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Salivary biomarkers in COVID-19 patients: the rabbit out of the hat!

Short title: Salivary biomarkers and COVID-19

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Abstract
Background: The ongoing outbreak of Coronavirus Disease 2019 (COVID-19) represents a major threat to human health, which impairs the functionality of several organs. One of the hardest challenges in the fight against COVID-19 is the development of wide-scale, effective, and rapid laboratory tests to control disease severity, progression, and possible sudden worsening. Monitoring patients in real-time is indeed highly demanded in this pandemic era when physicians need reliable and quantitative tools to prioritize patients' access to intensive care departments. In this regard, salivary biomarkers are extremely promising, as they allow for a fast and non-invasive specimens' collection, which can be repeated multiple times.
Methods: We compare salivary levels of immunoglobulin A subclasses (IgA1 and IgA2) and free-light chains (FLC k and λ) in a cohort of 29 SARS-CoV-2 patients and 21 healthy subjects.
Results: We found that each biomarkers differs significantly between the two groups, with *p*-values ranging from 10^{-8} to 10^{-4}. The performance ranking of these markers, shows that λFLC level (p=1.4e-8) is the best-suited candidate to discriminate the two groups, with an accuracy of 0.94 (0.87-1.00 95% CI), a precision of 0.91 (0.81-1.00 95% CI), a sensitivity of 1.00 (0.96-1.00 95% CI) and a specificity of 0.86 (0.70-1.00 95% CI).
Conclusion: These results suggest λFLC as an ideal indicator of patient conditions. This is more strengthened in consideration that λFLC half-life (approximately 6 hours) is significantly shorter than the IgA one (21 days): thus λFLC appears displaying the potential to effectively monitor patients fluctuation in real-time.

Key words: COVID-19, IgA, FLC, salivary biomarkers

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INTRODUCTION

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in late 2019 in the city of Wuhan, China, caused an outbreak of unusual viral pneumonia, named 'coronavirus disease 2019' (COVID-19), which threatens human health and public safety [1]. Being highly transmissible, this novel coronavirus disease dramatically spreads all over the world; due to the wide range of organ impairments paralleled by abnormalities in biochemical markers of inflammation, cardiac function, muscle integrity, coagulation parameters, kidney and liver function recorded in affected patients, COVID-19 could be considered a systemic pathology [2,3].

In these challenging times, worldwide there is the urgent need to perform broad-scale population-based testing to ameliorate COVID-19 prevention, diagnosis, and control of outcomes, understand and contain transmission dynamics and monitoring herd immunity. As saliva represents a suitable biological material to detect SARS-CoV-2 RNA as well as antibodies anti SARS-CoV-2 antibodies, this sample type could provide an important opportunity to monitor individual and population level SARS-CoV-2 transmission, infection, and seropositivity [2]. Salivary biomarkers are emerging as a clinically useful tool for the easy and non-invasive modality to collect specimens, not requiring a specialist training; in particular, saliva may be preferable to blood samples in those clinical study designs involving repeated sampling. Salivary immunoglobulin A (IgA) represents an important immune biomarker secreted by healthy people and patients in course of different diseases, even in response to acute mental stress and exercise. In the oral cavity it represents the key player among mucosal defense proteins with a central role in the first line of defense against the adhesion of pathogens and their penetration into the tissues [3,4]. The majority of IgA-secreting plasma cells are located within mucosal membranes lining the intestine; moreover, the occurrence of autoantibodies of the IgA class [5] in several autoimmune diseases suggests that IgAs play a wider role in the regulation of immune responses.

IgA class consists of 2 subclasses, IgA1 and IgA2, which are not equally distributed within the humoral and mucosal immune systems. IgA1 is the major (approximately 80%) subclass in serum while IgA2 is the major component in secretions [6].

The majority of IgA is secreted as a dimer into mucosal tissues, where regulates immune homeostasis, while the monomeric IgA in serum can mediate pro-inflammatory responses, through the release of cytokines and chemokines, and the activation of phagocytosis and degranulation; furthermore, IgA subclasses IgA1 and IgA2 act differently on neutrophils and macrophages. IgA2 promotes inflammatory reactions through neutrophil extracellular traps formation and inflammatory cytokines expression [7].

Recently, subclass-specific antisera have been applied in a turbidimetric assay to establish age-dependent reference values for serum concentrations of the two IgA subclasses in children and adults [6]. The biological variability of IgA makes it a challenging marker for the right significance, therefore it has been suggested that IgA should be integrated in a panel of biomarkers incorporated into diagnostic, monitoring and follow-up algorithms of immunological diseases [8,9].

Serological free light chains (FLC) of immunoglobulins appears as an interesting new candidate for inflammatory diseases. During the process of immunoglobulin synthesis, plasma cells produce light chains in excess of intact immunoglobulins that may be released into the circulation; FLCs should be considered bioactive molecules rather than a secondary product of the synthesis of Igs without any functional relevance. FLCs' role in course of autoimmune diseases has been deeply investigated [10–13].

The aim of the present study is to evaluate the diagnostic performance of saliva specimens measuring IgA and FLC levels to observe the trend of these markers in the course of the disease and to study the activation and involvement of oral immunity in COVID-19 patients. Thanks to the easy

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accessibility of saliva, these immunological markers can represent a useful and convenient tool for assessing the activity of the disease and monitoring it over time.

