Check for updates

BRIEF REPORT

REVISED The utility of salivary CRP and IL-6 as a non-invasive measurement evaluated in patients with COVID-19 with and without diabetes [version 3; peer review: 2 approved]

Endang Bachtiar[1], Boy M Bachtiar[1], Ardiana Kusumaningrum[2], Hari Sunarto[3,4], Yuniarti Soeroso[3], Benso Sulijaya[3], Efa Apriyanti[5], Citra Fragrantia Theodorea[1], Irandi Putra Pratomo[6], Yudhistira Yudhistira[7], Defi Efendi[8], Widya Lestari[9]

[1]Department of Oral Biology and Oral Sciences Research Center, Faculty of Dentistry Universitas Indonesia, Jakarta, Indonesia, 10430, Indonesia
[2]Department of Microbiology, Faculty of Medicine, Universitas Indonesia; Clinical Microbiology Medicine Staff Group, Universitas Indonesia Hospital, Jakarta, Indonesia, 10430, Indonesia
[3]Department of Periodontology, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia, 10430, Indonesia
[4]Dental Center, Universitas Indonesia Hospital, Depok, West Java, Indonesia
[5]Department of Pediatric Nursing, Faculty of Nursing Universitas Indonesia, and Paediatric Intensive Care Unit, Universitas Indonesia Hospital, West Java, Indonesia
[6]Department of Pulmonology and Respiratory Medicine, Faculty of Medicine, Universitas Indonesia, Salemba Raya 6, Jakarta, 10430, Indonesia
[7]Clinical Pathology Medicine Staff Group,, Universitas Indonesia Hospital., Depok, West Java, Indonesia
[8]Department of Pediatric Nursing, Faculty of Nursing Universitas Indonesia, and Neonatal Intensive Care Unit, Universitas Indonesia Hospital, Depok, West Java, Indonesia
[9]Oral Biology Unit, Fundamental Dental and Medical Sciences Kuala Lumpur, Malaysia International Islamic University Malaysia, Kuala Lumpur, Malaysia

v3 **First published:** 18 Apr 2023, **12**:419
https://doi.org/10.12688/f1000research.130995.1
Second version: 25 Oct 2023, **12**:419
https://doi.org/10.12688/f1000research.130995.2
Latest published: 11 Jan 2024, **12**:419
https://doi.org/10.12688/f1000research.130995.3

Abstract

Background

The available evidence suggests that inflammatory responses, in both systemic and oral tissue, contribute to the pathology of COVID-19 disease. Hence, studies of inflammation biomarkers in oral fluids, such as saliva, might be useful to better specify COVID-19 features.

Methods

In the current study, we performed quantitative real-time PCR to measure salivary levels of C-reactive protein (CRP) and interleukin-6

Open Peer Review

Approval Status ✓ ✓

	1	2
version 3 (revision) 11 Jan 2024		✓ view
version 2 (revision) 25 Oct 2023	✓ view	? view
version 1 18 Apr 2023	✗ view	

1. **Joel Schwartz**, University of Illinois Chicago, Chicago, USA

(IL-6) in saliva obtained from patients diagnosed with mild COVID-19, in a diabetic group (DG; n = 10) and a non-diabetic group (NDG; n = 13). All participants were diagnosed with periodontitis, while six participants with periodontitis but not diagnosed with COVID-19 were included as controls.

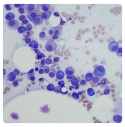
Results

We found increases in salivary total protein levels in both the DG and NDG compared to control patients. In both groups, salivary CRP and IL-6 levels were comparable. Additionally, the levels of salivary CRP were significantly correlated with total proteins, in which a strong and moderate positive correlation was found between DG and NDG, respectively. A linear positive correlation was also noted in the relationship between salivary IL-6 level and total proteins, but the correlation was not significant. Interestingly, the association between salivary CRP and IL-6 levels was positive. However, a moderately significant correlation was only found in COVID-19 patients with diabetes, through which the association was validated by a receiver operating curve.

Conclusions

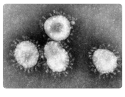
These finding suggest that salivary CRP and IL-6 are particularly relevant as potential non-invasive biomarker for predicting diabetes risk in mild cases of COVID-19 accompanied with periodontitis.

Keywords
Diabetes, COVID-19, periodontitis, C-reactive protein, interleukin-6



This article is included in the Cell & Molecular Biology gateway.

This article is included in the Emerging Diseases and Outbreaks gateway.



This article is included in the Coronavirus collection.

2. **Ghada Ibrahim Taha**, University of Baghdad, Baghdad, Iraq

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Endang Bachtiar (endang04@ui.ac.id)

Author roles: Bachtiar E: Conceptualization, Funding Acquisition, Methodology, Supervision, Writing – Original Draft Preparation; **Bachtiar BM**: Conceptualization, Data Curation, Methodology, Software, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Kusumaningrum A**: Data Curation, Validation; **Sunarto H**: Project Administration, Resources; **Soeroso Y**: Methodology, Supervision, Writing – Review & Editing; **Sulijaya B**: Investigation, Project Administration; **Apriyanti E**: Data Curation, Project Administration; **Theodorea CF**: Data Curation, Formal Analysis; **Putra Pratomo I**: Validation, Visualization; **Yudhistira Y**: Investigation, Validation; **Efendi D**: Data Curation, Investigation; **Lestari W**: Conceptualization, Resources, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This study was supported by Universitas Indonesia (Grant NKB-1126 /UN2.RST/HKP.05.00/2022).
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2024 Bachtiar E *et al*. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Bachtiar E, Bachtiar BM, Kusumaningrum A *et al.* **The utility of salivary CRP and IL-6 as a non-invasive measurement evaluated in patients with COVID-19 with and without diabetes [version 3; peer review: 2 approved]** F1000Research 2024, **12**:419 https://doi.org/10.12688/f1000research.130995.3

First published: 18 Apr 2023, **12**:419 https://doi.org/10.12688/f1000research.130995.1

REVISED **Amendments from Version 2**

Methods section: We amplified cDNA that had been converted from salivary RNA using the same procedure we used for the gingival crevicular sample for IL-6 using quantitative real-time polymerase chain reaction (qPCR; ABI StepOnePlus Real-Time PCR system) and SYBR Green I for gene expression analysis. Thus, in this study we used saliva instead of GCF as oral sample.

Subjects, saliva sampling, and in vitro methods; "all COVID-19 patients participated in this study received a diagnosis of periodontitis. The patients were further split into two groups: those with (n = 10) and those without (n = 13) diabetes. As controls, six periodontitis non-diabetic individuals and no COVID-19 were also recruited".

Methods section, under the subtitle of Data analysis, we explained; "The nonparametric Kruskal Wallis test was used to compare the diabetic group (DG), the non-diabetic group (NDG), and the control group. The Mann-Whitney test was used to compare the DG and NDG data".

We changed the explanation of the control group in the Methods section to read as follows: As controls, six non-diabetic individuals with periodontitis but no COVID-19 were also included.

Discussion: We added more detail about the limitation of this study.

Any further responses from the reviewers can be found at the end of the article

Introduction

There is a growing interest in measuring inflammatory biomarkers in saliva, as sampling this oral fluid is less invasive than blood.[1-3] Despite the promising results of saliva studies, the findings of salivary measures of inflammation have been inconsistent.

In this study, we used saliva derived from patients with COVID-19 to evaluate the association of selected inflammatory markers, interleukin-6 (IL-6), and C-reactive protein (CRP) in COVID-19 patients. The extent to which salivary IL-6 and CRP levels are associated with diabetes or non-diabetes events in this population still needs to be explored.

