



ORIGINAL ARTICLE

SARS-CoV-2 RNA in dental biofilms: Supragingival and subgingival findings from inpatients in a COVID-19 intensive care unit

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Abstract

Background: Saliva, salivary glands, gingival crevicular fluid, and supragingival biofilms may harbor SARS-CoV-2 RNA. This observational study aimed to investigate the presence and load of SARS-CoV-2 RNA in supragingival, and subgingival biofilms obtained from intensive care unit (ICU) patients.

Methods: A convenience sample, composed of 52 COVID-19+ participants (48.6 ± 14.8 years, 26.9% females), were evaluated for pre-existing comorbidities, number of teeth, and periodontal data [visible plaque (VPI), bleeding on probing (BOP), periodontal probing depth (PPD), and attachment loss (AL)]. Supragingival and subgingival samples (SubDeep: four sites with the deepest PPD; SubRemain: remaining shallower sites) were analyzed by RT-qPCR with corresponding cycle quantification (Cq). Statistical analyses considered the individual ($P = 5\%$).

Results: Twenty-six participants tested positive for dental biofilms (Biofilm+) with 96.2% of them being positive for subgingival samples. Pre-existing comorbidities, number of teeth examined, VPI, PPD, AL, and BOP were similar between Biofilm+ and Biofilm-. SubDeep PPD (3.72 ± 0.86), AL (4.34 ± 1.33), and % of BOP (66.0 ± 31.1) values were significantly greater compared to SubRemain values (2.84 ± 0.48 , 3.37 ± 0.34 , and 20.4 ± 24.1 , respectively). Biofilm+ Cqs showed no association with the periodontal condition. Cqs from Nasopharynx/Oropharynx (Naso/Oro; $n = 36$) were similar between Biofilm+ and Biofilm- participants. Length of time since ICU intake, last Naso/Oro RT-qPCR readings, onset of COVID-19 symptoms, and biofilm samplings were greater for Biofilm-.

Conclusions: ICU patients harbored SARS-CoV-2 RNA in supragingival and subgingival biofilms, irrespective of the periodontal condition, and systemic viral load. The high number of positive patients highlights the need to better understand this habit to provide adequate oral care.

KEYWORDS

COVID-19, dental plaque, periodontitis, SARS-CoV-2



1 | INTRODUCTION

Since its emergence, the SARS-CoV-2 virus has infected millions of individuals worldwide, leading to countless deaths and severe changes in the routines of nations.

Periods of social isolation, the large-scale use of face masks, and the implementation of hand hygiene measures are examples of the changes imposed by the pandemic to reduce interindividual contamination.¹⁻⁵ However, the infection rate of the virus is expressive, and variants have been detected worldwide.⁶⁻⁸ Since the beginning of the pandemic, efforts have been made to elucidate the transmission pathways of the virus and its penetration routes into human cells, in addition to the development of prevention, methods (including immunization) and therapeutic methods.

The oral cavity plays a vital role in SARS-CoV-2 infection, together with the upper airways, as viral particles are expelled by sneezing or through phlegm, speech, coughing, and sputum.⁹⁻¹² Saliva is an excellent example of a critical intraoral transmission source.¹³⁻¹⁶ Besides showing high sensitivity and specificity to the detection of SARS-CoV-2 RNA compared to throat samples,¹⁵ saliva harbors a high viral load.¹⁷ Additionally, SARS-CoV-2 RNA is present at the salivary glands,¹⁸ and the viral RNA has been detected in gingival crevicular fluid¹⁹ with a sensitivity of 63.64% [confidence interval (CI) 45.1% to 79.60%], considering the presence of SARS-CoV-2 RNA in the nasopharyngeal swab sampling as the gold standard.

Further, the oral cavity may act as a viral pathway. It has been suggested that the salivary glands¹⁸ and gingival tissue cells harbor angiotensin-converting enzyme receptors (ACEs), furin, and transmembrane serine protease 2 (TMPRSS2)²⁰ which are responsible for binding with the virus spike proteins and separating the S1 and S2 ends of the virus to penetrate human cells, respectively.^{21,22} According to Lloyd-Jones et al.,²³ gingival tissue may play a role in the penetration of the virus, which in turn affects the bloodstream and pulmonary cells. Interestingly, an evaluation of postmortem gingival specimens revealed five out of seven positive Quantitative Real-Time PCR (RT-qPCR) results.²⁴

In our initial study,²⁵ the presence of viral RNA in supragingival (Sup) biofilms was explored. A total of 18.9% of the participants, who tested positive for SARS-CoV-2 RNA in oropharyngeal and nasopharyngeal samples, also tested positive for the Sup biofilms. However, in that study, the viral RNA and viral load in the subgingival biofilms, estimated through the Cycle quantification values, could not be investigated. Once there is a close relationship between the supragingival and subgingival environments, the current hypothesis implies that SARS-CoV-2 RNA could be present in both biofilms. Therefore, the present

study was designed to evaluate the presence and load of the viral RNA in supragingival and subgingival biofilms of patients positive for COVID-19 in an intensive care unit (ICU).