MATERIALS AND METHODS

Patients and controls

We enrolled 29 COVID-19 patients (17 males and 12 females) for this monocentric prospective study among patients admitted to COVID-19 Unit at Fondazione Policlinico universitario "A.Gemelli", IRCCS, Rome; 21 healthy donators (HD) (10 Male and 11 Female) served as control group. Salivary specimens were collected from May 2020 to July 2020. The whole patient population consisted in hospitalized COVID-19 adult patients with a positive molecular assay test result for SARS-CoV-2 and characteristic symptoms of COVID-19: fever (temperature greater than> 37.5 °C), dry cough and pharyngodynia. Patients under the age of 18 were not included.

HD have been enrolled among the health personnel of our Institution, with a nasopharyngeal swab negative for the presence of SARS-CoV-2 virus performed no more than 24 hours before serum and saliva collection, and negative for anti-SARS-CoV-2 antibodies in the serum.

The clinical and demographic characteristics of the enrolled patients are reported in Table 1.

Patients displayed comorbidities: diabetes (n = 3), hypertension (n = 6), obesity (n = 4), oncology history (n = 2), heart disease (n = 4). Symptoms at onset: fever (n = 22), cough (n = 7), dyspnoea (n = 7), asthenia (n = 10). Several complications were experienced: 21/29 patients had bilateral pneumonia, 11 patients were treated with oxygen therapy, 3 were in intensive care, 6/29 patients were smokers. Salivary immune response biomarkers - including FLCs and IgA and IgA subclasses - were measured in both groups and summarized in Table 2.

Table 1. Clinical and demographic characteristics of the enrolled patients

	Patients N=29
Age (Mean±SD)	50.4±11.5
M/F (N)	17/12
Smoke (Y/N)	6/23
Diabetes (%)	10.7
Hypertension (%)	21.4
Obesity (%)	14.3
Cardiopathy (%)	14.3
Oncology History (%)	7.1
Fever (%)	78.6
Cough (%)	25
Dyspnea (%)	25
Asthenia (%)	26
Pneumonia (%)	75
Oxygen Teraphy (%)	39.3
Intensive care (%)	10.7

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Table 2. Salivary and blood biomarkers in COVID-19 patients and healthy control (CTRL).

	CTRL			Patients			
Analyte	**n**	**Median**	**IQR**	**n**	**Median**	**IQR**	***p*-value**
k (mg/L)	21	0.58	0.56	29	2.12	3.03	1.80E-04
λ (mg/L)	21	0.65	0.09	29	0.70	1.77	1.40E-08
k/λ	21	0.91	0.75	29	1.35	2.15	1.30E-02
k+λ (mg/L)	21	1.22	0.59	29	3.43	5.00	6.60E-05
IgA_1 (mg/L)	21	36.30	9.70	29	133.10	237.10	1.30E-05
IgA_2 (mg/L)	21	29.70	33.60	29	118.80	227.20	2.00E-05
Ferritin (ng/L)	–	–	–	27	432.00	286.00	–
IL-6 (ng/L)	–	–	–	22	11.45	10.22	–
PCR (mg/L)	–	–	–	25	28.60	42.50	–
D-Dimer (ng/mL)	–	–	–	28	437.50	393.75	–

Laboratory testing

In all subjects, one sample of serum and one sample of saliva were collected. The study was cross-sectional and none of the subjects contributed more than one saliva-serum paired sample.

Saliva was collected using the Sarstedt-Salivette® (Sarstedt AG & Co. KG Nümbrecht, Germany), and the samples were centrifuged to remove non-soluble material and processed according to manufacturer's instructions, finally stored at −80°C until analyzed.

Saliva samples were tested for salivary IgA, IgA subclasses and free k and λ chains. IgA subclasses were assessed by Optilite analyzer (Optlite IgA CSF kit, Optilite IgA1 and IgA2 subclasses kit, The Binding Site, UK; salivary IgA normal range: 8.7-20 mg/dl). Total IgA levels were also measured in serum samples in 20 out of 29 patients (serum normal range for IgA: 0.845-4.990 g/L; for IgA1: 760.81-3282.03 mg/L; for IgA2: 68.9-1142.5 mg/L).

FLCs were measured by means of the Optilite analyzer (Freelite™ Human Kappa and Lambda Free Kits, The Binding Site, UK; serum normal range: 3.3-19.4 mg/L for free k and 5.7-26.3 mg/L for free λ). A ratio of k/λ < 0.26 or >1.65 was considered abnormal.

Samples were thawed only once and immediately assayed in a single batch, following the manufacturer's instructions. Each sample was tested twice to minimize eventual discrepancies, and all tests were performed in the same laboratory with the same instruments.

Ethical Consideration

This study complied with the Ethical Principles for Medical Research Involving Human Subjects according to the World Medical Association Declaration of Helsinki and was certified by the Committee of the Applicable Institution of the Fondazione Policlinico Universitario Agostino Gemelli IRCCS (FPG), Rome.

The ethic committee of institution (FPG) approved the study (ID: 3222). All patients gave written informed consent to the use of their clinical and serological data in this study. The whole study was conducted according to the Declaration of Helsinki, as revised in 2013.

Statistical Analysis

Statistical analyses were performed using the software package R (4.0.2 release) [14]. A total of 50 subjects were recruited for the study (44% females), 29 diagnosed with COVID-19 (mean age 49.5, IQR 12 years) and 21 healthy control subjects (mean age 45.0, IQR 17 years).

