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BNT162b2 mRNA Vaccine—Induced Immune Response in Oral Fluids and Serum

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ABSTRACT

Objectives: The COVID-19 vaccine is currently being administered worldwide to address the ongoing pandemic. Although these vaccines have proven effective in preventing severe disease, the level of immunity required to prevent respiratory mucosal infection remains less well understood. Therefore, it is desirable to develop a noninvasive screening strategy such as oral fluid to monitor secreted antibodies longitudinally as potential surrogates of mucosal immunity.

Methods: We evaluated the anti-spike protein antibodies in gingival crevicular fluid (GCF) and saliva and compared them to immune responses in the blood of 50 healthy health care workers following 2 doses of intramuscular Pfizer/BioNTech-BNT162b2 vaccine.

Results: The antibodies to SARS-CoV-2 spike and subdomain proteins (RBD, S1, S2, and NTD) were significantly higher in serum than oral fluids but showed a greater detection rate and higher median titres in GCF than saliva. For all tested SARS-CoV-2 antigens, IgG in GCF (as opposed to saliva) showed a more significant and stronger correlation with IgG in serum. Serum-neutralising antibodies (Nab) titres also displayed a significant and stronger correlation with anti-spike protein and their subdomains in GCF than saliva. Interestingly, the time post−second dose of vaccine and sex had a similar influence on IgG in serum and GCF. However, interferon (IFN)-γ−producing T-cell responses showed no association with SARS-CoV-2 IgG antibodies in serum, GCF, or saliva and neutralisation antibodies in serum. The correlation matrix of all measured parameters grouped serum and GCF IgG parameters separately from salivary IgG parameters indicating that GCF better represents the humoural response in serum than saliva.

Conclusions: Within limitations, we propose that GCF could be a less invasive alternative to serum and more appropriate than saliva to detect antibody responses by current COVID-19 vaccines if the GCF collection procedure could be standardised. Further research is needed to investigate the suitability of GCF for community immune surveillance for vaccines.

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Introduction

Universal vaccination against COVID-19 is an effective strategy against severe COVID-19 infection and a viable gateway to normalcy.1 The BNT162b2 mRNA vaccine (Pfizer/BioNTech) has been shown to produce robust serum antibody responses and >95% efficacy against SARS-CoV-2.2,3 Unfortunately,
vaccine effectiveness has been shown to reduce after 6 months postvaccination, primarily due to waning immunity over time.12,13 Whilst screening of anti-SARS-CoV-2 titres are not mandatory for booster vaccination, various cutoffs for immune correlates have been helpful in the decision for various monoclonal antibody–based therapeutics in the treatment of COVID-19. Hence, quantifying vaccine-induced immune responses may have utility in clinical care and determining the net immunity against COVID-19 in individuals. This may have relevance in individualised treatment or vaccination strategies against COVID-19.

Unfortunately, determining a serum immunoglobulin level against SARS-CoV-2 involves venepuncture, which can cause pain and requires trained personnel to perform. Hence, it can be more desirable to utilise personalised monitoring of vaccine-induced immunity using noninvasive strategies, such as oral fluids. Currently, licensed mRNA vaccines have been shown to elicit detectable levels of antibodies in both serum and saliva.14 In convalescent patients, previous studies have demonstrated the persistence of salivary antibodies against SARS-CoV-2.6,7 Similarly, antibodies in self-collected gingival crevicular fluid (GCF) were consistent with those in serum. They showed comparable sensitivity and specificity in COVID-19 recovered and convalescent patients.8–11

Unfortunately, published COVID-19 studies have often used the terms “saliva” and “GCF” interchangeably, making distinctions between them unclear. Whilst the whole saliva refers to a biofluid containing mainly salivary and a fraction of nonsalivary elements, GCF is a serum transudate present in the gingival crevicular space.12,13 Past HIV studies have shown self-collected GCF (ie, harvested by rubbing the collection foam paddle along the gum line) to be enriched with antibodies and possess an antibody composition closely resembling serum.14 Therefore, we propose GCF as a less invasive alternative to the serum that is more appropriate than saliva to detect antibody responses post–COVID-19 vaccination. In the present study, we evaluated the humoral responses in GCF and saliva compared to immune responses (both humoral and cellular) in serum following 2 doses of intramuscular Pfizer/BioNTech BNT162b2 vaccine.

