosis of oral cancer. *Int J Dent*. 2013; 813756
9. Aziz S, Ahmed SS, Ali A, et al. Salivary Immunosuppressive Cytokines IL-10 and IL-13 Are Significantly Elevated in Oral Squamous Cell Carcinoma Patients. *Cancer Invest*. 2015; 33(7):318–328. [PubMed: 26046681]
10. Chang P-Y, Kuo Y-B, Wu T-L, et al. Association and prognostic value of serum inflammation markers in patients with leukoplakia and oral cavity cancer. *Clin Chem Lab Med*. 2013; 51(6): 1291–1300. [PubMed: 23154424]
11. Krishnan R, Thayalan DK, Padmanaban R, Ramadas R, Annasamy RK, Anandan N. Association of serum and salivary tumor necrosis factor- α with histological grading in oral cancer and its role in differentiating premalignant and malignant oral disease. *Asian Pac J Cancer Prev*. 2014; 15(17): 7141–7148. [PubMed: 25227804]
12. Fakhry C, Marks MA, Gilman RH, et al. Comparison of the immune microenvironment of the oral cavity and cervix in healthy women. *Cytokine*. 2013; 64(2):597–604. [PubMed: 24021705]
13. Beigi RH, Yudin MH, Cosentino L, Meyn LA, Hillier SL. Cytokines, pregnancy, and bacterial vaginosis: comparison of levels of cervical cytokines in pregnant and nonpregnant women with bacterial vaginosis. *J Infect Dis*. 2007; 196(9):1355–1360. [PubMed: 17922400]
14. Kemp TJ, Hildesheim A, García-Piñeres A, et al. Elevated systemic levels of inflammatory cytokines in older women with persistent cervical human papillomavirus infection. *Cancer Epidemiol Biomark Prev Publ Am Assoc Cancer Res Cosponsored Am Soc Prev Oncol*. 2010; 19(8):1954–1959.
15. Dezzutti CS, Hendrix CW, Marrazzo JM, et al. Performance of swabs, lavage, and diluents to quantify biomarkers of female genital tract soluble mucosal mediators. *PloS One*. 2011; 6(8):e23136. [PubMed: 21858008]

16. Marks MA, Viscidi RP, Chang K, et al. Differences in the concentration and correlation of cervical immune markers among HPV positive and negative perimenopausal women. *Cytokine*. 2011; 56(3):798–803. [PubMed: 22015106]
17. Koshiol J, Sklavos M, Wentzensen N, et al. Evaluation of a multiplex panel of immune-related markers in cervical secretions: a methodologic study. *Int J Cancer*. 2014; 134(2):411–425. [PubMed: 23824624]
18. Gravitt PE, Marks M, Kosek M, et al. Soil-Transmitted Helminth Infections Are Associated with an Increase in Human Papillomavirus Prevalence and a T-Helper Type 2 Cytokine Signature in Cervical Fluids. *J Infect Dis*. 2016; 213(5):723–730. [PubMed: 26486638]
19. Bio-Rad Laboratories I. Bio-Plex suspension array system tech note. Hercules, CA: 2005.
20. Marks MA, Eby Y, Howard R, Gravitt PE. Comparison of normalization methods for measuring immune markers in cervical secretion specimens. *J Immunol Methods*. 2012; 382(1–2):211–215. [PubMed: 22677266]
21. Bio-Rad Laboratories I. Bio-plex pro human cytokine, chemokine, and growth factor assays technical bulletin. 2009:1–4.
22. UCLA. Academic technology services SCG. Sidak-Holm: Adjusted p-values. Available at: http://www.ats.ucla.edu/stat/stata/code/sidak_holm.htm. Accessed on 29th September 2015
23. Qeadan F, Dwivedi S, Page K, Gravitt P. Modeling cytokines and chemokines through a rooted correlation network. *The Journal of Immunology*. 2016; 196(1):51.19.
24. Qeadan F, Gravitt P. Statistical Visualization for the Association between Soil Transmitted Helminth Infection and Changes in Immune Microenvironments at the Cervix. *Cancer Microenvironment*. The 7th International Conference on Tumor Microenvironment: Progression, Therapy and Prevention. 2015; 8(1):S91–S92. Abstr.
25. Saeed AI, Qeadan F, Sood A, et al. A novel cytokine profile associated with cancer metastasis to mediastinal and hilar lymph nodes identified using fine needle aspiration biopsy–A pilot study. *Cytokine*. 2017; 89:98–104. [PubMed: 27599390]
26. Daniels TE. Human mucosal Langerhans cells: postmortem identification of regional variations in oral mucosa. *J Invest Dermatol*. 1984; 82(1):21–24. [PubMed: 6228611]
27. Hovav A-H. Dendritic cells of the oral mucosa. *Mucosal Immunol*. 2014; 7(1):27–37. [PubMed: 23757304]
28. Lebendiger M, Lehner T. Characterization of mononuclear cells in the human oral mucosa. *Arch Oral Biol*. 1981; 26(12):1041–1049. [PubMed: 6951511]
29. Gibbons RJ. Adherent interactions which may affect microbial ecology in the mouth. *J Dent Res*. 1984; 63(3):378–385. [PubMed: 6583240]
30. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol*. 2005; 43(11):5721–5732. [PubMed: 16272510]
31. Nave H, Gebert A, Pabst R. Morphology and immunology of the human palatine tonsil. *Anat Embryol (Berl)*. 2001; 204(5):367–373. [PubMed: 11789984]
32. Mowat AM, Parker LA, Beacock-Sharp H, Millington OR, Chirido F. Oral tolerance: overview and historical perspectives. *Ann N Y Acad Sci*. 2004; 1029:1–8. [PubMed: 15806729]
33. Andersson J, Abrams J, Björk L, et al. Concomitant in vivo production of 19 different cytokines in human tonsils. *Immunology*. 1994; 83(1):16–24. [PubMed: 7821961]
34. Castro-Junior AB, Horta BC, Gomes-Santos AC, et al. Oral tolerance correlates with high levels of lymphocyte activity. *Cell Immunol*. 2012; 280(2):171–181. [PubMed: 23399844]
35. Gornowicz A, Bielawska A, Bielawski K, et al. Pro-inflammatory cytokines in saliva of adolescents with dental caries disease. *Ann Agric Environ Med AAEM*. 2012; 19(4):711–716. [PubMed: 23311795]
36. Tsybikov NN, Baranov SV, Kuznik BI, Malezhik LP, Isakova NV. Serum, oral and gingival fluid levels of heat shock protein-70, cytokines and their autoantibodies by periodontal disease [Article in Russian]. *Stomatologiya*. 2014; 93(1):16–18.
37. Lu R, Zhang J, Sun W, Du G, Zhou G. Inflammation-related cytokines in oral lichen planus: an overview. *J Oral Pathol Med Off Publ Int Assoc Oral Pathol Am Acad Oral Pathol*. 2015; 44(1):1–14.