Laboratory parameters were tested for normality by means of a visual inspection of the QQ-Plot, followed by a Shapiro-Wilk test, showing significant deviations from normality. Comparisons

between groups were performed with the Wilcoxon Unpaired Two-Sample test. The diagnostic performance of the investigated salivary markers in distinguishing the two groups was assessed by logistic regression followed by ROC curve analysis. Logistic regression was performed using the glm function from the R package stats to extract probabilities from the fitted models, either with a single biomarker or with several biomarkers used in combination. ROC curves and AUC values were calculated as previously described[15–17]and using the R package pROC. Stepwise backward logistic regression was used to select the best subset of biomarkers according to the Aikake's Information Criterium (AIC)[18]. The optimal cut-off is calculated by a maximization of the Youden's statistics J = sensitivity+specificity –1. The classifiers were evaluated with a bootstrap procedure carried on the logistic regression, which allowed us to find out the distribution of performance indicators such as accuracy, precision, sensitivity, and specificity. Correlations between variables were evaluated with the Spearman's correlation coefficients. Strength of correlation was evaluated considering coefficients >0.70 as strong correlation, 0.30 - 0.70 as moderate and <0.3 as weak correlation. Correlation heat maps were calculated with the package corrplot implemented in the software R.

RESULTS

In Figure 1A-F, we showed a box plot analysis of the selected salivary markers measuring subjects' immune response together with the result of a Wilcoxon Unpaired Two-Sample test. Data are represented using a logarithmic scale. A statistically significant increase in salivary levels in COVID-19 patients compared to healthy controls is detected for all the investigated biomarkers, namely FLC-k (p=0.00018, fig 1A), FLC-λ (p=1.4e-8, fig 1B), k+λ (p=6.6e-5, fig 1C), k/ λ (p=6.6e-5, fig 1D), IgA1 (p=1.3e-5, fig 1E) and IgA2 (p=2e-5, fig 1F). The number of subjects included in the analysis, median values, and interquartile ranges (IQR) are summarized in Table 2.

ROC curves were exploited to assess the performance of salivary biomarkers in distinguishing the two groups. These curves are well known statistical figures in which sensitivity, also referred to as true positive (TP) rate, is plotted against 1−specificity, which is the false positive (FP) rate. As specificity ranges between 0 and 1, ROC curves might be also reported in terms of sensitivity as a function of specificity, inverting the x-axis. In the present paper, the latter notation is used (Figure 2A). The diagonal line represents the y = x bisector, which indicates a completely random classifier. ROC curves of the selected salivary biomarkers, especially the FLC-λ curve, increase substantially for low values of the x-axis, showing their potential effectiveness in distinguishing the two groups. As expected from Figure 2A, large and significant AUC values were measured for each marker (Table 3). To improve the effectiveness of the model shown in Figure 2B, we performed a stepwise logistic regression including all the measured markers. To this purpose, firstly the complete model was obtained and then a stepwise backward selection was performed to highlight the most relevant subset of parameters k that minimizes the Akaike's Information Criterion (AIC)=2k-2 ln (L), where L is the maximum value of the likelihood function for the model.

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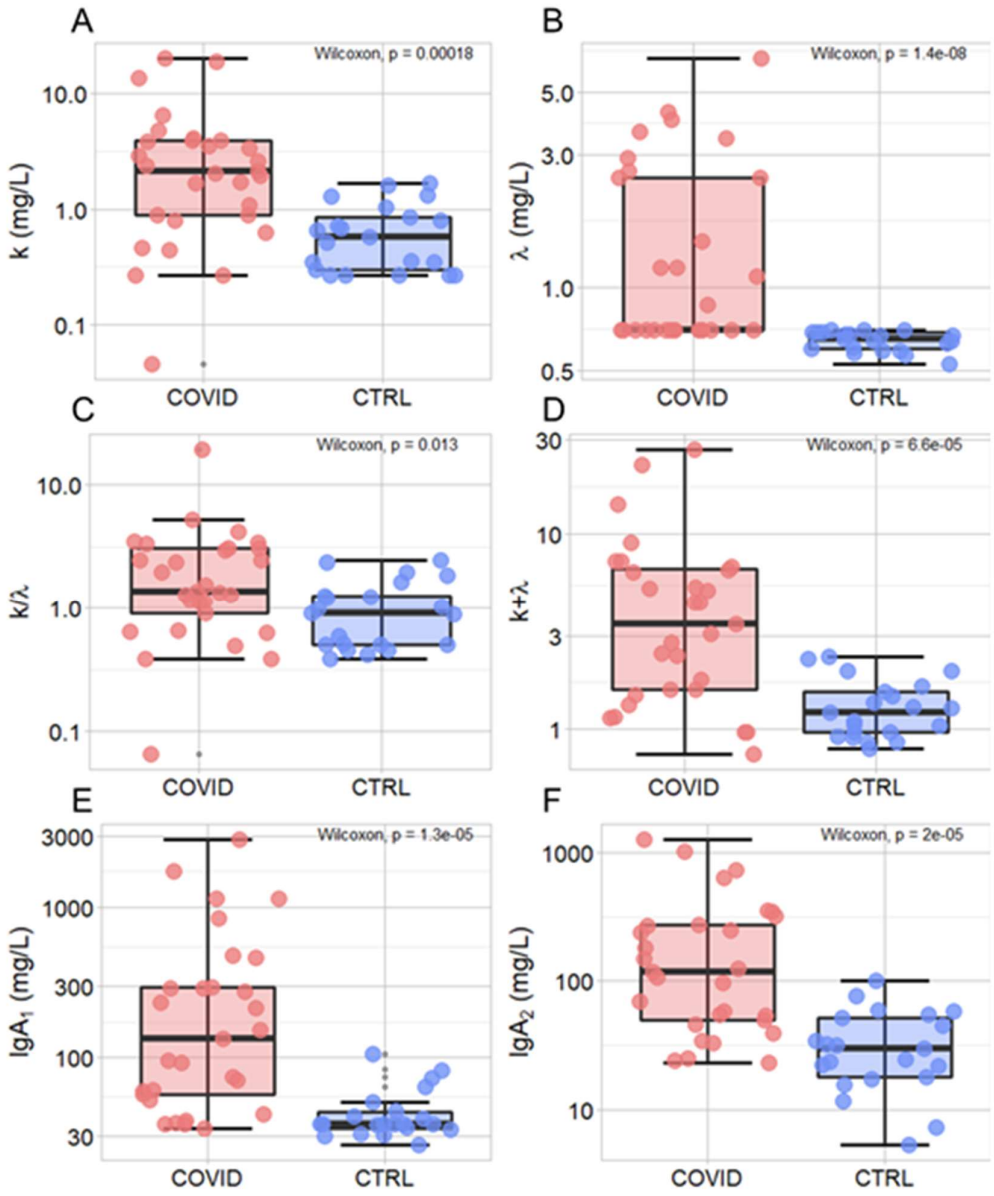