2 | MATERIALS AND METHODS

2.1 | Ethical considerations

This observational clinical study was conducted at the Hospital de Clínicas de Porto Alegre (HCPA), Porto Alegre, Brazil. The study protocol was approved by the HCPA Ethics Committee (CAAE: 41763220.8.0000.5327). This report followed the STARD statement guidelines.²⁶

2.2 | Sample selection

Patients from the HCPA's COVID-19 ICU (May-June 2021), and who had an informed consent form signed by a legal representative, were eligible for the study. The inclusion criteria were:

- Dentate participants.
- Not pronated.
- Had teeth that allowed for the experimental examination.
- Had a systemic condition and well-being that allowed for experimental examination.

Disavowal by legal representatives (even after provision of a signed informed consent form and/or data collection) and impossibility to sample dental biofilms (even if clinical examinations could be performed) served as exclusion criteria in the present study. Figure 1 displays the composition of the final study population.

2.3 | Data collection

The collection of the data occurred between May 1st and June 15th, 2021. Demographic data were collected from the HCPA records to define the characteristics of all the individuals enrolled in the study.

Additionally, the following data were obtained:

- Pre-existing comorbidities: diabetes, hypertension, obesity, and others [neurological, psychological, cardiovascular diseases (HIV; hepatitis B and C), cancer, autoimmune disease, metabolic disease, respiratory disease, history of organ transplantation]

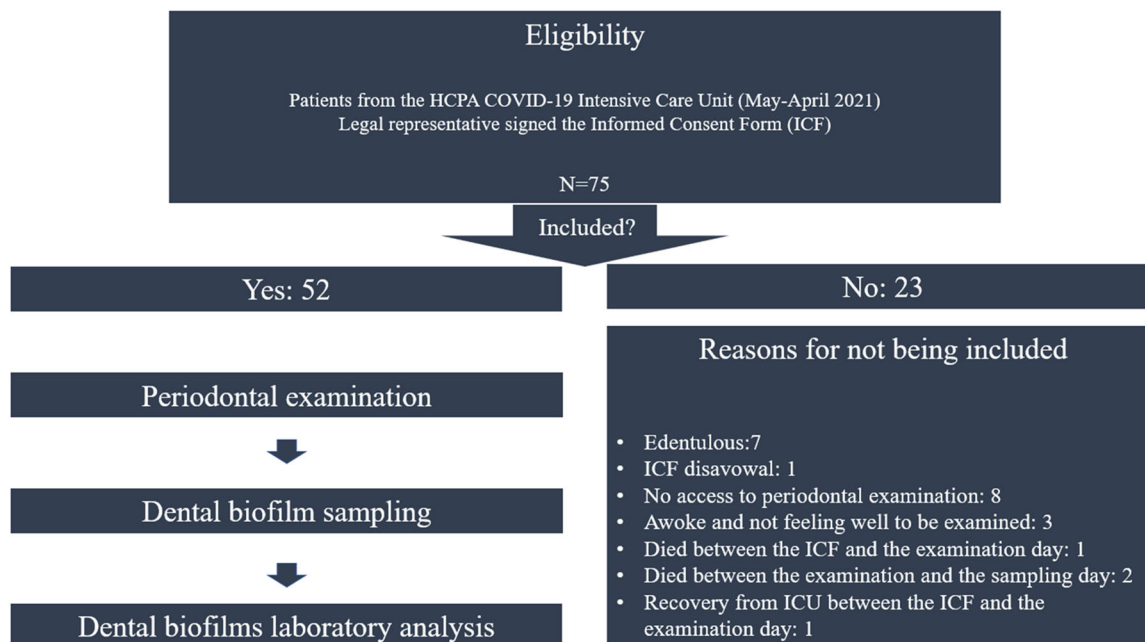


FIGURE 1 Flowchart of the study

- Time elapsed between the ICU intake and the dental biofilm sampling.
- Time elapsed between the last Nasopharyngeal and Oropharyngeal RT-qPCR and the dental biofilm sampling.
- Time elapsed between the COVID-19 symptom's onset and the dental biofilm sampling.

2.3.1 | Clinical examination

A trained and calibrated periodontist (LuMMi) performed all the clinical examinations. The intraclass correlation coefficient (ICC) for repeated periodontal probing depth (PPD) and attachment loss (AL) examinations were 0.82 and 0.78, respectively.

Even with the difficulties associated with a COVID-19 ICU, still it was possible to examine the participants in their beds. However, not all the teeth that were present were accessible for the experimental procedures. Moreover, no examinations were performed at the palatal/lingual sites.

The following examinations were performed at the buccal aspect:

- Visible plaque index (VPI)²⁷ from the distal, medial, and mesial sites
- Bleeding on probing (BOP), PPD, and AL from the distal and mesial sites. The PPD and AL were measured in millimeters (10 mm periodontal probe¹).

During the collection of the data, two additional periodontists (JF and LM) helped with the management of the participant in their beds, the data registration, and the handling of the instruments and sampling materials. The periodontal probe was disinfected with alcohol 70° between the sites to avoid possible SARS-CoV-2 cross-infection. The periodontal exams were dictated to a recorder to eliminate the use of periodontal sheets and other materials inside the ICU.

2.3.2 | Dental biofilm sampling

Because of the logistics of the COVID-19 ICU, including a high demand to reduce the length of stay of the personnel, the biofilm sampling was planned 3 days after the periodontal examination, which allowed time for select the sites to be sampled subgingivally. Thus, the examinations were performed on Saturdays and the sampling was conducted on Tuesdays. During the interval, Falcon tubes, sterile dental swabs^{2,25} and all the materials required for sampling were prepared outside the ICU.