38. Nishanian P, Aziz N, Chung J, Detels R, Fahey JL. Oral fluids as an alternative to serum for measurement of markers of immune activation. *Clin Diagn Lab Immunol.* 1998; 5(4):507–512. [PubMed: 9665958]
39. Khosravizamani M, Maliji G, Seyfi S, et al. Effect of the menstrual cycle on inflammatory cytokines in the periodontium. *J Periodontal Res.* 2014; 49(6):770–776. [PubMed: 24673464]
40. Navazesh M. Methods for collecting saliva. *Ann N Y Acad Sci.* 1993; 694:72–77. [PubMed: 8215087]
41. Dawes C. Considerations in the development of diagnostic tests on saliva. *Ann N Y Acad Sci.* 1993; 694:265–269. [PubMed: 8215062]

Figure 1.

On the x-axis is each immune marker measured with a specific color as denoted on x-axis legend. For each immune marker of interest, a boxplot is presented with median, 25th and 75th quartile as well as lower and upper range (line extending beyond the boxes). Additionally, outliers are indicated using circles or x's. The mean concentration is marked by '+' on the colored boxes. The concentrations are in ng/ml, logarithmically transformed and using standardized measure for graphic presentation. The immune markers are arranged in ascending order of mean concentration.

Figure 2.

As in Figure 1, the x-axis is each immune marker measured with a specific color as denoted on x-axis legend. For each immune marker of interest, a boxplot is presented with median, 25th and 75th quartile as well as lower and upper range (line extending beyond the boxes). Additionally, outliers are indicated using circles or x's. The mean concentration is marked by '+' on the colored boxes. The concentrations are in ng/ml, logarithmically transformed and using standardized measure for graphic presentation. The order of immune markers shown in Figure 1 is maintained in Figure 2 for comparison.

Figure 3.

The measured immune markers are on x-axis. On the y-axis is the mean difference in concentration between saliva and oral secretion samples. (The mean difference has been measured as concentration of immune marker in oral secretion subtracted by concentration of immune marker in saliva) The horizontal line in the center divides the graph into two parts. Values above the line denote values greater than zero (the concentrations in oral secretions are higher than in saliva). Values below the line indicate negative mean concentrations and that concentrations of the immune marker of interest in the saliva is higher as compared to the oral secretion sample. Since all concentration values are negative, then positive difference is an indication that saliva has smaller values but larger in absolute values.

Figure 4.

The immune markers are arranged on the x- axis and y-axis in same order. Each square corresponds to inter- marker correlation for that pair of immune markers present along x- and y-axis. Dark squares denote strong correlation (Sidak adjusted p-value<0.01) and light squares denote poor correlation.

Figure 5.

The immune markers are arranged on the x- axis and y-axis in same order. Each square corresponds to inter- marker correlation for that pair of immune markers present along x- and y-axis. Dark squares denote strong correlation (Sidak adjusted p-value<0.01) and light squares denote poor correlation.

Figure 6.

Canonical correlation using the first (left half of the plot) and second variates (right half of the plot) between the concentrations in the saliva samples and oral secretion samples have been denoted.

Figure 7.

Plot of canonical variables using the first and second variates between the concentrations in the saliva samples (left half of the plot) and oral secretion samples (right half of the plot) have been denoted.