**Methods**

**Recruitment and sample collection**

We enrolled 50 health care workers (HCWs) who had undergone vaccination with 2 intramuscular doses of the Pfizer/BioNTech BNT162b2 from the Singapore Health Services institutions. Matched serum, saliva, and GCF samples were collected between 2 and 24 weeks following administration of the second dose of the vaccine (Supplementary Figure 1). The demographic features of the study participants are shown in Supplementary Table S1. The blood samples were collected by venepuncture, and the serum was extracted using serum separator tube tubes. The GCF was self-collected by gently brushing the gumline with an Oracol S14 saliva collection device (Malvern Medical Developments) for 2 minutes until saturation. Unstimulated saliva was collected via spit technique according to published protocols.15 GCF and saliva samples were centrifuged for 10 minutes at 1,500 ×g and stored at −80 °C until further use. This study was approved by the SingHealth Centralized Institutional Review Board (CIRB Ref: 2021/2170).

**SARS-CoV-2 bead-based Luminex Immunoassay**

IgG and IgA responses in serum, GCF, and saliva samples were measured using previously described bead-based Luminex Immunoassay with slight modifications.16 IgG and IgA binding to antigen were measured as Median Fluorescence Intensity (MFI) using a Magpix instrument (Luminex). MFI values of samples were converted to antibody quantity (ie, mg/mL) using anti-spike IgG and IgA antibody standards (AcroBiosystems). To fit the MFI values in the range of antibody standards, before testing, samples were diluted in block buffer (ie, serum, saliva, and GCF were diluted to 1:100, 1:10, and 1:10, respectively).

**Surrogate Virus Neutralisation Test (sVNT)**

sVNT assay is the proxy measure of antibodies that inhibit the binding of SARS-CoV-2 virus to the host cell receptor human angiotensin-converting enzyme 2 (hACE2) and was determined using cPass SARS-CoV-2 Neutralisation Antibody Detection Kit (GenScript).17 All samples were diluted to 1:20 in the kit sample buffer, respectively, and sVNT was measured per manufacturer guidelines. Percentage inhibition of RBD-hACE2 binding was computed using the following equation: % inhibition=[1−(OD with sample)/(OD with Negative Control)]×100

**SARS-CoV-2 Interferon-Gamma Release Assay (IGRA)**

The cellular SARS-CoV-2 immunity was determined indirectly by measuring the concentration of interferon-gamma (IFN-γ) secreted by activated T cells in serum using SARS-CoV-2 IGRA kit by EUROIMMUN. First, T cells were stimulated with S1 subunit of the SARS-CoV-2 protein, and after 24 hours of in vitro stimulation, IFN-γ secreted by T cells were measured using enzyme-linked immunosorbent assay. Magellan software (version 6.5) was employed to estimate IFN-γ concentration in serum samples.

**Statistical analysis**

Descriptive and inferential statistics were performed in R software (R Core 2020) and GraphPad Prism software (Graphpad Software Inc., version number 6.04). Fairwise comparisons for IgG and IgA antibodies in GCF and saliva with serum were performed using the Mann–Whitney U test. Spearman correlation coefficients were used to estimate correlations between serum vs GCF and serum vs saliva for all the biomarkers. GCF and saliva samples with antibody titres below the detection limit were assigned the value of the detection limit of that assay to be included in the statistical analysis. Statistical significance was set at P < .05.
### Results

**Greater detection of SARS-CoV-2-specific antibodies in GCF than saliva**

IgG antibodies to SARS-CoV-2 spike (S)-protein and its subdomains (RBD, S1, S2, and NTD) were detected in the serum of all participants, of which one serum sample was excluded from subdomain analysis owing to technical failure. GCF of all participants also showed the presence of IgG antibodies to S-protein, whilst salivary antibodies were detected in all but one participant. In contrast to serum, IgG antibodies to subdomain proteins were not detected in the GCF and saliva of all participants. RBD, S1, and S2-specific IgG antibodies were detected in GCF of 16% to 20% more participants than saliva, whilst NTD IgG was detected in GCF of 42% more participants. Additionally, the detection rate of IgG antibodies to S-protein and its subdomains were sustained in GCF even more than 3 months post