Supragingival samples (Sup): One pool was obtained from the dento-gingival area, as follows:

- One dental swab per quadrant was used for all the accessible teeth, that is, all buccal sites.
- After sampling, one cotton roll was used to remove reminescent supragingival biofilm to grant an adequate

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subgingival sampling. An additional roll was used to avoid saliva during the dental biofilm sampling.

- 1. Subgingival samples: One dental swab per site was used, which were pooled accordingly:
- Deepest sites (SubDeep): In each participant the deepest PPD at four different teeth of all examined teeth were identified as SubDeep sampling sites. If more than one site showed the same PPD, the presence of BOP was decisive. However, if no differences could be observed regarding the PPD and BOP, the higher AL was decisive.
- Remaining sites (SubRemain): all the other shallower sites sampled subgingivally.

One Falcon tube containing 1 mL of saline solution was used for each pooled sample, therefore, there were three tubes for each participant (one Sup, one SubDeep, and one SubRemain).

All samples were stored at -80°C until analysis in the laboratory.

2.4 | Laboratory analysis

2.4.1 | Preparation of the samples

A total of 2 mL of saline solution was added to each tube, and the tubes were refrigerated until processing. All the samples were handled in a security level II—B2 chamber following the recommendations for viral diagnosis set out by the Brazilian Ministry of Health. During processing, the samples were vortexed, and three 1 mL aliquots were extracted. Two of the aliquots were stored at -80°C as reserve samples, whereas a MagMax™ Viral Pathogen II Nucleic Acid Isolation Kit³ was used to extract viral genetic material from the third aliquot (according to the manufacturer's instructions). The extracted RNA samples were stored at -80°C . Before RNA isolation, 200 μL of buffer was added to each sample.

2.4.2 | Real-time quantitative polymerase chain reaction (RT-qPCR)

Determination of the presence of the SARS-CoV-2 virus was conducted using real-time quantitative polymerase chain reaction (RT-qPCR). The Charité protocol²⁸ was used in addition to the AgPath-ID One-Step RT-qPCR Reagents kit[‡]. Additionally, a control assay was conducted using ribonuclease P (RNase P), according to the protocol of the US Centers for Disease Control and Prevention.²⁹

< 38 for viral and control genes were positive controls for SARS-CoV-2. The samples in which only one of the viral genes was amplified were classified as inconclusive, and the RT-qPCR was repeated. The samples that did not show amplification of either gene were considered inadequate, and the RNA extraction and RT-qPCR were repeated. A total of three samples required RT-qPCR repetition.

2.5 | Data analysis

Initially, aiming to calculate the percentage of sites positive to VPI and BOP, the total number of teeth examined per participant was multiplied by a factor of 3 (i.e., distal, medial, and mesial sites of the buccal surface). The obtained number represented the total number (i.e., 100%) of all sites present. Thereafter, the number of positive sites per participant was identified (thus making it possible to calculate the percentage of affected sites for each individual). The averages of PPD and AL, in turn, were reported in millimeters. Afterwards, the means for each variable were calculated to the study sample.

The mean (standard deviation) or median (interquartile range) was calculated for each numeric variable, according to its distribution. Distribution frequencies were determined for the categorical variables.

Through the Shapiro–Wilk test and the F test, the assumptions of normality and homoscedasticity of the data were evaluated to decide whether parametric or nonparametric analyses would be performed. Regarding the quantitative variables, for comparisons of independent groups, the *t* test or Mann–Whitney U test were used. To assess the differences between paired groups, the *t* test for paired samples or the Wilcoxon matched-pair signed-rank test was used. To measure the relationship between the periodontal clinical variables and Cq values, the Pearson and Spearman correlation coefficients were used, according to the distributions of the variables. Fisher's Exact Test was used to test the associations between the qualitative variables.

To explore the temporal presence of the viral RNA in dental biofilms, three timeframes (based on the sampling date of dental biofilms) were considered:

- The number of days since ICU intake
- The number of days elapsed since the last Nasopharynx/Oropharynx (Naso/Oro) RT-q-PCR
- The number of days since the onset of COVID-19 symptoms

When the Naso/Oro Cq results could be assessed (data retrieved from 36 participants, constituting the Naso/Oro^{Gp}), this information was used for comparisons with the Cq from the dental biofilms.

³ Thermo Fisher Scientific, Waltham, MA, USA.

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS⁴) for Windows (version 18.0). The analyses were conducted at the level of the individual and the significance level was set at 5%.

3 | RESULTS

3.1 | Demographics, pre-existing comorbidities, and the presence of the viral RNA in dental biofilms

Seventy-five informed consent forms were obtained and a consecutive sample of 52 individuals was composed (48.6 ± 14.8 years, 26.9% females). A flowchart of the study is depicted in Figure 1.

A total of 26 participants (50% of the total sample) showed at least one positive RT-qPCR result for the presence of SARS-CoV-2 RNA in dental biofilms, that is, of supra- or subgingival origin. The demographics, pre-existing comorbidities, and periodontal characteristics of all the participants ($n = 52$) and for those who tested positive (Biofilm⁺: $n = 26$) or negative (Biofilm⁻: $n = 26$) in the dental biofilms are shown in Table 1, with no differences between them. However, the number of days since the ICU intake, the last Naso/Oro RT-qPCR examination and the COVID-19 symptoms' onset, in relation to the dental biofilm sampling day, were always greater for Biofilm⁻ participants ($P = 0.012, 0.006, \text{ and } 0.011$, respectively).