Methods

Subjects, saliva sampling, and in vitro methods

This study was part of a project on COVID-19 and its association with the oral ecosystem. Thus, the current report was based on data from our previous study, in which data from 23 people with mild severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection were requested from Rumah Sakit Universitas Indonesia (RSUI), a university hospital, between June and August 2021.[4] In addition to COVID-19, all patients were diagnosed with periodontitis. Patients were further divided into two groups: with (n = 10) and without (n = 13) diabetes. As controls, six non-diabetic individuals with periodontitis but no COVID-19 were also included. Sampling methods and information regarding the inclusion and exclusion criteria of patients, how consent was obtained from participants, and obtaining medical reports were performed in accordance with the guidelines provided by the ethics committee of RSUI (protocol number: 2021/04/052). All of the participants signed the written informed consent form. Additionally, this study was performed in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guidleines.[5] Information regarding clinical status, such as age, sex, and chronic medical history of comorbidities, was obtained from the medical reports of mildly symptomatic patients with COVID-19 (not shown). Only subjects who had no respiratory symptom for more than 2 weeks were included in this study. However, our focus was on patients with COVID-19, with and without diabetes. Thus, only diabetic status (type 2 diabetes), as reported in the medical records, was included as a comorbidity variable in the data analysis, while all COVID-19 patients were diagnosed with periodontitis (moderate to severe) according to the criteria described by the American Academic of Periodontology Classification of Periodontal Disease,[6] without dental radiographic assessment.

The oral specimen was obtained by spitting unstimulated whole saliva into a sterile Falcon tube, placed on ice, and transferred to the laboratory for subsequent processing.[4]

The total protein level in saliva was determined using the Bradford assay method, as reported elsewhere.[7] The concentration of CRP in saliva samples was determined using an enzyme-linked immunosorbent assay kit (Elabscience Biotechnology Inc., Wuhan, China) according to the manufacturer's instructions. For IL-6, we used quantitative real time-polymerase chain reaction (qPCR; ABI StepOnePlus Real-Time PCR system), using SYBR Green I for gene expression analysis, to amplify cDNA that had been converted from salivary RNA using the method we used for the gingival crevicular sample.[8] The PCR was run in triplicate using primers (IL-6 and housekeeping gene/GAPDH) and the PCR program as reported previously.[8] The $2^{-\Delta\Delta CT}$ method was used to analyze the relative expression of mRNA.[9]

Data analysis

There was a strong correlation between mRNA and protein expression levels.[10] In this study, owing to the observed variations in saliva concentrations, both CRP (protein) and IL-6 (transcription level) were adjusted for total salivary protein. Statistical analyses were performed using GraphPad Prism 9.4 software. Data are presented as mean ± SD or median. The nonparametric Kruskal Wallis test was used to compare the diabetic group (DG), the non-diabetic group (NDG), and the control group. The Mann-Whitney test was used to compare the DG and NDG data. The statistical significance level was set at $p < 0.05$. Spearman's correlation coefficient (r) with two-tailed p-values was calculated, and linear regression was used to generate the line of best fit with 95% confidence intervals. Receiver operating characteristic (ROC) curve analysis was also performed to evaluate this association.

Results

As shown in Figure 1A, compared to the control group, the mean values of the total protein concentration in the saliva of the DG and the NDG were significantly increased ($p < 0.05$). However, the concentrations were comparable in both groups.

We further noted that in the DG, salivary CRP was detectable in 9/10 (90%) patients, while IL-6 was only measured in 8/10 (80%) patients. In the NDG, the concentration of CRP and the transcription level of IL-6 in saliva samples were detected in all (100%) and 12/13 (92.31%), respectively. The median values of CRP and IL-6 in saliva are shown in Figure 1B, C. The protein concentrations of CRP and the transcription level of IL-6 mRNA detected in saliva among the patient groups were comparable ($p > 0.05$).

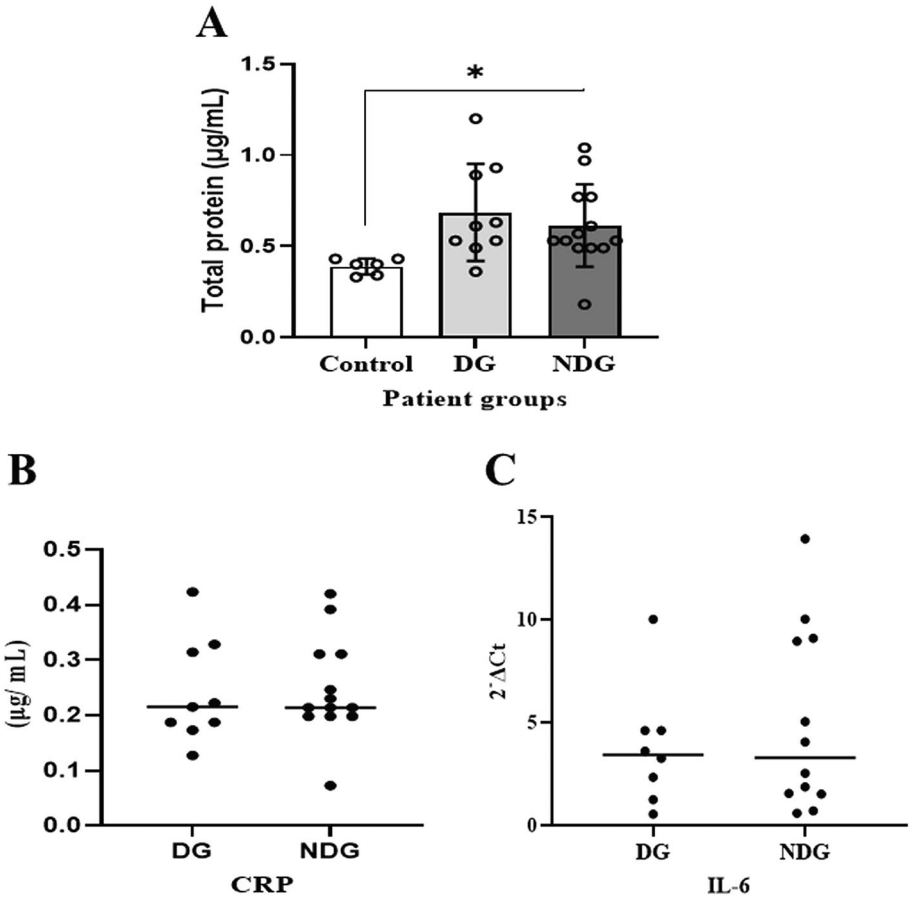


Figure 1. Comparison of total salivary protein concentration in patients with COVID-19 with and without diabetes. Significant differences in salivary total proteins concentration were observed in both DG and NDG groups compared to control (A). However, in each group tested, the salivary level of either CRP (B) or IL-6 (C) was comparable, as assessed by Bredford protein assay and quantification of mRNA expression, respectively. *$p < 0.05$. CRP = C-reactive protein, Il-6 = Interleukin-6. DG and NDG are diabetes and non-diabetes group, respectively.