Figure 1. Box plot analysis of the salivary markers measured in COVID-19 patients (pink) and control subjects (blue). Comparison between the two groups is performed using the Wilcoxon Unpaired Two-Sample test.

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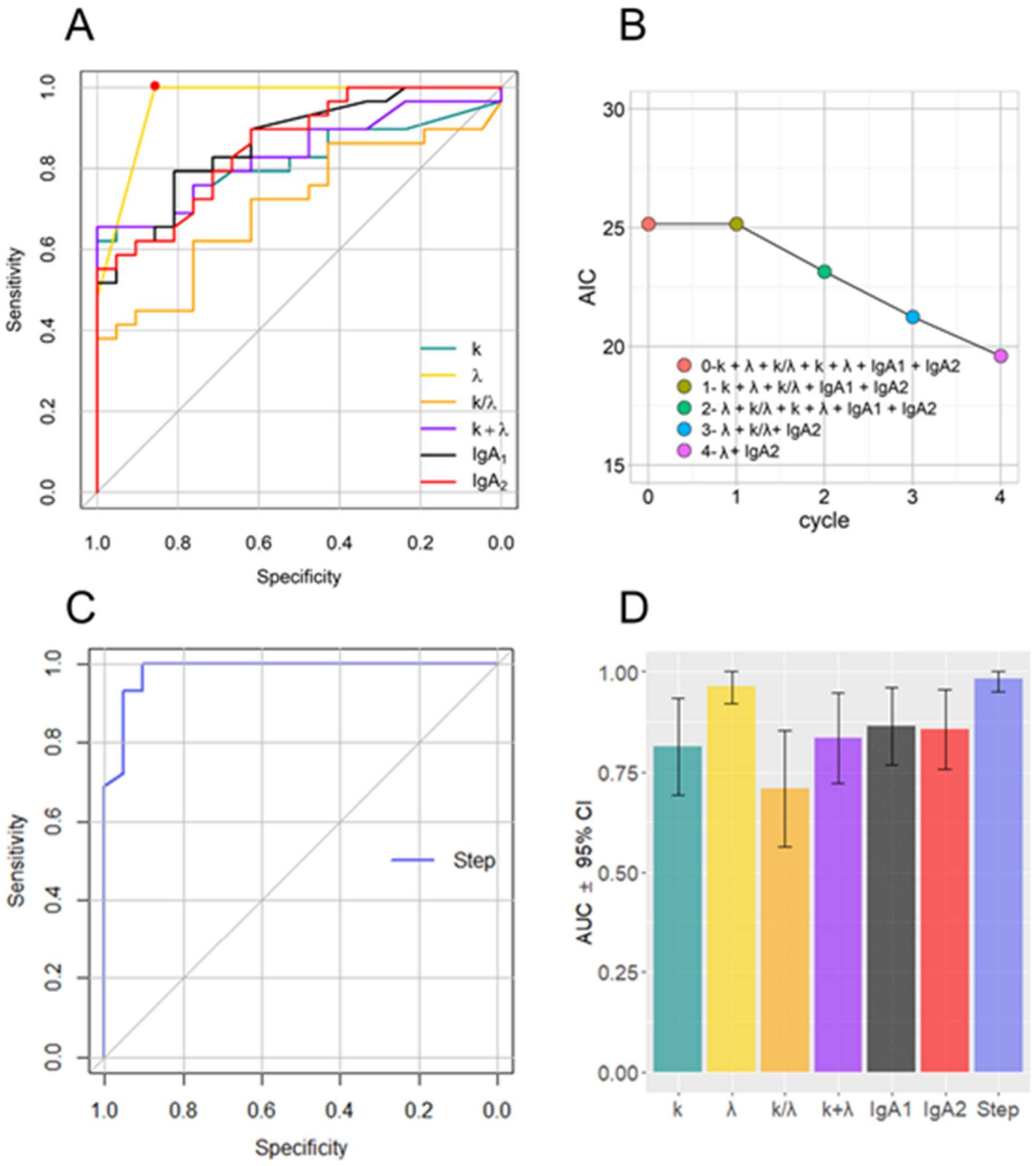

Figure 2. ROC curves for the selected salivary biomarkers (A); Evolution of the Aikake Information Criterion during a stepwise logistic regression performed on all the measured parameters (B); ROC curve calculated using the selected variables (C); AUC values and 95% confidence intervals for all the computed ROC curves.

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Table 3. AUC values (95% CI) computed for each ROC curve reported in Figure 2.