|                  | No. GCF, No. (%) | Saliva, No. (%) | Serum, No. (%) | % Difference in GCF vs saliva |
|------------------|-----------------|----------------|----------------|-----------------------------|
| **Spike**        |                 |                |                |                             |
| <3 mo            | 10 (100%)       | 10 (100%)      | 10 (100%)      | 0%                          |
| >3 mo            | 40 (100%)       | 39 (97.5%)     | 40 (100%)      | 3%                          |
| Total            | 50 (100%)       | 49 (98%)       | 50 (100%)      | 2%                          |
| **RBD**          |                 |                |                |                             |
| <3 mo            | 10 (100%)       | 9 (90.0%)      | 10 (100%)      | 10%                         |
| >3 mo            | 40 (100%)       | 30 (75%)       | 39 (97.5%)     | 18%                         |
| Total            | 50 (100%)       | 39 (78%)       | 39 (97.5%)     | 16%                         |
| **S1**           |                 |                |                |                             |
| <3 mo            | 10 (100%)       | 10 (100%)      | 10 (100%)      | 0%                          |
| >3 mo            | 40 (100%)       | 30 (75%)       | 39 (97.5%)     | 20%                         |
| Total            | 50 (100%)       | 40 (80%)       | 49 (98%)       | 16%                         |
| **S2**           |                 |                |                |                             |
| <3 mo            | 10 (90%)        | 6 (60%)        | 10 (100%)      | 30%                         |
| >3 mo            | 40 (85%)        | 27 (67.5%)     | 39 (97.5%)     | 18%                         |
| Total            | 50 (86%)        | 33 (66%)       | 49 (98%)       | 20%                         |
| **NTD**          |                 |                |                |                             |
| <3 mo            | 10 (70%)        | 1 (10%)        | 10 (100%)      | 60%                         |
| >3 mo            | 40 (55%)        | 7 (17.5%)      | 39 (97.5%)     | 38%                         |
| Total            | 50 (58%)        | 8 (16%)        | 49 (98%)       | 42%                         |

Fig. 1 – IgG responses to SARS-CoV-gcf-2 S-protein and its subdomains in gingival crevicular fluid (GCF), saliva, and serum samples. IgG-specific to spike (S) protein (A), RBD (B), S1 (C), S2 (D), and NTD (E) of SARS-CoV-2 (ancestral strain) were measured using a bead-based Luminex immunoassay. Humoral responses between groups were statistically compared using Mann–Whitney U tests, and all P values >.05 are marked on the graph. ****P < .0001, ***.001 < P < .001, **.01 < P < .001, *.05 < P < .01. 
second dose of vaccine and were more consistent than saliva samples. IgA antibodies in GCF and saliva of a majority of vaccinated participants were below the limit of detection, despite the detection being 100% in serum of matched study participants (Supplementary Figure 2).

Within the tested study population, anti-S IgG protein median titres were ~600-fold higher in serum than GCF ($P < .001$) and ~1200-fold higher than in saliva ($P < .001$) (Figure 1A). Interestingly, the anti-spike IgG protein showed approximately 2-fold higher titres in GCF than in saliva ($P < .001$). Likewise, IgG responses to the different S subdomains showed a similar trend, with GCF consistently containing significantly greater IgG levels than saliva (Figure 1B–E).

IgG antibody titres in serum strongly correlate with IgG in GCF

IgG antibodies specific to spike-S, RBD, S1, and S2 proteins showed a strong correlation between serum and GCF ($r > 0.6$, $P < .001$), whilst a moderate correlation was observed with NTD protein ($r = 0.5$, $P < .001$) (Figure 2). Salivary IgG antibodies to RBD and S1 proteins correlated strongly with serum IgG antibodies ($r > 0.6$, $P < .001$), whilst spike and S2 IgG showed weak correlation ($r = 0.5$, $P < .01$) and NTD showed poor correlation ($r = 0.3$, $P = .01$). In conclusion, for all tested SARS-CoV-2 antigens, IgG in GCF (as opposed to saliva) showed a more significant and stronger correlation with IgG in serum.

Interestingly, parameters such as time post–second dose of vaccine and sex influenced IgG in serum and GCF similarly. Specifically, both serum and GCF showed significantly lower S-, S1-, RBD-, and NTD-specific IgG in the >3 months than the <3 months postvaccination group, whilst IgG in saliva displayed no statistical differences between the groups (Supplementary Figure 3). No sex-specific differences were observed in humoral responses of serum, GCF, or saliva (Supplementary Figure 4). These data further imply similar trends in the SARS-CoV-2–specific humoral responses detected in serum and GCF.