3.2 | Distribution of the viral RNA in supragingival or subgingival samples, the periodontal condition from subdeep and subremain sites, and the cycle quantification values

Twenty-four Biofilm⁺ participants (92.31%) tested positive in supragingival biofilm samples, with only one participant testing solely in supragingival biofilm. Of the 25 participants who tested positive subgingivally (97.2% of the Biofilm⁺ participants), 19 (73.8%) tested positive for the SubDeep and SubRemain samples simultaneously. Six patients, however, tested positive for only one of these subcategories (Table 2). The Cq values observed for the total sample were 33.34 ± 3.5 .

SubDeep showed significantly higher PPD [3.72 ± 0.86 mm versus 2.84 ± 0.48 mm, respectively; $P < 0.001$] (Table 3) compared with SubRemain sites. Also, the AL means, and the mean percentage of BOP positive sites were significantly greater for SubDeep sites. Interestingly, the

correlation coefficients showed that the Cq values were not influenced by the periodontal parameters ($P > 0.05$).

The Cq values from the Naso/Oro RT-qPCR were available for part of the study population ($n = 36$) (Table 4). Additionally, the prevalence of positive and negative results from the Sup, SubDeep, and SubRemain samples can be seen in Table 4. The Cq means were greater for the dental biofilm samples when compared to the Naso/Oro^{Gp} Cq values ($P < 0.001$). The Naso/Oro^{Gp} Cq values did not differ between the Biofilm⁺ and Biofilm⁻ participants ($P > 0.05$).

4 | DISCUSSION

During the present study, 50% of the patients from the COVID-19 ICU of a university and public hospital (HCPA) harbored SARS-CoV-2 RNA in dental biofilms, either supra- or subgingivally (SubDeep and SubRemain). In general, when an individual tested positive for one of the three biofilm samples, at least one other sample was also positive. Interestingly, the presence and load of the viral RNA were independent of the periodontal condition. Besides, the number of biofilm-positive participants decreased over time in the ICU.

This study is the second of its kind to investigate the presence of SARS-CoV-2 RNA in dental biofilms. The first study only assessed the viral RNA in supragingival samples, and the participants were younger and periodontally healthy.²⁵ In the present study, the mean age of the participants was 48.6 ± 15.0 years, and they were predominantly male. In contrast, in the study conducted by Gomes et al.,²⁵ a mean age of 40 ± 9.8 years was reported, and the participants were mostly female. In the present investigation, access to periodontal examinations was limited, either by the presence of intraoral medical devices or by the general COVID-19 condition of the participants. In this respect, only the data regarding the number of teeth that could be examined per participant are shown (Table 1). For the same reason, only the buccal sites could be examined. However, the periodontal examination was performed by a periodontist, measuring subgingival parameters suitable for periodontal diagnosis (PPD, AL, and BOP). Overall, no differences were observed between the Biofilm⁺ and Biofilm⁻ participants regarding the demographics and all the periodontal parameters computed (the mean percentage of the sites that were positive for VPI and BOP, and the PPD and AL millimeter means). Each participant showed AL of at least 2 mm in two interproximal sites from different teeth (data not shown). Thus, even though a complete investigation of the periodontal condition could not be performed, it could be inferred that all participants showed signs of periodontitis.³⁰

⁴ SPSS Inc., Chicago, IL, USA.



TABLE 1 Summary of the sociodemographic data, time frames in relation to the dental biofilm sampling, the presence of systemic comorbidities, number of teeth, and the periodontal examinations for the total sample (Total) and for the participants who tested positive (Biofilm⁺) or negative (Biofilm⁻) for SARS-CoV-2 RNA in dental biofilm samples

Parameters	Total (n = 52)	Biofilm ⁺ (n = 26)	Biofilm ⁻ (n = 26)	P-value
Age (years) ^a	48.6 [15.0]	48.2 [15.5]	49.0 [14.6]	0.885 ^b
Female participants ^c	14 (26.9)	7 (26.9)	7 (26.9)	1.000 ^d
Time frames				
Days in the ICU ^a	17.5 [12.3]	13.2 [7.5]	21.7 [14.4]	0.012 ^b
Time elapsed between the Oro/Naso RT-qPCR and the dental biofilm sampling ^a	18.6 [10.6]	14.5 [7.2]	22.6 [12.0]	0.006 ^b
Time since the onset of COVID-19 symptoms ^a	27.1 [13.9]	22.1 [9.4]	32.2 [16.1]	0.011 ^b
Presence of comorbidities^c				
Diabetes	12 (23.1)	4 (15.4)	8 (30.8)	0.324 ^d
Hypertension	25 (48.1)	11 (42.3)	14 (53.8)	0.579 ^d
Obesity	23 (44.2)	12 (46.2)	11 (42.3)	1.000 ^d
Others	42 (80.8)	21 (80.8)	21 (80.8)	1.000 ^d
Number of teeth (contributed)^a	5.9 [1.1]	5.8 [0.6]	6.0 [1.4]	0.734 ^e
Periodontal examinations^a				
Visible plaque (%)	82.7 [18.1]	86.5 [15.2]	78.9 [20.3]	0.169 ^e
PPD	3.1 [0.5]	3.1 [0.5]	3.2 [0.6]	0.744 ^b
BOP (%)	36.9 [22.9]	32.7 [20.9]	40.7 [24.0]	0.202 ^e
AL	3.7 [0.9]	3.7 [0.9]	3.8 [0.9]	0.583 ^b

P = comparison between the Biofilm⁺ and Biofilm⁻ participants.