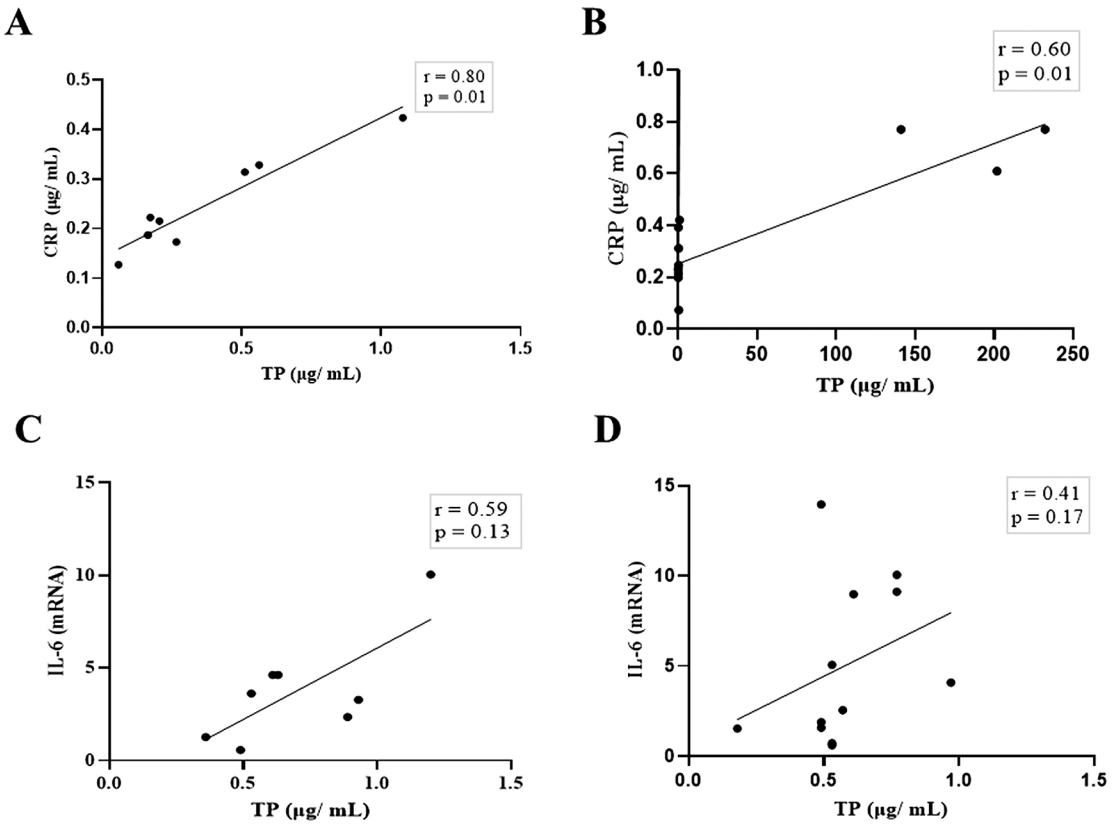


Figure 2. Relationship between salivary levels of CRP/IL-6 and total protein in patients with COVID-19 with and without diabetes. Spearman correlation analysis indicates a statistically significant positive correlation between salivary levels of CRP and total protein, A strong and moderate correlations was observed in DG (A) and NDG (B), respectively. The positive relationship between IL-6 salivary levels and total salivary proteins were also found in DG (C) and NDG (D). However, the correlations were not significant. CRP = C-reactive protein, IL-6 = Interleukin-6. DG and NDG. Spearman correlation coefficient (r) and exact p-value are shown.

Next, we compared the association between total salivary proteins and each of the two inflammation markers detected in the saliva. As shown in Figure 2A–D, IL-6 and CRP levels were identical in their association with total salivary proteins. Spearman's correlation showed a strongly significant positive correlation between salivary CRP and total salivary proteins in the DG ($r = 0.80$, $p = 0.01$), while a moderately significant correlation was observed in the NDG ($r = 0.60$, $p = 0.01$). A similar trend was observed for the correlation between salivary IL-6 and total salivary protein levels. In the DG ($r = 0.59$, $p = 0.13$) and the NDG ($r = 0.41$, $p = 0.17$), the correlation was positive, but not significant. Finally, we compared the correlation between the two inflammation markers in each group. In both groups, the correlation was positive, but a significant association was only observed in the DG. The correlation coefficients were $r = 0.52$, $p = 0.03$, and $r = 0.38$, $p = 0.21$ in the DG and NDG, respectively (Figure 3A,B). Hence, since a significant relationship was only shown in the DG, we made inferences about association accuracy by performing ROC analysis. The area under the curve of the CRP/IL-6 association was 0.983 (95% confidence interval [CI]: 0.9451–1, $p < 0.0001$; Figure 3C).

Discussion

The most obvious finding of this study is that salivary CRP and IL-6 are potential markers of systemic inflammation in mild cases of COVID-19 with and without diabetes. With regard to CRP, our results were not consistent with a recent report, which showed that an increased concentration of salivary CRP was only found in severe cases of COVID-19.[11] We reasoned that this inconsistency could be related to the eligibility criteria in recruiting participants. In the cited report, the authors excluded participants with periodontitis, whereas in our study, periodontitis was an inclusion criterion.[8] However, for IL-6, the results of this study are in line with a previous report, where the concentration of salivary IL-6 was elevated in periodontitis patients with and without diabetes.[12]

Current literatures show that IL-6 is a pleiotropic cytokine with complex role in inflammation and metabolic disease. Its biological activities include B-lymphocyte differentiation, T-lymphocyte proliferation,[13] pro and anti-inflammatory

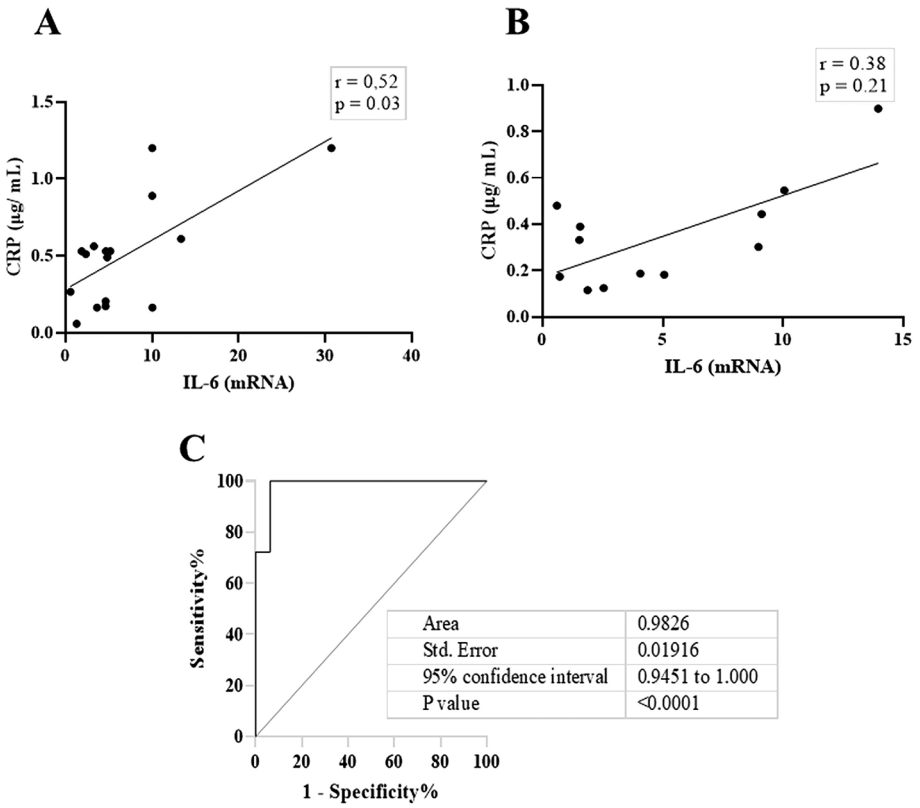


Figure 3. The scatter diagram denotes the relationship between salivary levels of CRP and IL-6. These data indicate that in DG (A), the correlation was significantly moderate, while a weak not significant correlation was noted in NDG (B). Receiver operating characteristic (ROC) was used to illustrate the plot and best cut-off of significant relationship between salivary levels of CRP and Il-6 in DG group (C). CRP = C-reactive protein, IL-6 = Interleukin-6. DG and NDG. Spearman correlation coefficient (r) and exact p-value are displayed.

activities,[14] the development of the nervous and hematopoietic system, and the regulation of metabolism.[15] As a proinflammatory cytokine, it induces insulin resistance and periodontal disease in the process of bone resorption.[16] Taken together, Il-6, which is considered an adipokine has an important role in the pathogenesis of localized oral inflammation (periodontitis), and may play a pivotal role in metabolic disease,[17] such as diabetes.