Parameter	AUC (95%CI)
k	0.81 (0.69-0.93)
λ	0.96 (0.92-1.00)
k/λ	0.70 (0.56-0.85)
k+λ	0.83 (0.72-0.94)
IgA_1	0.86 (0.76-0.96)
IgA_2	0.85 (0.75-0.95)
Step	0.98 (0.95-1.00)

The computed AIC value for each cycle of the procedure is reported in Figure 2B, showing the progressive removal of less informative biomarkers. The minimization procedure selects λ and IgA2, while discards the other parameters. In Figure 2C, we show the computed ROC curve for the step model, based on the two selected variables. All the computed AUC values, together with the corresponding 95% CIs, are compared in Figure 2D. An analysis of this figure suggests that the combined use of λ and IgA2 does not provide significant improvement with respect to λ alone. A more in-depth comparison of the two models was performed by means of a bootstrap procedure aimed at estimating the statistical distribution of selected performance parameters, including accuracy, precision, sensitivity, and specificity. To this purpose, we performed 5000 bootstrap samples of the investigated datasets, and we used them to compute the logistic regression parameters with the corresponding confusion matrix. Ultimately, the ensemble of generated confusion matrixes was used to derive the corresponding distribution of the desired statistics. In Figure 3 we show the computed distribution of accuracy (3A, E), precision (3B, F), sensitivity (3C, G) and specificity (3D, H) for λ (upper panels) and λ +IgA2 (lower panels). Despite both models show very good classification abilities, the computed distribution appears to be very similar. Therefore, to keep the number of parameters in the model as low as possible, we conclude that the use of λ alone is preferable over the step model in Figure 2C. For this model, the following parameters (95% CIs) can be derived, accuracy = 0.94 (0.87-1.00), precision = 0.91(0.81-1.00), sensitivity = 1.00 (0.96-1.00), and specificity = 0.86 (0.70-1.00). Mean parameters and confidence intervals were derived fitting the retrieved distribution with a Gaussian curve (Figure S1).

To investigate more in-depth the relationship existing between immune response salivary markers and inflammatory blood markers in the pathological subjects, we performed a correlational analysis between the two groups of variables. To this purpose, the Spearman's correlation coefficients were computed and visualized as a correlation map (Figure 4).

This map does not display the typical symmetric form, as we decided to plot all the calculated correlations below the main diagonal and only the significant ones above the main diagonal (α=0.05). Significant Spearman's correlations are summarized in Table 4 together with the corresponding *p*-values. As we show (Table 4 and Figure 4) correlation among salivary markers and between selected salivary and blood markers are strong and positive, thus hinting at a global and

coordinated response of the immune systems and inflammatory process during the COVID-19 infections. Notably, ferritin levels appear to be negatively correlated with IgA2 levels, a result that deserves a better in-depth study.

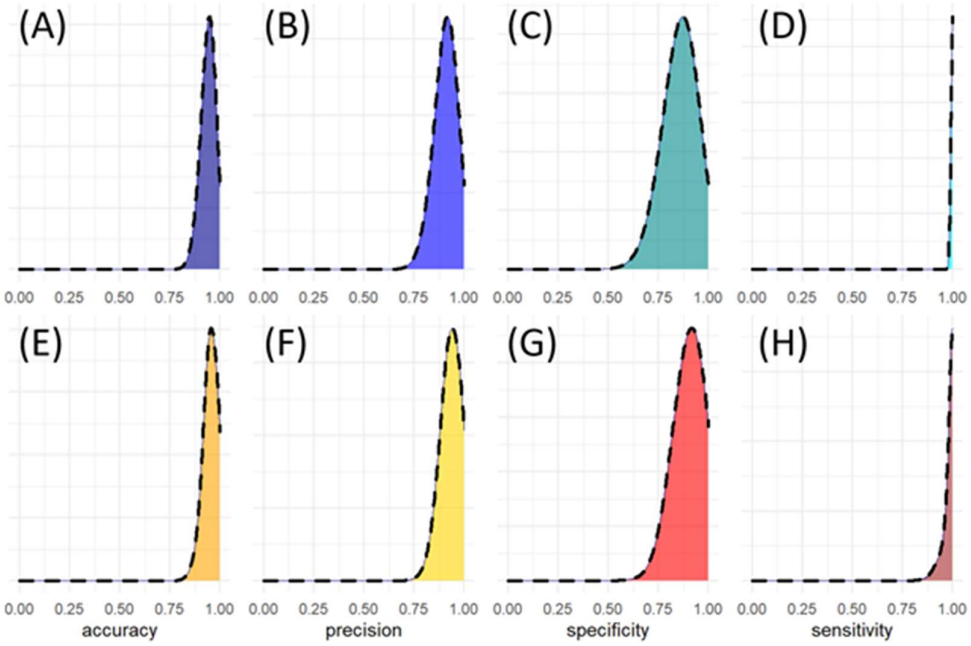

Figure 3. Bootstrap distribution of accuracy (3A,E), precision (3B,F), sensitivity (3C,G) and specificity (3D,H) for λ (upper panels) and λ +IgA2 (lower panels).