^amean [standard deviation].

^bindependent samples *t* test.

^cnumber (percentage).

^dFisher's exact test.

^eMann-Whitney test.

Biofilm⁺: all the participants who showed a positive RT-qPCR result for the presence of SARS-CoV-2 RNA in a dental biofilm, irrespective of whether it was a supragingival or subgingival sample.

Number of teeth (contributed): mean number of teeth examined per participant; PPD: periodontal probing depth; BOP: bleeding on probing; AL: attachment loss. Others: neurological, psychological, cardiovascular diseases (HIV; hepatitis B and C), cancer, autoimmune disease, metabolic disease, respiratory disease, transplant.

TABLE 2 Dental biofilm origin and the number and percentage of participants who tested positive for the presence of SARS-CoV-2 RNA considering all possible combinations of positive results, with the corresponding mean cycle quantification (Cq)

Biofilm origin	Number of positive participants	Cumulative percentage	Cq [sd]
Sup	1	3.85	35.8
SubDeep	1	3.85	32.2
SubRemain	1	3.85	37.3
Sup + SubDeep	2	7.70	36.39 [0.1]
SubDeep + SubRemain	2	7.70	35.72 [0.3]
Sup + SubDeep + SubRemain	19	73.08	32.5 [3.6]
Total	26	100	33.34 [3.5]

Abbreviations: Sup: supragingival biofilm samples; SubDeep: subgingival biofilm samples from the deepest sites; SubRemain: subgingival biofilm samples from all remaining shallower sites; sd: standard deviation.

TABLE 3 Means [sd] of the periodontal probing depth (PPD; in millimeters), bleeding on probing (BOP; in percentage), and attachment loss (AL; in millimeters) considering the subgingival deepest sites (SubDeep) and subgingival remaining shallower sites (SubRemain) for the total sample ($n = 52$), irrespective of whether a participant tested positive (Biofilm⁺) or negative (Biofilm⁻) for the presence of SARS-CoV-2 RNA in dental biofilm samples

Periodontal parameters	SubDeep ^a	SubRemain ^a	P-value ^b	Cq SubDeep	Cq SubRemain
PPD ($n = 52$)	3.72 [0.86]	2.84 [0.48]	< 0.001	NA	NA
PPD Biofilm ⁺	3.59 [0.67]	2.86 [0.51]	< 0.001	$r = 0.032$ ($P = 0.886$)	$r = -0.407$ ($P = 0.060$)
PPD Biofilm ⁻	3.85 [1.01]	2.82 [0.45]	< 0.001	NA	NA
P-value ^c	0.270	0.767			
BOP ($n = 52$)	66.0 [31.1]	20.4 [24.1]	< 0.001 ^d	NA	NA
BOP Biofilm ⁺	62.5 [30.2]	16.1 [20.1]	< 0.001 ^d	$r = 0.219$ ($P = 0.328$)	$r = 0.011$ ($P = 0.962$)
BOP Biofilm ⁻	69.6 [32.2]	24.6 [27.3]	< 0.001 ^d	NA	NA
P-value ^e	0.402	0.266			
AL ($n = 52$)	4.34 [1.33]	3.37 [0.74]	< 0.001	NA	NA
AL Biofilm ⁺	4.11 [1.18]	3.39 [0.80]	< 0.001	$r = -0.034$ ($P = 0.879$)	$r = -0.224$ ($P = 0.316$)
AL Biofilm ⁻	4.58 [1.44]	3.34 [0.68]	< 0.001	NA	NA
P-value ^e	0.198	0.843			

^amean [sd].

^bpaired-samples *t* test.

^cindependent samples *t* test.

^drelated samples Wilcoxon test.

^eMann-Whitney test; Pearson correlation coefficient or Spearman correlation coefficient values (*r*) and significance (*p*).

TABLE 4 Number of participants with RT-qPCR results for Nasopharynx and Oropharynx samples (Naso/Oro^{Gp}, $n = 36$) who tested positive (Biofilm⁺) or negative (Biofilm⁻) in dental biofilm samples and considering the origin of the samples (Sup, SubDeep, and SubRemain) with the respective Cycle Quantification values

Naso/Oro ^{Gp}	N	Naso/Oro ^{Cqs}	Biofilm ^{Cqs}
Naso/Oro ^{Gp}	36	23.9 [5.3]	NA
Biofilm ⁺	17	23.0 [4.0]	33.1 [3.9] ^a
Biofilm ⁻	19	24.7 [6.2]	NA
		0.330 ^b	
Sup ⁺	15	23.1 [4.0]	31.9 [4.5] ^a
Sup ⁻	21	24.4 [6.1]	NA
		0.429 ^b	
SubDeep ⁺	16	22.8 [4.0]	33.5 [4.0] ^a
SubDeep ⁻	20	24.7 [6.1]	NA
		0.271 ^b	
SubRemain ⁺	14	22.5 [3.6]	32.8 [3.9] ^a
SubRemain ⁻	22	24.8 [6.0]	NA
		0.164 ^b	

mean [sd]; Naso/Oro^{Cqs}: nasopharyngeal and oropharyngeal samples; Cq Biofilm: Cq from the biofilm samples; NA: not available.