The current study is an extension of our primary study involving the same patients with COVID-19 to evaluate the inflammatory conditions in periodontal microenvironment.[18] We found that the transcription levels of both IL-6 and complement C3 (the central component of innate immune system) in gingival crevicular fluid (GCF) were markedly higher in COVID-19 patients with diabetes (DG) compared to the non-diabetes patients (NDG). We also noticed that in periodontal niche, the mRNA upregulation of host receptor for SARS-CoV-2 (angiotensin-converting enzyme 2/ACE2)[19] was positively associated with the transcription levels of either inflammation marker tested (IL-6 or C3). Thus, our previous results along with other work by other investigators[20,21] suggest that the virus receptor (ACE2) can be detected in oral tissue. Additionally, certain molecules (furin and TMPRSS2) that are involved in promoting the SARS-CoV-2 entry and infection can be detected in oral cavity.[22] This means that the essential molecules for SARCOV-2 infection are abundant in the oral cavity, and the infected virus may lead to localized inflammation and loss of taste (dysgeusia) as well as dry mouth, which are the most frequently reported symptom in COVID-19 patient.[23–25] The literature search also indicated that the relationship between COVID-19 and diabetes mellitus is complicated and bidirectional.[26] In addition to diabetes, all patients with COVID-19 included in the current study also had periodontitis, a common comorbidity observed in patients with COVID-19.[26]

Consequently, it is necessary to consider the influence of confounding variables on the oral ecology. Therefore, to optimize the utility of salivary CRP and Il-6 as inflammation markers in separating patients with COVID-19 with and without diabetes, we used total salivary protein concentration to normalize the tested analyte levels (CRP and IL-6) in saliva.[27]

Compared to the control group (non-SARS-CoV-2 infected participants), we observed a highly significant increase in the salivary total protein concentration in both patient groups tested. These results suggest that elevated total salivary protein concentration occurred in all patients with COVID-19. Therefore, we assumed that the rising total salivary protein levels in our patients with COVID-19 might include increasing levels of both salivary inflammation markers (CRP and IL-6).

Based on the design mentioned above, we noticed the association between salivary CRP/IL-6 and total salivary proteins was consistent, but the strength of the correlation varied. In both patient groups, a significantly positive association was found only for CRP. A strong and moderate correlation was observed in the DG and the NDG. Further, moderate, and weak positive correlations between IL-6 and total protein were found in the DG and the NDG, respectively. Overall, these results indicates that the highest correlation (0.80) was found between salivary CRP and total protein of hospitalized COVID-19 patients with diabetes, while the non-diabetic patients had a lower correlation of 0.60. Since CRP in oral fluids reflects its level in circulation,[28] our result might indicate that in COVID-19 patients with diabetes, the proportion of salivary CRP was greater when serum levels were raised.

However, unlike salivary CRP, IL-6 levels in saliva have been reported to not correlate with those in plasma or serum.[29,30] In this regard, the elevated levels observed in this study may indicate inflammation induced by periodontitis-associated bacteria, particularly *Porphyromonas gingivalis*, as its components (dipeptidyl peptidase-4/DPP4), that mimic human dpp4, involved in postprandial glycemic control in individuals with type 2 diabetes,[31,32] that according to the medical record, were the participants included in the current study.

Moreover, it seems likely that IL-6 is a key stimulator of the hepatic synthesis of CRP.[33] In contrast, the salivary levels of CRP and IL-6 appeared to be independently regulated by diabetes status. As indicated in this study, a positive correlation between the two cytokines was observed in patients with and without diabetes.

This result was also corroborated by the ROC curve, and we were able to attain nearly 100% sensitivity and specificity. We believe that defining the relationship between CRP and IL-6 in patients with COVID-19 is unique to separate diabetes and non-diabetes in patients with COVID-19 suffering from periodontitis as a comorbidity.

Taken together, as mentioned above, the salivary CRP level reflects the CRP level in circulation, and it is likely that our data may reflect a positive correlation between IL-6 and CRP levels in the serum. Therefore, the results of the current study suggest that salivary levels of IL-6 might be overshadowed by local inflammation (periodontitis) instead of inflammation-related diabetes. Indeed, our findings provide additional information regarding the unequivocal results with either the presence[34,35] or the absence[36] of a relationship between the status of periodontal disease and salivary CRP.

Considering that CRP is a key cytokine that plays an important role in the progression of various inflammatory diseases,[37] we assumed that both periodontitis and diabetes are related to the pathophysiology of COVID-19. This association could be linked to the existence of two unique protein structures in CRP.[38] The first isoform is a CRP monomer that is activated by local signals of inflammation and tissue injury, and the other is the pentameric isoform synthesized by the liver.[39,40] As our participants were patients with COVID-19 with and without diabetes accompanied by periodontitis, we assumed that the salivary CRP detected in this study is the monomeric isoform that might have been activated by periodontal inflammation. Its presence in saliva may have been independently regulated by the diabetes status of our patients. Further studies are required to clarify this assumption.

Alternative explanations for our results warrant further discussion. First, SARS-CoV-2 infection is closely related to depressive disorder,[41,42] and the positive correlation between salivary CRP and IL-6 levels observed in this study may indicate a characteristic inflammatory response in depressive disorder that is commonly found in COVID-19 patients.[43] Second, since diabetes is among the common comorbidities noted in patients diagnosed with COVID-19,[44] the similar effect of the depression-associated inflammatory response (salivary CRP and IL-6) found in the two tested groups (DG and NDG) might be a consequence of the impact of depression on SARS-CoV-2 infection rather than diabetes itself. Thus, it is possible that the subtle elevation of the selected salivary biomarkers observed in the DG and the NDG (shown in Figure 2B, C) was no longer statistically significant. Further studies are required to confirm this possibility.

This observational study is subject to numerous limitations. First, the small sample size meant that the findings could not accurately represent the entire COVID-19-affected population. A bigger sample size and more investigation are required. Second, changes in salivary proteins that occur during the saliva collecting procedure were not taken into consideration in the study design. The results of the study do not definitively demonstrate a relationship between the level of inflammatory determinant in saliva and the systemic inflammatory mechanism of COVID-19. Therefore, more research is needed to assess dynamic salivary changes. Lastly, we cannot totally rule out the chance that the amount of salivary flow rate affects

the salivary levels of CRP and IL-6. Consequently, it is advised to validate the findings of this study and incorporate this confounding variable as a patient-specific component in subsequent research.

Conclusions

According to the current study, salivary levels of CRP and IL-6, which have a low correlation, seems to be a valuable oral fluid biomarker that could be useful in differentiating between patients with COVID-19 who have diabetes and those who do not. Thus, corroborate a prior study that found a connection between periodontal diseases and the expression of proinflammatory cytokines (IL-6 and CRP) associated with hyperglycemia.[45] Furthermore, the results of saliva analytes (IL-6 and CRP) align with the presence of oral dysbiosis, which is indicated by the presence of periodontitis.[4] This implies that oral dysbiosis in diabetics may raise the risk of diabetes-associated inflammation in situations of mild SARS-CoV-2 infection, This suggests that in cases of mild SARS-CoV-2 infection, oral dysbiosis in individuals with diabetes might increase the probability of diabetes-associated inflammation, which is characterized by increased levels of salivary CRP and IL-6.

Data availability

Underlying data

Open Science Framework: The utility of salivary CRP and IL-6 as a non-invasive measurement evaluated in patients with COVID-19 with and without diabetes, https://doi.org/10.17605/OSF.IO/PXD6R.[46]

This project contains the followign underlying data:

- Excel data TP-CRP-IL-6 F1000 Jan 23.xlsx

Data are available under the terms of the Creative Commons Zero "No rights reserved" data waiver (CC0 1.0 Public domain dedication).

Acknowledgements

All the authors would like to acknowledge all the study participants, the Director of Universitas Indonesia Hospital (RSUI), and the Ethical clearance committee for providing permission and ethical clearance to conduct this research project.