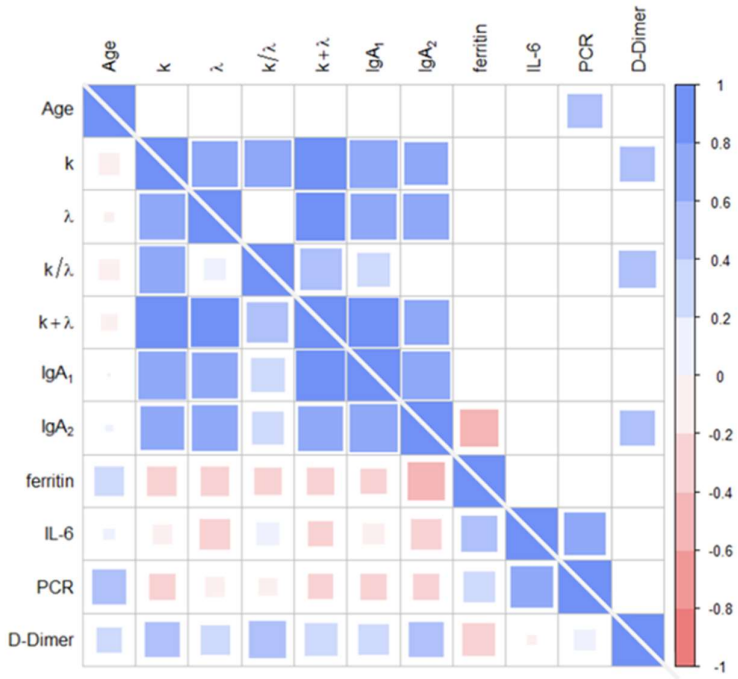

Figure 4. Heat map of Spearman's correlation coefficients between salivary and blood biomarkers.

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Table 4. Statistically significant Spearman's correlation coefficients between salivary and blood biomarkers

Groups	Coefficient	*p*
k vs λ	7.16E-01	1.25E-05
k vs IgA_1	7.79E-01	6.31E-07
k vs IgA_2	6.33E-01	2.22E-04
k vs D-Dimer	4.03E-01	3.34E-02
λ vs IgA_1	7.07E-01	1.75E-05
λ vs IgA_2	7.17E-01	1.17E-05
IgA_2 vs Ferritin	-4.76E-01	1.20E-02
IgA_2 vs D-Dimer	4.20E-01	2.60E-02
IL6-PCR	6.01E-01	5.02E-03

Ultimately, the potential effect of comorbidities, symptoms at onset and complications on the salivary and plasmatic biomarkers levels is displayed in Table S1 for patient group, showing the absence of statistically significant difference among patients for almost any considered factors with the following exceptions: patients diagnosed with diabetes or hypertension showed a significantly decreased k/λ ratio; patients with oncology history or undergoing oxygen therapy showed a significantly increased k/λ ratio.

DISCUSSION

We hypothesize that oral mucosal surfaces are possible sites of initial triggering events for COVID-19, as first line of defense against pathogens. IgA antibodies play an essential component of the adaptive immune system to fight pathogens at lining mucosal surfaces, capable of both pro- and anti-inflammatory effects [19,20] and the two subclasses, IgA1 and IgA2, display different levels in serum and mucosal fluids [7]. Actually, there is a large and increasing interest in researching salivary markers for SARS-Cov-2 diagnosis and monitoring. In this context, patients' IgA levels in saliva were recently evaluated in two clinical papers [21,22] To the best of our knowledge, this is the first study to date aimed to evaluate salivary IgA1 and IgA2 subclasses, as well as salivary FLCs levels in COVID-19 patients in comparison to heathy subjects, not yet vaccinated

As known, these molecules are important key player of the first line of non-specific immune defenses acting in lining membranes, where viral infection starts and triggers, therefore could represent alarm sentinel of increased morbidity and mortality. Notably, we found a statistically significant increase of all these biomarkers in the COVID-19 group when compared to HS.

Additionally, we also analysed the overall amount of FLCs (k+λ) and the FLC-k/FLC-λ ratio (k/λ), which were also significantly different between the two groups. We measured a very significant increase associated to remarkably low *p*-values that range from 10^{-8} to 10^{-4}, except for k/λ (p=0.013) even still significant. The strength of this increase is even clearer considering that data in Figure 1 are represented in a logarithmic scale, due to the large data range, and that there is no or little superposition between interquartile ranges. These findings suggested us to investigate the possible introduction of the selected biomarkers in diagnostic management. Major limitation however is that these markers are not COVID-19 specific and, therefore, their application is meaningful only for monitoring purposes. Our analysis (Figure 2D) suggests that λFLC level is the best suited candidate to distinguish between the two population with an accuracy of 0.94 (0.87-1.00 95% CI), a precision of 0.91(0.81-1.00 95% CI), a sensitivity of 1.00 (0.96-1.00 95% CI), and specificity of 0.86 (0.70-1.00

95% CI). Additionally, we have some evidence that a combined use of λFLC and IgA2 might improve classification performances, but we have not enough information to prove it statistically (Figure 3). A further study conducted on a larger sample size would help, on the one hand, to confirm our results on λFLC, and, on the other hand, to decide whether a combined model would be more informative over the use of a single parameter.

This highly correlated behaviour is not surprising as saliva FLC secretion has been shown to exhibit daily variation that reflects IgA fluctuation [4]. Despite the aforementioned complexity, a more in-depth evaluation of IgA2 role is highly interesting because of its role and bio-distribution. IgA1 and IgA2, show different concentrations in serum and mucosal fluids [7]. While IgA1 strongly dominates in serum with an IgA1/IgA2 ratio of 9/1, in other biological fluids - including saliva - higher levels of IgA2 are reported [19,20]. A larger p-value is detected for IgA2 compared to IgA1, a finding that deserves a more in-depth study. From our results, the possible use of salivary IgAs, in particular of IgA2 seems to be very attractive and reliable even because the correspondent serological level of total IgAs measured on 20/29 patients recruited in the pathological group were within the range of normality (mean 2.32 g/l and standard deviation 0.92, data none shown).

Aside from IgAs, the large difference in salivary FLCs between the two groups is particularly intriguing as the immunological role of this molecular class is still not fully understood and it is a matter of debate in literature.