^apaired-samples *t* test ($P < 0.001$).

^bindependent samples *t* test.

The results of the present study showed a close periodontal condition between the Biofilm⁺ and Biofilm⁻ participants. Of particular interest is the high percentage of

sites that were positive for VPI in both groups of participants (Biofilm⁺ and Biofilm⁻). Thus, it is conceivable that the amount of supragingival biofilm was not decisive for the presence of viral RNA and most likely reflects the difficulties experienced by the nursing team in performing the oral hygiene protocol set out by the HCPA (12-12 hours tooth brushing with chlorhexidine 0.12% solution). The COVID-19 ICU conditions, such as the presence of facial respiratory masks, pronate position of the patients, urgent maneuvers, the number of urgent interments, and the risk of the spread of the virus, are important limitations for oral hygiene practices. Moreover, intraoral manipulation difficulties were easily perceived by the authors of the present study during the experimental procedures. Therefore, every effort was made to avoid saliva contamination during the sampling of dental biofilms using cotton rolls. Additionally, before the subgingival sampling was conducted, the supragingival biofilm was removed using cotton rolls. However, cross-infection via saliva cannot be fully disregarded. Conversely, the risk of cross-contamination was equally distributed among the 52 participants and not solely among those who tested positive for the viral RNA. In addition, some participants who tested positive had another one or two samples that showed negative results. Thus, even if cross-contamination of the saliva did occur, a definitive response would depend on the results of a saliva RT-qPCR test from the same day of the dental biofilm sampling. However,



this was not the case and is a limitation of the present study.

Interestingly, 19 of 26 participants (73.7%) tested positive for all the biofilms, irrespective of whether they were supragingival or subgingival in origin. The exceptions were one participant who tested positive only for the supragingival sample, four participants who tested positive for the supragingival sample and one of the subgingival samples (SubDeep or SubRemain), and two participants who tested positive only for the subgingival samples. To the best of our knowledge, this is the first study to test the hypothesis regarding the presence of the viral RNA, aside from supragingival, from subgingival biofilms. The results revealed a trend which indicated that, once a participant tested positive for one of the biofilms, he/she would also test positive for other samples. With respect to the sites sampled subgingivally, these results are particularly interesting as they cannot be attributed to site-to-site cross-infection once the periodontal probe was cleaned and disinfected with cotton and alcohol 70°. The present findings, in which 25 participants tested positive for at least one of the subgingival samples (97.15% of Biofilm⁺), show that there is no requirement to explore SubDeep and SubRemain sampling sites in future studies if the objective is to investigate the prevalence of positive subjects. In contrast, the pooling of samples from all sites would be more appropriate. In the present study, the aim was also to investigate the potential relationship between the periodontal condition and the presence and load of the viral RNA. Therefore, separate evaluations of the SubDeep and SubRemain sites were required. In general, the criteria that were applied concerning the selection of the SubDeep and SubRemain provided consistent information regarding the periodontal status from these sites: the deepest sites represented the most affected sites in terms of inflammation, that is, PPD and BOP, and attachment loss.

As shown in Table 4, no significant correlations were observed between the viral load and the periodontal condition. So, the viral load of the subgingival biofilm seemed to be independent of the periodontal condition, which is particularly important for a discussion regarding periodontal disease and COVID-19 outcomes. Marouf et al.³¹ reported an association of radiographic periodontal bone loss and COVID outcomes. In our study we did not find a correlation between clinical inflammatory parameters, PPD and BOP, and the presence of the viral RNA. These observations together with the results reported by Larvin et al.³² that obese COVID patients who experienced gingival pain and bleeding gums or teeth with mobility had an increased risk of death (CI 95% 1.91-5.06) grants further investigations on the potential of dental biofilm plaque sampling to be used as detection tool for SARS-CoV-2.

Considering that 18.9% of patients tested positive in supragingival biofilm at the beginning of the onset of COVID-19 symptoms, as shown previously,²⁵ and that 46.1% tested positive in the present study during the ICU stay, it seems that the presence of the viral RNA in dental biofilms may increase from the peak of the symptoms, which generally occurs approximately 5 days after the initial exposure to the virus.⁹ Moreover, a recognized difficulty regarding SARS-CoV-2 is understanding the potential of the virus to survive in different types of human samples.³³ Therefore, the next step was to explore whether there was signaling that could provide information regarding the amount of time that the viral RNA remains in dental biofilms. The number of days that elapsed between the ICU intake, the onset of COVID-19 symptoms and the last RT-qPCR were always greater for the Biofilm⁻ participants. These results suggest that as time passes, the viral RNA does not remain in the dental biofilm. However, because of the similar medical condition as well of periodontal conditions among the study population, it remains unclear why certain subjects tested positive, and others tested negative within the same timeframes. Besides, the similarity observed for the Naso/Oro Cqs, as shown in Table 4, makes difficult to draw conclusions about the prevalence of biofilm positive and negative individuals.