References

1. Granger DA, Johnson SB, Szanton SL, *et al.*: **Incorporating salivary biomarkers into nursing research: an overview and review of best practices.** *Biol. Res. Nurs.* Oct 2012; **14**(4): 347–356.
PubMed Abstract | Publisher Full Text | Free Full Text

2. Engeland CG, Bosch JA, Rohleder N: **Salivary Biomarkers in Psychoneuroimmunology.** *Curr. Opin. Behav. Sci.* Aug 2019; **28**: 58–65.
PubMed Abstract | Publisher Full Text | Free Full Text

3. Rathnayake N, Akerman S, Klinge B, *et al.*: **Salivary biomarkers for detection of systemic diseases.** *PLoS One.* 2013; **8**(4): e61356.
PubMed Abstract | Publisher Full Text | Free Full Text

4. Bachtiar BM, Bachtiar EW, Kusumaningrum A, *et al.*: **Porphyromonas gingivalis association with inflammatory markers and exosomal miRNA-155 in saliva of periodontitis patients with and without diabetes diagnosed with COVID-19.** *Saudi Dent. J.* Dec 16 2022; **35**: 61–69.
PubMed Abstract | Publisher Full Text | Free Full Text

5. von Elm E, Altman DG, Egger M, *et al.*: **The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) Statement: guidelines for reporting observational studies.** *Int. J. Surg.* Dec 2014; **12**(12): 1495–1499.
Publisher Full Text

6. Papapanou PN, Sanz M, Buduneli N, *et al.*: **Periodontitis: Consensus report of workgroup 2 of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions.** *J. Periodontol.* 2018 Jun; **89**(Suppl 1): S173–S182.
PubMed Abstract

7. Bachtiar EW, Gusliana DS, Bachtiar BM: **Correlation between the extent of smoking, salivary protein profiles, and dental caries in young adult smokers.** *Saudi Dent. J.* Nov 2021; **33**(7): 533–537.
PubMed Abstract | Publisher Full Text | Free Full Text

8. Bachtiar EW, Bachtiar BM, Kusumaningrum A, *et al.*: **ACE2 expression in saliva of patients with COVID-19 and its association with *Candida albicans* and *Aggregatibacter actinomycetemcomitans*.** *F1000Res.* 2022; **11**: 557.
PubMed Abstract | Publisher Full Text | Free Full Text

9. Bachtiar EW, Bachtiar BM: **Effect of cell-free spent media prepared from *Aggregatibacter actinomycetemcomitans* on the growth of *Candida albicans* and *Streptococcus mutans* in co-species biofilms.** *Eur. J. Oral Sci.* Oct 2020; **128**(5): 395–404.
PubMed Abstract | Publisher Full Text

10. Koussounadis A, Langdon SP, Um IH, *et al.*: **Relationship between differentially expressed mRNA and mRNA-protein correlations in a xenograft model system.** *Sci. Rep.* Jun 8 2015; **5**: 10775.
PubMed Abstract | Publisher Full Text | Free Full Text

11. Alwafi HA, Ali SS, Kotha SB, *et al.*: **Elevated Salivary Inflammatory Biomarkers are Associated with SARS-CoV-2 Infection Severity.** *Can. J. Infect. Dis. Med. Microbiol.* 2022; **2022**: 1543910–1543918.
PubMed Abstract | Publisher Full Text | Free Full Text

12. Balaji A, Chandrasekaran SC, Subramanium D, *et al.*: **Salivary Interleukin-6 - A pioneering marker for correlating diabetes and chronic periodontitis: A comparative study.** *Indian J. Dent. Res.* Mar-Apr 2017; **28**(2): 133–137.
PubMed Abstract | Publisher Full Text

13. Brocker C, Thompson D, Matsumoto A, *et al.*: **Evolutionary divergence and functions of the human interleukin (IL) gene family.** *Hum. Genomics.* 2010 Oct; **5**(1): 30–55.
PubMed Abstract | Publisher Full Text | Free Full Text

14. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S: **The pro- and anti-inflammatory properties of the cytokine interleukin-6.** *Biochim. Biophys. Acta.* 2011 May; **1813**(5): 878–888.
PubMed Abstract | Publisher Full Text

15. Hirano T: **Interleukin 6 and its receptor: ten years later.** *Int. Rev. Immunol.* 1998; **16**(3-4): 249–284.
PubMed Abstract | Publisher Full Text

16. Lima RPE, Belem FV, Abreu LG, *et al*. **Effect of Periodontal Therapy on Serum Levels of IL-6 in Type 2 Diabetics: A Systematic Review.** *Int. J. Periodontics Restorative Dent.* 2019 Jan/Feb; **39**(1): e1–e10.
PubMed Abstract | Publisher Full Text

17. Makki K, Froguel P, Wolowczuk I: **Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines.** *ISRN Inflamm.* 2013 Dec **2013**: 139239.
PubMed Abstract | Publisher Full Text | Free Full Text

18. Bachtiar BM, Bachtiar EW, Kusumaningrum A, *et al*. **ACE2 gene expression and inflammatory conditions in periodontal microenvironment of COVID-19 patients with and without diabetes evaluated by qPCR.** *MedRxiv [Preprint].* 2022; March 14, 2022. [accessed 2023 October 7]:32.
Publisher Full Text

19. Li W, Zhang C, Sui J, *et al*. **Receptor and viral determinants of SARS-coronavirus adaptation to human ACE2.** *EMBO J.* 2005 Apr 20; **24**(8): 1634–1643.
PubMed Abstract | Publisher Full Text | Free Full Text

20. Alonso GC, Pavarina AC, Sousa TV, *et al.*: **A quest to find good primers for gene expression analysis of Candida albicans from clinical samples.** *J. Microbiol. Methods.* 2018 Apr; **147**: 1–13.
PubMed Abstract | Publisher Full Text

21. Xu H, Zhong L, Deng J, *et al*. **High expression of ACE2 receptor of 2019-nCoV on the epithelial cells of oral mucosa.** *Int. J. Oral Sci.* 2020 Feb 24; **12**(1): 8.
PubMed Abstract | Publisher Full Text | Free Full Text

22. Sakaguchi W, Kubota N, Shimizu T, *et al*. **Existence of SARS-CoV-2 Entry Molecules in the Oral Cavity.** *Int. J. Mol. Sci.* 2020 Aug 20; **21**(17).
PubMed Abstract | Publisher Full Text | Free Full Text

23. Finsterer J, Stollberger C: **Causes of hypogeusia/hyposmia in SARS-CoV2 infected patients.** *J. Med. Virol.* 2020 Oct; **92**(10): 1793–1794.
PubMed Abstract | Publisher Full Text | Free Full Text

24. Elangovan S **Taste Disorders and Xerostomia Are Highly Prevalent in Patients with Covid-19.** *J. Evid. Based Dent. Pract.* 2022 Mar; **22**(1): 101687. Epub 20211224.
PubMed Abstract | Publisher Full Text | Free Full Text

25. Kumar H, Nishat R, Desai A: **A review on oral manifestations of COVID-19 disease.** *J. Family Med. Prim. Care.* 2022 Oct; **11**(10): 5879–86.
PubMed Abstract | Publisher Full Text | Free Full Text

26. Marouf N, Cai W, Said KN, *et al.*: **Association between periodontitis and severity of COVID-19 infection: A case-control study.** *J. Clin. Periodontol.* Apr 2021; **48**(4): 483–491.
PubMed Abstract | Publisher Full Text | Free Full Text

27. Pay JB, Shaw AM: **Towards salivary C-reactive protein as a viable biomarker of systemic inflammation.** *Clin. Biochem.* Jun 2019; **68**: 1–8.
PubMed Abstract | Publisher Full Text

28. Santa Cruz A, Mendes-Frias A, Oliveira AI, *et al.*: **Interleukin-6 Is a Biomarker for the Development of Fatal Severe Acute Respiratory Syndrome Coronavirus 2 Pneumonia.** *Front. Immunol.* 2021; **12**: 613422.
PubMed Abstract | Publisher Full Text | Free Full Text

29. Fernandez-Botran R, Miller JJ, Burns VE, *et al.*: **Correlations among inflammatory markers in plasma, saliva and oral mucosal transudate in post-menopausal women with past intimate partner violence.** *Brain Behav. Immun.* Feb 2011; **25**(2): 314–321.
PubMed Abstract | Publisher Full Text | Free Full Text

30. Minetto M, Rainoldi A, Gazzoni M, *et al.*: **Differential responses of serum and salivary interleukin-6 to acute strenuous exercise.** *Eur. J. Appl. Physiol.* Mar 2005; **93**(5-6): 679–686.
PubMed Abstract | Publisher Full Text

