Salivary markers and coronavirus disease 2019: insights from cross-talk between the oral microbiome and pulmonary and systemic low-grade inflammation and implications for vascular complications

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To date, coronavirus disease 2019 (COVID-19) has affected over 6.2 million individuals worldwide, including 1.46 million deaths. COVID-19 complications are mainly induced by low-grade inflammation-causing vascular degeneration. There is an increasing body of evidence that suggests that oral dysbiotic taxa are associated with worse prognosis in COVID-19 patients, especially the Prevotella genus, which was retrieved from nasopharyngeal and bronchoalveolar lavage samples in affected patients. Oral dysbiosis may act by increasing the likelihood of vascular complications through low-grade inflammation, as well as impairing respiratory mucosal barrier mechanisms against SARS-CoV-2. Salivary markers can be used to reflect this oral dysbiosis and its subsequent damaging effects on and the lungs and vasculature. Salivary sampling can be self-collected, and is less costly and less invasive, and thus may be a superior option to serum markers in risk stratification of COVID-19 patients. Prospective studies are needed to confirm such

Introduction

Coronavirus disease 2019 (COVID-19) is one of the largest public health pandemics since the Spanish flu of 1918. With a wide spectrum of clinical manifestations, COVID-19 has caused major setbacks in healthcare worldwide and challenged the capabilities of some of the most accomplished health care systems in the world. Although up to 80% of patients are initially asymptomatic,

a nonnegligible percentage eventually require hospitalization and intensive care admission (ICU) [1].

Currently, the worst complication of COVID-19 is vascular inflammation and degeneration [2]. Dysbiosis, being closely related to vascular inflammation and degeneration, is one of the theories which may explain the interpersonal variability of COVID-19's wide spectrum of manifestations [3]. Shen *et al.* [4] previously conducted serum proteomic profiling of COVID-19 patients and established a blood proteomic risk score (PRS) for the forecast of the patients' clinical results. Kageyama *et al.* [5] showed that this proteomic profile is related to a particular set of proinflammatory intestinal microbiota. Chakraborty *et al.* [6] outlined the association of periodontopathic oral bacteria with unfavorable outcomes in COVID-19 patients.

Salivary markers, such as C-reactive protein (CRP) and leukotrienes (exclusively perceptible in saliva), are simple and affordable tests that can be used to determine oral dysbiosis and periodontopathic strains. In this review, we summarize the available evidence, which suggests the role of oral microbiome dysbiosis in pulmonary and vascular inflammatory complications and the potential for salivary markers as a prognostic tool in COVID-19.

Low-grade inflammation association with coronavirus disease 2019 mortality and its relation to oral dysbiosis, the oral cavity-systemic inflammation axis

How oral microbiota contributes to low-grade inflammation?

According to recent studies, oral dysbiosis leads to chronic low-grade local and systemic inflammation. Periodontitis is one of the causes that increases the risk of cardiovascular disease, as well as contributes to the pathogenesis of atherosclerosis. Periodontitis patients have increased markers of systemic inflammation, including CRP, interleukin 6 (IL-6), haptoglobin and fibrinogen [7]. Rai *et al.* [8] found a link between oral dysbiosis and increased levels of interleukin 8 (IL-8), interleukin 6 (IL-6), tumor necrosis factor (TNF-α), granulocyte monocyte colony-stimulating factor (GM-CSF) and interferon γ (IFN-γ), which also increase the susceptibility to developing oral cancer.

Little is known about how oral dysbiosis induces systemic inflammation. The major depression model attempts to outline the systemic effects of dysbiosis [9]. Socransky *et al.* [10] investigated the mechanisms by which oral dysbiosis contributes to systemic inflammation. It is increasingly that a relationship between chronic periodontitis major depression exists. This relationship is the result of (1) stimulation of the immune system to secrete proinflammatory cytokines, mainly IL-6 and IL-8, (2) secretion of proteases that activate the associated complement system, (3) inflammation evoked by direct invasion of periodontal pathogen and their inflammatory products and (4) disturbances in the hypothalamic-pituitary-thyroid axis which alter periodontal status by affecting the immune system.