**IFN-γ–producing T-cell responses in vaccinated participants did not correlate with humoral responses**

We then investigated vaccine-induced cellular immunity by determining the ability of T cells in whole blood to produce IFN-γ when stimulated with SARS-CoV-2 spike antigen. Correlation analysis showed no association between IFN-γ–producing T-cell responses in blood and SARS-Cov-2 IgG antibodies in serum, GCF, or saliva and neutralisation antibodies in serum (Figure 3).

Neutralizing antibody activity in serum positively correlates with IgG in GCF

Neutralising antibody (Nab) titres were detected in serum from all the participants, whilst very weak or no SARS-CoV-2
neutralisation was detected in GCF and saliva (Figure 4A). As expected, serum Nab titres showed strong correlations with SARS-CoV-2 S- and subdomain- (except S2 proteins) binding IgG titres in serum \( (r = 0.8, P < .001; \text{Figure 4B}) \). Interestingly, serum Nab titres also displayed a significant and strong correlation with anti-spike protein and their subdomains (except S2 proteins) in GCF \( (r = 0.6, P < .001; \text{Figure 4C}) \). Salivary IgG antibodies only correlated strongly with sera Nab for anti-spike, RBD, and S1 antigens \( (r = 0.6, P < .001; \text{Figure 4D}) \).

The correlation between all measured parameters was then analysed using unsupervised hierarchical clustering (Figure 4E). A correlation matrix with hierarchical clustering identified 4 similar clusters, where measured serum and GCF IgG parameters were grouped in one cluster and saliva IgG parameters in a separate cluster. This further indicates that GCF provides a better representative of humoural responses in the serum than saliva.

**Discussion**

In the present study, we comparatively evaluated the BNT162b2 mRNA vaccine–induced immune responses in circulatory fluids (specifically serum) and oral fluids (specifically GCF and saliva). Our results indicated that antibodies to SARS-CoV-2 spike and subdomain proteins could be detected in self-collected GCF in vaccine recipients. Saliva has been suggested as a noninvasive tissue to track for vaccine-induced immune responses.\(^4,5\) Whilst the present study supports the recently published data on the detection of SARS-CoV-2 antibodies in saliva, it also provides quantifiable evidence suggesting that GCF may be superior to saliva in terms of SARS-CoV-2 antibody detection and correlation with serologic responses.

GCF is a serum exudate that contains key immune components and locally generated tissue breakdown products.\(^13\) On the other hand, saliva is secreted by the acinar cells of salivary gland secretion and contains an influx of a large proportion of the GCF from the periodontal pockets.\(^12\) Because saliva is made of 99% water, there is considerable dilution in the biomarker concentrations, rendering it challenging to detect at times.\(^18\) Nevertheless, studies have detected high levels of antibody response in saliva in vaccination recipients.\(^19–21\) Given the time-dependent decay in the antibody levels and the salivary dilution, the antibodies in saliva can be expected to diminish to undetectable levels with time. In this context, the significantly higher proportions of SARS-CoV-2 IgG antibodies in GCF than in saliva appears to be a utilitarian observation in our study. As most participants (35/50) were 20 to 24 weeks after the second vaccination dose, our data demonstrate comparatively long-term persistence of antibodies against SARS-CoV-2 in GCF.
Fig. 4—SARS-CoV-2–neutralizing antibody activity in serum, gingival crevicular fluid (GCF), and saliva. A, Neutralizing antibodies against the ancestral strain of SARS-CoV-2 was assessed in samples using the surrogate virus neutralisation assay. B–D, The correlation of neutralizing antibodies in serum with binding IgG in serum (B), GCF (C), or saliva (D). Neutralizing
IgG accumulation in the GCF occurs mainly via passive transport or transudation from blood, whilst a fraction of it escapes into the saliva.\textsuperscript{22,23} Nahass et al\textsuperscript{2} recovered the highest number of SARS-CoV-2 antibody isotypes and subtypes in the oral fluid samples collected by the device that required mucosal contact near the gumline compared to the whole saliva.\textsuperscript{3} Accordingly, we observed significantly higher IgG antibody titres to spike protein and its subdomain in GCF than saliva, although the titres were hundreds-fold higher in serum. Because IgG in GCF is mostly transuded from blood, it probably explains why GCF correlated well with spike proteins and its subdomains, whilst saliva correlated strongly only with RBD and S1 proteins. Recently, Rao et al developed a GCF-based, rapid point-of-care test (CovAbTM test) to detect previous SARS-CoV-2 infection and initial adaptive immune response to vaccination.\textsuperscript{24} Although the test performed well to detect antibodies in convalescent GCF of convalescent patients, there was considerable heterogeneity in the magnitude of antibody titres achieved following Oxford-AstraZeneca vaccination. Nevertheless, their preliminary data using the CovAbTM test showed that GCF is the major source of the SARS-CoV-2 antibodies compared to saliva. Thus, GCF seems to be an appropriate antibody detection system to determine seroprevalence. However, the immune correlates that would provide adequate protection against SARS-CoV-2 is yet to be determined. Determining the baseline efficacy limits of a vaccine can guide need-based revaccination and demands further investigations.