31. Ohara-Nemoto Y, Nakasato M, Shimoyama Y, *et al*. **Degradation of Incretins and Modulation of Blood Glucose Levels by Periodontopathic Bacterial Dipeptidyl Peptidase 4.** *Infect. Immun.* 2017 Sep; **85**(9). Epub 20170818.
PubMed Abstract | Publisher Full Text | Free Full Text

32. Deacon CF: **Physiology and Pharmacology of DPP-4 in Glucose Homeostasis and the Treatment of Type 2 Diabetes.** *Front. Endocrinol (Lausanne).* 2019; **10**: 80. Epub 20190215.
PubMed Abstract | Publisher Full Text | Free Full Text

33. Papanicolaou DA, Wilder RL, Manolagas SC, *et al.*: **The pathophysiologic roles of interleukin-6 in human disease.** *Ann. Intern. Med.* Jan 15 1998; **128**(2): 127–137.
Publisher Full Text

34. Nethravathy RR, Alamelu S, Arun KV, *et al.*: **Evaluation of circulatory and salivary levels of heat shock protein 60 in periodontal health and disease.** *Indian J. Dent. Res.* May-Jun 2014; **25**(3): 300–304.
PubMed Abstract | Publisher Full Text

35. Shojaee M, Fereydooni Golpasha M, Maliji G, *et al.*: **C - reactive protein levels in patients with periodontal disease and normal subjects.** *Int. J. Mol. Cell Med.* Summer 2013; **2**(3): 151–155.

36. Redman RS, Kerr GS, Payne JB, *et al.*: **Salivary and serum procalcitonin and C-reactive protein as biomarkers of periodontitis in United States veterans with osteoarthritis or rheumatoid arthritis.** *Biotech. Histochem.* 2016; **91**(2): 77–85.
PubMed Abstract | Publisher Full Text | Free Full Text

37. Luan YY, Yao YM: **The Clinical Significance and Potential Role of C-Reactive Protein in Chronic Inflammatory and Neurodegenerative Diseases.** *Front. Immunol.* 2018; **9**: 1302.
PubMed Abstract | Publisher Full Text | Free Full Text

38. Rajab IM, Hart PC, Potempa LA: **How C-Reactive Protein Structural Isoforms With Distinctive Bioactivities Affect Disease Progression.** *Front. Immunol.* 2020; **11**: 2126.
PubMed Abstract | Publisher Full Text | Free Full Text

39. Eisenhardt SU, Thiele JR, Bannasch H, *et al.*: **C-reactive protein: how conformational changes influence inflammatory properties.** *Cell Cycle.* Dec 2009; **8**(23): 3885–3892.
PubMed Abstract | Publisher Full Text

40. Thiele JR, Habersberger J, Braig D, *et al.*: **Dissociation of pentameric to monomeric C-reactive protein localizes and aggravates inflammation: in vivo proof of a powerful proinflammatory mechanism and a new anti-inflammatory strategy.** *Circulation.* Jul 1 2014; **130**(1): 35–50.
PubMed Abstract | Publisher Full Text

41. Diez-Quevedo C, Iglesias-Gonzalez M, Giralt-Lopez M, *et al.*: **Mental disorders, psychopharmacological treatments, and mortality in 2150 COVID-19 Spanish inpatients.** *Acta Psychiatr. Scand.* Jun 2021; **143**(6): 526–534.
PubMed Abstract | Publisher Full Text | Free Full Text

42. Vai B, Mazza MG, Delli Colli C, *et al.*: **Mental disorders and risk of COVID-19-related mortality, hospitalisation, and intensive care unit admission: a systematic review and meta-analysis.** *Lancet Psychiatry.* Sep 2021; **8**(9): 797–812.
PubMed Abstract | Publisher Full Text | Free Full Text

43. Sommer IE, Bakker PR: **What can psychiatrists learn from SARS and MERS outbreaks?** *Lancet Psychiatry.* Jul 2020; **7**(7): 565–566.
PubMed Abstract | Publisher Full Text | Free Full Text

44. Patrick DM, Van Beusecum JP, Kirabo A: **The role of inflammation in hypertension: novel concepts.** *Curr. Opin. Physio.* Feb 2021; **19**: 92–98.
PubMed Abstract | Publisher Full Text | Free Full Text

45. Salvi GE, Yalda B, Collins JG, *et al.*: **Inflammatory mediator response as a potential risk marker for periodontal diseases in insulin-dependent diabetes mellitus patients.** *J. Periodontol.* 1997 Feb; **68**(2): 127–135.
PubMed Abstract | Publisher Full Text

46. Bachtiar EW: **The utility of salivary CRP and IL-6 as a non-invasive measurement evaluated in patients with COVID-19 with and without diabetes.** 2023, January 31.
Publisher Full Text

Open Peer Review

Current Peer Review Status: ✔ ✔

Version 3

Reviewer Report 23 January 2024

https://doi.org/10.5256/f1000research.161123.r237285

© **2024 Taha G.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

✔ **Ghada Ibrahim Taha**
University of Baghdad, Baghdad, Baghdad Governorate, Iraq

Revised accepted

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: microbiology and Clinical immunologist

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 04 January 2024

https://doi.org/10.5256/f1000research.157516.r232990

© **2024 Taha G.** This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

? **Ghada Ibrahim Taha**
University of Baghdad, Baghdad, Baghdad Governorate, Iraq

Dear Author

I have carefully reviewed your manuscript and would like to bring the following points to your attention for clarification and enhancement:

Sample Clarification: The manuscript initially refers to the use of saliva samples; however, later sections mention a 'Gingival crevicular fluid (GCF)' in the context of interleukin-6 detection. Could you please clarify the exact nature of the samples used? If the GCF is indeed the sample of choice, kindly detail its collection method and the protocol employed in your study.

Sample Size Justification: The number of samples utilized in the study appears quite limited. Could you provide a rationale for this sample size? An explanation of how it's statistically sufficient to support the study's conclusions would be beneficial.

Study Group Composition: The research seems to encompass three distinct groups: individuals with gum disease, those with diabetes, and those affected by Covid. This approach appears somewhat broad. Could you elucidate whether the aim is to establish a correlation among these conditions? If so, references to prior studies establishing such a relationship would significantly strengthen your argument.

Control Group Discrepancy: There seems to be a contradiction regarding the control group's condition; the summary indicates they suffer from gum disease, whereas the methods section suggests they are free from any ailments. A consistent definition of the control group across all sections is crucial for the clarity and integrity of the study.

Supporting Evidence for Conclusions: The current conclusions would benefit greatly from additional supporting evidence from relevant studies. Incorporating references and discussions around existing research would provide a more robust context for your findings.

Exploration of Limitations: I noticed an absence of a detailed discussion on the limitations of your study. Understanding and acknowledging these limitations is vital for a comprehensive view of the research's scope and impact.

Your attention to these matters would greatly enhance the clarity and depth of your study. I look forward to your revisions.

Best regards,

Is the work clearly and accurately presented and does it cite the current literature?
No

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: microbiology and Clinical immunologist

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 07 Jan 2024

Endang Bachtiar

Dear Reviewer,

We appreciate the time and effort that you dedicated to providing feedback on our manuscript in this journal. We are grateful for the insightful comments on valuable improvements to our paper.
We have incorporated most of your suggestions. Please see below, the point-by-point response to the reviewer's comments and concerns

Reviewers' comments (RC) and Author Response (AR)

1. **RC**: I have carefully reviewed your manuscript and would like to bring the following points to your attention for clarification and enhancement:

AR: Thank you.

Sample Clarification: The manuscript initially refers to the use of saliva samples; however, later sections mention a 'Gingival crevicular fluid (GCF)' in the context of interleukin-6 detection. Could you please clarify the exact nature of the samples used? If the GCF is indeed the sample of choice, kindly detail its collection method and the protocol employed in your study.