An additional observation regarding the Naso/Oro Cq values (a mean of 23.9 ± 5.3 , Table 4) from participants inside the Naso/Oro^{Gp} showed a lower viral RNA load than what has previously been reported (15.99 ± 6.9).²⁵ This suggests that, more than the systemic viral load itself, the permanence of the systemic viral RNA possibly increases the probability of detecting the viral RNA in dental biofilms. The biofilm sampling conducted by Gomes et al.²⁵ occurred during the onset of the initial COVID-19 symptoms. In the present study, the amount of time that elapsed since the onset of symptoms was 27.1 ± 13.9 days. Moreover, as time elapsed, a greater number of negative participants was observed. However, this information remains incomplete. It would be of utmost importance to reassess the dental biofilms, in special the subgingival ones, from those Biofilm⁺ participants who survived COVID-19.

The results shown here do not allow inferring whether the viral RNA in the biofilm is a consequence of contamination by crevicular fluid nor about the possibility of participating in an eventual recontamination of the periodontal tissues.^{18,19,22,23} In this context, it is important to emphasize that the presence of the viral RNA in supra- and subgingival biofilms does not imply that the virus is vital. This information would be important to trace dental care strategies to COVID-19 patients. Besides, other

molecular analysis could help understand how the virus relates with the dental biofilm or whether the presence of SARS-CoV-2 implies to a dental biofilm dysbiosis. Even though samples are available, and this analysis has been foreseen in the study protocol, this research could not be performed because of limited laboratory facilities. Other limitations of the present investigation are related to the small sample size, that may respond to some lack of precision in the results, and the limited access to the Naso/Oro Cqs. Regardless of these limitations, we believe that the present results add important information for the necessary research trajectory aiming to better understand periodontal condition and COVID-19.

5 | CONCLUSION

The presence of SARS-CoV-2 RNA in dental biofilms most likely reflects the extent to which the virus can spread throughout the body. Thus, it is crucial to analyze dental biofilms, whether of supra- or subgingival origin, and irrespective of the periodontal condition, as potential SARS-CoV-2 habitats. Furthermore, the high number of positive patients observed in the present study highlights the need to better understand this habitat to facilitate the provision of adequate oral care. Moreover, the mechanical disruption of dental biofilms in patients from the COVID-19 ICU should be strongly considered.

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We would like to dedicate this work to all the participants and their legal representatives. May all efforts help to mitigate the effects of this terrible pandemic and provide guidance for future treatments.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in connection with this article.

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DATA AVAILABILITY STATEMENT

The data supporting the present findings are available from the corresponding author upon reasonable request.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions to conception and design of the study. JGF, LMM, and LM

were responsible for clinical data collection. PDMA was involved with the data collection from the hospital records. MLL and ISB were responsible for the laboratory steps. LNN was responsible for the statistical analysis, data interpretation, and drafting the manuscript. SCG was involved with the study conception, data collection, and interpretation, and drafting the manuscript. All authors revised the manuscript critically and have given final approval of the version to be published.

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REFERENCES

1. Bendavid E, Oh C, Bhattacharya J, Ioannidis JPA. Assessing mandatory stay-at-home and business closure effects on the spread of COVID-19. *Eur J Clin Invest.* 2021;51:e13484. <https://doi.org/10.1111/eci.13484>.
2. Li Y, Liang M, Gao L, et al. Face masks to prevent transmission of COVID-19: a systematic review and meta-analysis. *Am J Infect Control.* 2021;49:900-906. <https://doi.org/10.1016/j.ajic.2020.12.007>.
3. Tabatabaeizadeh SA. Airborne transmission of COVID-19 and the role of face mask to prevent it: a systematic review and meta-analysis. *Eur J Med Res.* 2021;26:1. <https://doi.org/10.1186/s40001-020-00475-6>.
4. Ma QX, Shan H, Zhang HL, Li GM, Yang RM, Chen JM. Potential utilities of mask-wearing and instant hand hygiene for fighting SARS-CoV-2. *J Med Virol.* 2020;92:1567-1571. <https://doi.org/10.1002/jmv.25805>.
5. Kahler CJ, Hain R. Fundamental protective mechanisms of face masks against droplet infections. *J Aerosol Sci.* 2020;148:105617. <https://doi.org/10.1016/j.jaerosci.2020.105617>.
6. Raman R, Patel KJ, Ranjan K. COVID-19: unmasking emerging SARS-CoV-2 variants, vaccines and therapeutic strategies. *Biomolecules.* 2021;11. <https://doi.org/10.3390/biom11070993>.
7. Zhou W, Wang W. Fast-spreading SARS-CoV-2 variants: challenges to and new design strategies of COVID-19 vaccines. *Signal Transduct Target Ther.* 2021;6:226. <https://doi.org/10.1038/s41392-021-00644-x>.
8. World Health Organization (WHO). Update on Omicron. 2021. Available at:
9. Wölfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature.* 2020;581:465-469. <https://doi.org/10.1038/s41586-020-2196-x>.
10. Bao L, Zhang C, Dong J, Zhao L, Li Y, Sun J. Oral microbiome and SARS-CoV-2: beware of lung co-infection. *Front Microbiol.* 2020;11:1840. <https://doi.org/10.3389/fmicb.2020.01840>.
11. Karia R, Gupta I, Khandait H, Yadav A, Yadav A. COVID-19 and its modes of transmission. *SN Compr Clin Med.* 2020; 1-4. <https://doi.org/10.1007/s42399-020-00498-4>.
12. Harrison AG, Lin T, Wang P. Mechanisms of SARS-CoV-2 transmission and pathogenesis. *Trends Immunol.* 2020;41:1100-1115. <https://doi.org/10.1016/j.it.2020.10.004>.