Consistent with previous studies, we also observed that anti-SARS-CoV-2 IgA exhibited much lower serum titres than IgG and was less uniformly detected in the oral fluids in response to vaccination.\textsuperscript{5,25} Spike-specific serum IgA levels decay more rapidly than IgG antibodies, resulting in lower serum titres over time.\textsuperscript{26} The salivary glands can generate local antibody responses involving secretory IgA (sIgA) that can persist at low levels for up to 6 months after the second dose of the vaccine.\textsuperscript{25} In line with this observation, we detected anti-spike IgA at a much higher rate in saliva than in GCF. As the detection level of IgA antibodies was relatively low in our samples, investigation of sIgA correlates and its neutralisation capacity was considered beyond the scope of this study.

Determination of Nab is highly predictive of immune protection following vaccination.\textsuperscript{17} Contrary to serum, Nabs titres were low in GCF and saliva in our study. However, the serum Nabs correlated significantly with antibodies in oral fluid, albeit with most IgG subdomain proteins in GCF and few in saliva. Though not much is known about GCF, the salivary Nab levels tend to decline after remaining high for approximately 120 days post-second dose of vaccination.\textsuperscript{28} Since most of the participants in our study had completed more than 120 days after the second dose, low titres of Nabs could be expected. Furthermore, higher salivary neutralizing activity has been reported in individuals with high levels of IgA in their saliva, but our study showed very low detection of IgA.\textsuperscript{5} Even the newly emerging variants of SARS-CoV-2 have demonstrated significant differences in RBD binding and neutralisation.\textsuperscript{19} In addition to Nab, cell-mediated immunity is also elicited in vaccinated recipients.\textsuperscript{16} Although T-cell response in GCF or saliva could not be measured, IFN-\textgamma in serum showed no correlation with IgG and Nab in serum, GCF, or saliva.

The limitations of the study are the cross-sectional study design that hindered the evaluation of temporal associations in post-vaccine recipients and the lack of paired saliva and serum samples from unvaccinated and infected groups. However, this study was not designed to assess the dynamics of the antibody response or to compare immune responsiveness between vaccinated and non-vaccinated groups. The purpose of this study was to show that GCF could be a pivotal specimen for assessing vaccine efficacy in a larger population. GCF is commonly isolated using micropipettes or precut methylcellulose filter paper strips.\textsuperscript{29} Although paper strips are considered to be very efficient in GCF collection,\textsuperscript{30} these approaches are cumbersome from the patient and operator perspectives. As shown in the present study, GCF can be self-collected noninvasively by swabbing along the gumline. Although, compared to paper strips, the self-collected GCF samples may invariably contain some salivary components, our results have shown that this approach captures the antibodies profile more efficiently than sampling saliva. Besides, GCF collection does not require strict protocols to be followed before sample collection, unlike saliva. Nevertheless, since venepuncture is the gold standard for immunoassays, the GCF method could be a possible alternative when venepuncture is not feasible, particularly in large-scale screening in resource-poor areas. As a noninvasive alternative to venepuncture for serum samples, it stands to reason that GCF could be investigated as a potential diagnostic fluid for vaccine-induced immunity if the GCF collection method could be more standardised.

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**Author contributions**

CJS, PB, RDA, and JSXY contributed to conception, design, data acquisition, analysis, and interpretation and drafted and critically revised the manuscript. NU, TH, and JZT contributed to design and data analysis and interpretation and drafted and critically revised the manuscript. GBT, EEO, and JLGH contributed to conception, design, and data acquisition and drafted and critically revised the manuscript. CJS, PB, and
RDA contributed equally to this work. All authors read and approved the final manuscript.

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Conflict of interest

None disclosed.

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