AR: We appreciate the feedback provided by the reviewer. We therefore need to be clear that, as we explained in the Methods section, we amplified cDNA that had been converted from salivary RNA using the same procedure we used for the gingival crevicular sample for IL-6 using quantitative real-time polymerase chain reaction (qPCR; ABI StepOnePlus Real-Time PCR system) and SYBR Green I for gene expression analysis.[8] Thus, in this study we used saliva instead of GCF as oral sample.

1. **RC**: Sample Size Justification: The number of samples utilized in the study appears quite limited. Could you provide a rationale for this sample size? An explanation of

how it's statistically sufficient to support the study's conclusions would be beneficial.

AR: The number of patients included in the study was limited as we adhered to the committee's ethical guidelines that only patients with mild COVID-19 infection were allowed to have oral samples taken. We analyzed the collected data using a non-parametric test, as we indicated in the Methods section.

1. **RC**: Study Group Composition: The research seems to encompass three distinct groups: individuals with gum disease, those with diabetes, and those affected by Covid. This approach appears somewhat broad. Could you elucidate whether the aim is to establish a correlation among these conditions? If so, references to prior studies establishing such a relationship would significantly strengthen your argument.

AR: This study's primary goal was to determine whether salivary CRP and IL-6 may be used as possible indicators of systemic inflammation in people with COVID-19-diagnosed periodontitis who have diabetes or not. As can be seen in Methods section, under the subtitle of

Subjects, saliva sampling, and in vitro methods; "all COVID-19 patients participated in this study received a diagnosis of periodontitis. The patients were further split into two groups: those with (n = 10) and those without (n = 13) diabetes. As controls, six periodontitis non-diabetic individuals and no COVID-19 were also recruited". Additionally, in Methods section, under the subtitle of Data analysis, we explained; "The nonparametric Kruskal Wallis test was used to compare the diabetic group (DG), the non-diabetic group (NDG), and the control group. The Mann-Whitney test was used to compare the DG and NDG data".

Therefore, the diabetes group (DG), the non-diabetic group (NDG), and the control group were compared using the nonparametric Kruskal Wallis test, as we detailed in the Methods section.

4. RC: Control Group Discrepancy: There seems to be a contradiction regarding the control group's condition; the summary indicates they suffer from gum disease, whereas the methods section suggests they are free from any ailments. A consistent definition of the control group across all sections is crucial for the clarity and integrity of the study.

AR: I greatly appreciate this crucial correction. As a result, we changed the explanation of the control group in the Methods section to read as follows: As controls, six non-diabetic individuals with periodontitis but no COVID-19 were also included.

5. RC: Supporting Evidence for Conclusions: The current conclusions would benefit greatly from additional supporting evidence from relevant studies. Incorporating references and discussions around existing research would provide a more robust context for your findings.

AR: Thank you for your suggestions. Therefore, in the revised version we changed the sentence as following:

According to the current study, salivary levels of CRP and IL-6, which have a low correlation, seems to be a valuable oral fluid biomarker that could be useful in differentiating between patients with COVID-19 who have diabetes and those who do not. Thus, corroborate a prior study that found a connection between periodontal diseases and the expression of proinflammatory cytokines (IL-6 and CRP) associated with hyperglycemia.[45] Furthermore, the results of saliva analytes (IL-6 and CRP) align with the presence of oral dysbiosis, which is indicated by the presence of periodontitis.[4] This implies that oral dysbiosis in diabetics may raise the risk of diabetes-associated inflammation in situations of mild SARS-CoV-2 infection, This suggests that in cases of mild SARS-CoV-2 infection, oral dysbiosis in individuals with diabetes might increase the probability of diabetes-associated inflammation, which is characterized by increased levels of salivary CRP and IL-6.

6. RC: Exploration of Limitations: I noticed an absence of a detailed discussion on the limitations of your study. Understanding and acknowledging these limitations is vital for a comprehensive view of the research's scope and impact.

AR: We agree to reviewer's comments. Therefore, the related paragraph has been changed accordingly, as following:
There are certain restrictions on this observational study. Firstly, the subjects who were included were limited, therefore these results could not be indicative of the total population impacted by COVID-19. More research with a larger sample size is needed. Secondly, the study design did not account for the alterations in salivary proteins during the saliva collection process. Future research is required to evaluate dynamic salivary changes because the study's findings do not conclusively suggest a correlation between the level of inflammatory determinants in saliva and the systemic inflammatory mechanism of COVID-19. Finally, we cannot completely rule out the possibility that salivary flow rate influences the levels of IL-6 and CRP in saliva. Consequently, it is advised to validate the findings of this study and incorporate this confounding variable as a patient-specific component in subsequent research.
7. RC: Your attention to these matters would greatly enhance the clarity and depth of your study. I look forward to your revisions.
AR: Thank you.

Competing Interests: No competing interests were disclosed.

Reviewer Report 02 November 2023

https://doi.org/10.5256/f1000research.157516.r218232

© 2023 Schwartz J. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Joel Schwartz

University of Illinois Chicago, Chicago, USA

I stated previously and still think this study although only involving a small cohort does provide important data which needs additional study. The authors did attempt to incorporate my conceptual concerns in the discussion.

Please index this study without additional edits.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral biology, molecular biology, biochemistry, pathology, mucosal immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 22 September 2023

https://doi.org/10.5256/f1000research.143798.r208201

© 2023 Schwartz J. This is an open access peer review report distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Joel Schwartz

University of Illinois Chicago, Chicago, USA

Rationale for the study is pro-inflammatory markers such as IL-6 and CRP derived from the saliva are identifiable and associated with the presence of COVID-19 and/or possible periodontal disease.. Results show correlations to be present in the data rejecting the null hypothesis with a high level of probability.

However, the problem with these results are these.

(relatively low number) 23 Samples (10 with diabetes mellitus (DM) and 13 with no (DM). We need information about the phase of disease pathogenesis and determine possible inflammatory behavior. regarding DM (e.g., Type 1 /Type 2, intermediate, active, control, uncontrolled, medication used? etc.) Periodontal disease (e.g., mild-moderate, sever, generalized, localized? How was PDS assessed: periodontal bone probing, indexing (plaque, gingival, bleeding, CAL/ALOSS), radiology? all or PSR shortcut?)

Saliva is a great source of inflammatory makers. Some of these are transudate or exudate products from the serum. Therefore, coincident levels between the saliva and serum can vary. The should clearly state in the discussion is SARS-CoV-2 is an oral virus. Its tropism is targeting oral epithelial cells populations based on the expression pattern of AVCE/TMPRSS2 which include keratinocytes, salivary ductal cells of various types which includes myoepithelial cells, tuft cells, gustatory and supporting cells of the tongue. Using saliva samples without gingival crevicular fluid as alluded to in the discussion increases difficulty to assess contribution by gingival tissues compared to other tissues. In addition, there are citations available that indicate there is a hierarchy of ACE2/TMPRSS2 expression with the highest levels in tongue and salivary gland coinciding with clinical signs such as loss of taste and dry mouth. These types of signs and symptoms need to be described to provide some degree of characterization of inflammation related to tissue site..

Additionally, there is lacking a immunologic description of the functional activity of IL-6. This cytokine which is also described as a adipokine is suppressible by adiponectin derived from the saliva. IL-6 has a variety of function requiring a mention in the discussion. For example, regulation of loss of tolerance and B cell differentiation while also effecting innate responses particularly in the periodontal tissues. Above I mentioned a possible association with adiponectin which acts as a protector against DM while increasing glucose uptake. There is another association overlooked in the discussion periodontal pathogens release metallo-peptidases and one of these mimics dipeptidyl-peptidase IV (DDPIV) which is diabetogenic. These types of relationship validate previous reports that periodontal disease is linked to DM.

I suggest providing additional information in methods section about the health conditions of the subjects.

Try to provide additional markers from the serum of saliva identifying SARS-CoV_2 presence in the oral cavity.