Although salivary FLCs do not have antigen-binding specificity comparable to complete antibodies, they are thought to play a relevant role in the immune adaptive system[23]. In a previous paper, we indeed hypothesized that FLCs could behave similarly to auto-antibodies shaping or skewing antibody reactivity [10,11]. Their active role in modulating the immune response is also supported by the overall expression of these molecules, only 60% of which is incorporated into whole Igs and the remaining fraction is released into serum as well as in other biological fluids [24]. Moreover, it is also possible that antigen specificity is not strictly necessary in salivary FLCs as it happens in light chain-mediated hypersensitivity-like responses [25].

Interestingly, our Spearman's coefficient map in Figure 4 does not highlight a significant correlation between FLC levels and age. This result is intriguing as other studies report a significant age-related increase of FLC in saliva. For instance, Heaney et al. demonstrated that older adults show higher FLC salivary levels compared with young adults as a consequence of an intense physical exercise [26]. In our opinion the absence of any correlation with age in our dataset has two possible explanations: firstly, subjects included by Heaney et al. [26] Span a larger age range, which goes from 18 to 80 years; secondly, and more interesting, this result is likely to indicate that age-related effects are negligible in terms of immune activation in comparison with pathological states, particularly in COVID-19 patients. The strong correlation existing between k and λ levels is not surprising and it is likely to mimic FLCs behavior in serum, where both light chains increase at the same time in response to inflammations/infections. A different behavior is often observed in other pathologies, such as cancer, where one chain type increases in excess compared to the other one. In this case the k/λ ratio is a clinically reliable biomarker as it is not altered is nonspecific inflammatory processes. Consistently, we observe a modest and slightly significant difference in the k/λ ratio between the two groups ($p=0.013$), further confirming that the FLC increase is likely to occur because of a nonspecific B-cell activation in the inflammatory process induced by SARS-CoV-2.

Taken together, our results can positively impact on the development of effective protocols for monitoring SARS-CoV-2 patients in a timely manner. In this context, it is worth stressing that molecular testing of naso-pharyngeal swabs has some limitations: although needful for diagnosis it is time consuming, and it requires specialized personnel as well as expensive equipment and probably not so suitable for monitoring on time. Conversely, saliva is extremely easily accessible, which showed good performance with accuracy, precision, sensitivity and specificity, and can be

sampled multiple times with no discomfort for the patients, thus providing an effective alternative to monitor the disease and follow up the infected patients. FLC levels outperform IgA in terms of performances in our database. This is likely to be correlated to the half-life of FLCs (a few hours) which is significantly shorter than IgA (21 days) [27]. Therefore, FLCs are like to provide an effective and real-time indicator of patient fluctuations, a particularly relevant issue in a pandemic era, as not all the patients can access to intensive care departments simultaneously.
However, the study has some limitations: the number of patients studied was limited, the results of ongoing studies still not provide evidence of immune protection of salivary IgA, but saliva present potential utility for monitoring local adaptive immunity.
In the pandemic era, tests of dynamic immune response segregating patients from controls could be a rifle sniper for COVID 19 patient's management.

Statement of contribution: UB, CN, CC, GP: designed the study; AF, ET, TDC, GCP: enrolled patients and collected clinical data; CN, CC: collected patients' saliva and serum samples; RDS, GC: performed the analysis; CN, MP, CC, AF, TDC, ET: analyzed data; UB, GC, MP, GLR, GP, AU, MF: drafted the article and/or revised it critically; UB,GC and GP: approved the final version of the article to be published.
Statement of conflict of interest: The Authors declare that there is no conflict of interest.
Acknowledgments: The authors thank the Binding Site for providing technical support to the specimens' analysis.
Abbreviations: SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; COVID-19: coronavirus disease 2019; IgA: immunoglobulin A; FLC: free light chains; FPG: Fondazione Policlinico Universitario Agostino Gemelli IRCCS; TP: true positive; FP: false positive; AIC: Akaike's Information Criterion; IQR: interquartile ranges.

REFERENCES

[1] Hu, B., Guo, H., Zhou, P., and Shi, Z.-L. (2021) Characteristics of SARS-CoV-2 and COVID-19. *Nature Reviews Microbiology*. 19 (3), 141–154.
[2] Pisanic, N., Randad, P.R., Kruczynski, K., Manabe, Y.C., Thomas, D.L., Pekosz, A., et al. (2020) COVID-19 Serology at Population Scale: SARS-CoV-2-Specific Antibody Responses in Saliva. *Journal of Clinical Microbiology*. 59 (1), JCM.02204-20, e02204-20.
[3] Nurkka, A., Obiero, J., Käyhty, H., and Scott, J. a. G. (2003) Effects of sample collection and storage methods on antipneumococcal immunoglobulin A in saliva. *Clinical and Diagnostic Laboratory Immunology*. 10 (3), 357–361.
[4] Rapson, A., Collman, E., Faustini, S., Yonel, Z., Chapple, I.L., Drayson, M.T., et al. (2020) Free light chains as an emerging biomarker in saliva: Biological variability and comparisons with salivary IgA and steroid hormones. *Brain, Behavior, and Immunity*. 83 78–86.
[5] Hansen, I.S., Baeten, D.L.P., and den Dunnen, J. (2019) The inflammatory function of human IgA. *Cellular and Molecular Life Sciences: CMLS*. 76 (6), 1041–1055.
[6] Schauer, U., Stemberg, F., Rieger, C.H.L., Borte, M., Schubert, S., Riedel, F., et al. (2003) Establishment of age-dependent reference values for IgA subclasses. *Clinica Chimica Acta; International Journal of Clinical Chemistry*. 328 (1–2), 129–133.
[7] Steffen, U., Koeleman, C.A., Sokolova, M.V., Bang, H., Kleyer, A., Rech, J., et al. (2020) IgA subclasses have different effector functions associated with distinct glycosylation profiles. *Nature Communications*. 11 (1), 120.
[8] Campbell, J.P. and Turner, J.E. (2018) Debunking the Myth of Exercise-Induced Immune Suppression: Redefining the Impact of Exercise on Immunological Health Across the Lifespan. *Frontiers in Immunology*. 9 648.
[9] Gleeson, M., Pyne, D.B., Elkington, L.J., Hall, S.T., Attia, J.R., Oldmeadow, C., et al. (2017) Developing a multi-component immune model for evaluating the risk of respiratory illness in athletes. *Exercise Immunology Review*. 23 52–64.