13. Yoon JG, Yoon J, Song JY, et al. Clinical significance of a high SARS-CoV-2 viral load in the saliva. *J Korean Med Sci.* 2020;35:e195. <https://doi.org/10.3346/jkms.2020.35.e195>.
14. Chen L, Zhao J, Peng J, et al. Detection of SARS-CoV-2 in saliva and characterization of oral symptoms in COVID-19 patients. *Cell Prolif.* 2020;53:e12923. <https://doi.org/10.1111/cpr.12923>.
15. Pasomsub E, Watcharananan SP, Boonyawat K, et al. Saliva sample as a non-invasive specimen for the diagnosis of coronavirus disease 2019: a cross-sectional study. *Clin Microbiol Infect.* 2021;27:285 e281-285 e284. <https://doi.org/10.1016/j.cmi.2020.05.001>.
16. Shamsoddin E. Saliva: a diagnostic option and a transmission route for 2019-nCoV. *Evid Based Dent.* 2020;21:68-70. <https://doi.org/10.1038/s41432-020-0104-8>.
17. To KK, Tsang OT, Yip CC, et al. Consistent detection of 2019 novel coronavirus in saliva. *Clin Infect Dis.* 2020;71:841-843. <https://doi.org/10.1093/cid/ciaa149>.
18. Huang N, Perez P, Kato T, et al. Integrated single-cell atlases reveal an oral SARS-CoV-2 infection and transmission axis. *medRxiv.* 2020. <https://doi.org/10.1101/2020.10.26.20219089>. medRxiv.
19. Gupta S, Mohindra R, Chauhan PK, et al. SARS-CoV-2 detection in gingival crevicular fluid. *J Dent Res.* 2021;100:187-193. <https://doi.org/10.1177/0022034520970536>.
20. Sakaguchi W, Kubota N, Shimizu T, et al. Existence of SARS-CoV-2 entry molecules in the oral cavity. *Int J Mol Sci.* 2020;21. <https://doi.org/10.3390/ijms21176000>.
21. Berger I, Schaffitzel C. The SARS-CoV-2 spike protein: balancing stability and infectivity. *Cell Res.* 2020;30:1059-1060. <https://doi.org/10.1038/s41422-020-00430-4>.
22. Örd M, Faustova I, Loog M. The sequence at Spike S1/S2 site enables cleavage by furin and phospho-regulation in SARS-CoV2 but not in SARS-CoV1 or MERS-CoV. *Sci Rep.* 2020;10:16944. <https://doi.org/10.1038/s41598-020-74101-0>.
23. Lloyd-Jones G, Molyem S, Pontes CC, Chapple I. The COVID-19 pathway: a proposed oral-vascular-pulmonary route of SARS-CoV-2 infection and the importance of oral healthcare measures. *J Oral Med and Dent Res.* 2021;2:1-25. Available at: <https://www.genesispub.org/j-oral-med-and-dent-res/the-covid-19-pathway-a-proposed-oral-vascular-pulmonary-route-of-sars-cov-2-infection-and-the-importance-of-oral-healthcare-measures>.
24. Fernandes Matuck B, Dolhnikoff M, Maia GVA, et al. Periodontal tissues are targets for Sars-Cov-2: a post-mortem study. *J Oral Microbiol.* 2020;13:1848135. <https://doi.org/10.1080/20002297.2020.1848135>.
25. Gomes SC, Fachin S, da Fonseca JG, et al. Dental biofilm of symptomatic COVID-19 patients harbours SARS-CoV-2. *J Clin Periodontol.* 2021;48:880-885. <https://doi.org/10.1111/jcpe.13471>.
26. Cohen JF, Korevaar DA, Altman DG, et al. STARD 2015 guidelines for reporting diagnostic accuracy studies: explanation and elaboration. *BMJ Open.* 2016;6:e012799. <https://doi.org/10.1136/bmjopen-2016-012799>.
27. Ainamo J, Bay I. Problems and proposals for recording gingivitis and plaque. *Int Dent J.* 1975;25:229-235.
28. Corman VM, Landt O, Kaiser M, et al. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill.* 2020;25. <https://doi.org/10.2807/1560-7917.ES.2020.25.3.2000045>.
29. Centers for Disease Control and Prevention (CDC). Division of Viral Diseases. CDC 2019-Novel Coronavirus (2019-nCoV) real-time RT-PCR diagnostic panel. Available at: <https://www.fda.gov/media/134922/download>.
30. 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 Clin Periodontol.* 2018;45 Suppl 20:S162-S170. <https://doi.org/10.1111/jcpe.12946>.
31. Marouf N, Cai W, Said KN, et al. Association between periodontitis and severity of COVID-19 infection: a case-control study. *J Clin Periodontol.* 2021;48:483-491. <https://doi.org/10.1111/jcpe.13435>.
32. Larvin H, Wilmott S, Kang J, Aggarwal VR, Pavitt S, Wu J. Additive effect of periodontal disease and obesity on COVID-19 outcomes. *J Dent Res.* 2021;100:1228-1235. <https://doi.org/10.1177/00220345211029638>.
33. Walsh KA, Jordan K, Clyne B, et al. SARS-CoV-2 detection, viral load and infectivity over the course of an infection. *J Infect.* 2020;81:357-371. <https://doi.org/10.1016/j.jinf.2020.06.067>.

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