Update and edit the discussion to comply with knowledge of SARS-CoV_2 and COVID-19 presentation effecting oral tissues.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral biology, molecular biology, biochemistry, pathology, mucosal immunology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 13 Oct 2023

Endang Bachtiar

Dear Reviewer,

Thank you for giving us the opportunity to submit a revised version of the manuscript "The utility of salivary CRP and IL-6 as a non-invasive measurement evaluated in patients with COVID-19 with and without diabetes". We appreciate the time and effort that the Reviewer dedicated to providing feedback on our manuscript and are grateful for the insightful comments on and valuable improvements to our paper. We have incorporated most of the suggestions made by the reviewer Those changes are highlighted in green within the manuscript.

Please see below, for a point by point response to the reviewer's comments and concerns. All page numbers refer to the revised manuscript with tracked changes.

Reviewer's Comments (**RC**)

1. Rationale for the study is pro-inflammatory markers such as IL-6 and CRP derived

from the saliva are identifiable and associated with the presence of COVID-19 and/or possible periodontal disease.. Results show correlations to be present in the data rejecting the null hypothesis with a high level of probability.

However, the problem with these results are these.

(relatively low number) 23 Samples (10 with diabetes mellitus (DM) and 13 without no (DM).

Author's Response (**AR**): Yes, we agree. As we mentioned in the limitation of our study; **page 9 line 33**. "This observational study has some limitations. First, the included participants were restricted. Additionally, as mentioned in Methods (under the subtitle: Subjects, saliva sampling, and in vitro methods). .....we performed the study in accordance with the guidelines provided by the ethics committee of RSUI, including "how consent was obtained from participants" (**Page 4, line 10**). Thus, only participants gave her consent was included.

1. **RC**: We need information about the phase of disease pathogenesis and determine possible inflammatory behaviour. regarding DM (e.g., Type 1 /Type 2, intermediate, active, control, uncontrolled, medication used? etc.) Periodontal disease (e.g., mild-moderate, sever, generalized, localized? How was PDS assessed: periodontal bone probing, indexing (plaque, gingival, bleeding, CAL/ALOSS), radiology? all or PSR shortcut?)

AR: Thank you for pointing this out. As can be seen in Methods section, all information about systemic condition of the patient, we obtained from the medical records. In the revised version, we have added some important information regarding "clinical status", such as age, sex, and chronic medical history of comorbidities, that was obtained from the medical reports (restricted) of mildly symptomatic patients with COVID-19 (not shown).

Only subjects who had no respiratory symptom for more than 2 weeks were included in this study, However, our focus was on patients with COVID-19, with and without diabetes. Thus, only diabetic status (type 2 diabetes), as reported in the medical records, was included as a comorbidity variable in the data analysis, while all COVID-19 patients were diagnosed with periodontitis (moderate to severe) according to the criteria described by the American Academic of Periodontology Classification of Periodontal Disease[6]. In this study, it would not possible to take dental radiology. Therefore, in revised manuscript, we added "without dental radiographic assessment following "patient diagnosed with periodontitis.....," mentioned above (**Page 4, Line 16-18**).

1. **RC**: Saliva is a great source of inflammatory makers. Some of these are transudate or exudate products from the serum. Therefore, coincident levels between the saliva and serum can vary.

The should clearly state in the discussion is SARS-CoV-2 is an oral virus. Its tropism is targeting oral epithelial cells populations based on the expression pattern of AVCE/TMPRSS2 which include keratinocytes, salivary ductal cells of various types which includes myoepithelial cells, tuft cells, gustatory and supporting cells of the tongue.

Using saliva samples without gingival crevicular fluid as alluded to in the discussion increases difficulty to assess contribution by gingival tissues compared to other tissues. In addition, there are citations available that indicate there is a hierarchy of ACE2/TMPRSS2 expression with the highest levels in tongue and salivary gland coinciding with clinical signs such as loss of taste and dry mouth. These types of signs and symptoms need to be

described to provide some degree of characterization of inflammation related to tissue site..

Additionally, there is lacking a immunologic description of the functional activity of IL-6. This cytokine which is also described as a adipokine is suppressible by adiponectin derived from the saliva. IL-6 has a variety of function requiring a mention in the discussion.

For example, regulation of loss of tolerance and B cell differentiation while also effecting innate responses particularly in the periodontal tissues. Above I mentioned a possible association with adiponectin which acts as a protector against DM while increasing glucose uptake.

There is another association overlooked in the discussion periodontal pathogens release metallo-peptidases and one of these mimics dipeptidyl-peptidase IV (DDPIV) which is diabetogenic. These types of relationship validate previous reports that periodontal disease is linked to DM.

AR: We agree and thank the reviewer to underline this important issue. Accordingly, in the revised manuscript, we have added explanations (**Page 9, Line 9-34)** regarding the tropism of SARS-CoV-2 as an oral virus, the hierarchy of ACE2/TMPRSS2 expression in oral tissue, as well as the oral sign and symptoms related to COVID-19, the immunologic description of IL-6 function as mentioned by the Reviewer, as follows;

Current literatures show that IL-6 is a pleiotropic cytokine with complex role in inflammation and metabolic disease. Its biological activities include B-lymphocyte differentiation, T-lymphocyte proliferation[13], pro and anti-inflammatory activities[14], the development of the nervous and hematopoietic system, and the regulation of metabolism[15]. As a proinflammatory cytokine, it induces insulin resistance and periodontal disease in the process of bone resorption[16]. Taken together, Il-6, which is considered an adipokine has an important role in the pathogenesis of localized oral inflammation (periodontitis), and may play a pivotal role in metabolic disease[17], such as diabetes.

The current study is an extension of our primary study involving the same patients with COVID-19 to evaluate the inflammatory conditions in periodontal microenvironment[18]. We found that the transcription levels of both IL-6 and complement C3 (the central component of innate immune system) in gingival crevicular fluid (GCF) were markedly higher in COVID-19 patients with diabetes (DG) compared to the non-diabetes patients (NDG). We also noticed that in periodontal niche, the mRNA upregulation of host receptor for SARS-CoV-2 (angiotensin-converting enzyme 2/ ACE2)[19]was positively associated with the transcription levels of either inflammation marker tested (IL-6 or C3). Thus, our previous results along with other work by other investigators[20, 21] suggest that the virus receptor (ACE2) can be detected in oral tissue. Additionally, certain molecules (furin and TMPRSS2) that are involved in promoting the SARS-CoV-2 entry and infection can be detected in oral cavity[22]. This means that the essential molecules for SARCOV-2 infection are abundant in the oral cavity, and the infected virus may lead to localized inflammation and loss of taste (dysgeusia) as well as dry mouth, which are the most frequently reported symptom in COVID-19 patient[23-25]. The literature search also indicated that the relationship between COVID-19 and diabetes mellitus is complicated and bidirectional[26]. In addition to diabetes,

all patients with COVID-19 included in the current study also had periodontitis, a common comorbidity observed in patients with COVID-19[27].

For the involvement of peptidase produced by periodontopathic pathogen, and mimics DDP4/DDP1V molecule of eukaryote cells, we have added explanations that can be found in the Discussion section (**Page 8, Line 8-13**), as follows;

However, unlike salivary CRP, IL-6 levels in saliva have been reported to not correlate with those in plasma or serum[30,31]. In this regard, the elevated levels observed in this study may indicate inflammation induced by periodontitis-associated bacteria, particularly *Porphyromonas gingivalis*, as its components (dipeptidyl peptidase-4 /DPP4), that mimic human dpp4, involved in postprandial glycemic control in individuals with type 2 diabetes[32,33], that according to the medical record, were the participants included in the current study.

1. **RC**: I suggest providing additional information in methods section about the health conditions of the subjects. Try to provide additional markers from the serum of saliva identifying SARS-CoV_2 presence in the oral cavity. Update and edit the discussion to comply with knowledge of SARS-CoV_2 and COVID-19 presentation effecting oral tissues.

AR: As mentioned in the above point 2, in the revised version of this manuscript, the additional information about participant's health condition have been added. As suggested by the reviewer, we also added the additional marker for the serum of saliva (complement C3) by referring to our previous study[18]. It can be seen in the above author response (point 3). Moreover, we have added the suggested content to the Discussion section, as explained in the above point 3.

Competing Interests: No competing interests were disclosed.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research