[10] Gulli, F., Napodano, C., Marino, M., Ciasca, G., Pocino, K., Basile, V., et al. (2020) Serum immunoglobulin free light chain levels in systemic autoimmune rheumatic diseases. *Clinical and Experimental Immunology*. 199 (2), 163–171.

[11] Napodano, C., Pocino, K., Rigante, D., Stefanile, A., Gulli, F., Marino, M., et al. (2019) Free light chains and autoimmunity. *Autoimmunity Reviews*. 18 (5), 484–492.

[12] Basile, U., Marino, M., Gragnani, L., Napodano, C., Gulli, F., Pocino, K., et al. (2020) Sentinel biomarkers in HCV positive patients with mixed cryoglobulinemia. *Journal of Immunological Methods*. 476 112687.

[13] Basile, U., Bruno, C., Napodano, C., Vergani, E., Pocino, K., Brunetti, A., et al. (2018) Plasmatic free light chains as inflammatory marker in insulin resistance: comparison of metabolic syndrome with adult growth hormone deficiency: Plasmatic free light chains as inflammatory marker in insulin resistance. *BioFactors*. 44 (5), 480–484.

[14] Team, R.D.C. and R Development Core Team, R. (2016) R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing*.

[15] Minelli, E., Ciasca, G., Sassun, T.E., Antonelli, M., Palmieri, V., Papi, M., et al. (2017) A fully-automated neural network analysis of AFM force-distance curves for cancer tissue diagnosis. *Applied Physics Letters*. 111 (14), 143701.

[16] Ciasca, G., Sassun, T.E., Minelli, E., Antonelli, M., Papi, M., Santoro, A., et al. (2016) Nano-mechanical signature of brain tumours. *Nanoscale*. 8 (47), 19629–19643.

[17] BASILE, U., MIELE, L., NAPODANO, C., CIASCA, G., GULLI, F., POCINO, K., et al. (2021) The diagnostic performance of PIVKA-II in metabolic and viral hepatocellular carcinoma: A pilot study. *European Review for Medical and Pharmacological Sciences*.

[18] Pocino, K., Napodano, C., Gragnani, L., Ciasca, G., Colantuono, S., Marri, S., et al. (2021) Revealed the mystery of HBV related mixed cryoglobulinemia: potential biomarkers of disease progression . *Rheumatology*.

[19] Brandtzaeg, P. (2013) Secretory IgA: Designed for Anti-Microbial Defense. *Frontiers in Immunology*. 4 222.

[20] Roos Ljungberg, K., Börjesson, E., Martinsson, K., Wetterö, J., Kastbom, A., and Svärd, A. (2020) Presence of salivary IgA anti-citrullinated protein antibodies associate with higher disease activity in patients with rheumatoid arthritis. *Arthritis Research & Therapy*. 22 (1), 274.

[21] Aita, A., Basso, D., Cattelan, A.M., Fioretto, P., Navaglia, F., Barbaro, F., et al. (2020) SARS-CoV-2 identification and IgA antibodies in saliva: One sample two tests approach for diagnosis. *Clinica Chimica Acta; International Journal of Clinical Chemistry*. 510 717–722.

[22] Varadhachary, A., Chatterjee, D., Garza, J., Garr, R.P., Foley, C., Letkeman, A.F., et al. (2020) Salivary anti-SARS-CoV-2 IgA as an accessible biomarker of mucosal immunity against COVID-19. *MedRxiv: The Preprint Server for Health Sciences*.

[23] Nakano, T., Matsui, M., Inoue, I., Awata, T., Katayama, S., and Murakoshi, T. (2011) Free immunoglobulin light chain: Its biology and implications in diseases. *Clinica Chimica Acta*. 412 (11–12), 843–849.

[24] Redegeld, F.A., van der Heijden, M.W., Kool, M., Heijdra, B.M., Garssen, J., Kraneveld, A.D., et al. (2002) Immunoglobulin-free light chains elicit immediate hypersensitivity-like responses. *Nature Medicine*. 8 (7), 694–701.

[25] Bryce, P.J., Miller, M.L., Miyajima, I., Tsai, M., Galli, S.J., and Oettgen, H.C. (2005) Immune sensitization in the skin is enhanced by antigen-independent effects of IgE on mast cells. *Novartis Foundation Symposium*. 271 15–24; discussion 24-38, 95–99.

[26] Heaney, J.L.J., Gleeson, M., Phillips, A.C., Taylor, I.M., Drayson, M.T., Goodall, M., et al. (2016) Salivary immunoglobulin free light chains: reference ranges and responses to exercise in young and older adults. *Exercise Immunology Review*. 22 28–41.

[27] Basile, U., Gulli, F., Gragnani, L., Napodano, C., Pocino, K., Rapaccini, G.L., et al. (2017) Free light chains: Eclectic multipurpose biomarker. *Journal of Immunological Methods*. 451 11–19.