Within the periodontal pocket, bacteria exist in a stratified dental biofilm, which consists mainly of microorganisms and their components (endotoxins and lipopolysaccharides). When inflammation in the periodontal pocket occurs, it becomes a significant source of inflammatory and pathogen-derived mediators. In a healthy individual, the majority of bacteria are Gram-positive aerobes, whereas, in periodontitis individuals, around 85% of the bacteria are Gram-negative, according to Socransky *et al.* [10]. These bacteria invade the intact pocket epithelium and subsequently gain access to the circulation resulting in systemic dissemination of bacterial products.

Association of coronavirus disease 2019 complications with disease states related to low-grade inflammation

Low-grade inflammation (LGI), characterized by a chronic increase in inflammatory cytokines (IL-6, TNF-α and IL-1-β), is the cornerstone of all the high-risk classifications for developing severe COVID-19. Hyperglycemia, obesity and hypertension, all features of metabolic syndrome (MetS), are believed to increase the risk of cardiovascular disease and type 2 diabetes mellitus and are accompanied by LGI. In severe acute respiratory syndrome (SARS-CoV-2) patients, target tissues including the lungs, brain, gut and kidneys are infiltrated with proinflammatory macrophages and lymphocytes, which lead to hypercytokinaemia that supplements LGI in MetS patients. It has been found that patients with IL-6 levels of ≥80 pg/ml had a greater risk of developing respiratory failure by 22 times than that for patients with lower IL-6 level [11].

In high-risk patients with chronic LGI and SARS-CoV-2 infection, cellular immune response is mediated mainly by T-helper cells (Th) 1, Th-17, and proinflammatory macrophages. Therefore, a 'normal' proinflammatory response may intensify cytokine levels above those of the underlying LGI, increasing the risk of developing fatal forms of COVID-19. This suggests that in cases of SARS-Cov-2 infection, a 'normal' antiviral immune response in combination with LGI may trigger fatal consequences [12].

Evidence of association of oral dysbiosis with disorders characterized by low-grade inflammation such as obesity

Obesity and related complications

Evidence suggests that obesity may be induced by alterations in the oral microbiota. Data from Tam *et al.* [13] series revealed that there are discriminative features of microbiota between obese and nonobese patients. Bacteroidetes and Spirochaetes were more dominant in subgingival plaques on nonobese populations, while Proteobacteria, Chloroflexi and Prevotella/Firmicutes were more dominant in the obese population, with the noted absence of representatives from the phylum Bacteroidetes. The phylum Firmicutes was identified in obese patients as an independent, significantly discriminative feature, with an abundance of over four orders of magnitude. These findings in the oral cavity coincide with the observed microbiota of the gut in obese individuals, which is characterized by a reduced abundance of Bacteroidetes paralleled by an increased abundance of Firmicutes; a lower ratio of Bacteroidetes to Firmicutes (B:F) exists in obese patients [14,15]. Verdam *et al.* [16] demonstrated that the low B:F ratios and increased predominance of Prevotella observed in obese individuals contributed to intestinal inflammation (as shown by high levels of calprotectin), as well as increased the likelihood of systemic inflammation associated with obesity.

Vascular degeneration and oral dysbiosis

The severity of COVID-19 has been linked to a state of vascular degeneration, as evidenced by the increased risk of thromboembolic events seen in severely ill patients. Evidence suggests that oral dysbiosis is associated with a greater risk of vascular degeneration [17].

Grau *et al.* [18] found that individuals with periodontitis had a greater risk of developing stroke by 400% than those with caries. Another study found a correlation between intimal thickening (used as a surrogate measure of atherosclerosis) and periodontal pathogens, but not health-compatible organisms [19]. Systemic antibody levels have been linked to periodontal pathogens, as well as the incidence of coronary heart disease and subclinical atherosclerosis. This data suggests a connection between periodontal pathogens and the incidence of cardiovascular disease [19].

Chhibber-Goel et al. [20] used next-generation sequencing to analyze bacterial species in atherosclerotic plaques; the most characteristic taxa were Prevotella and Veillonella.

Periodontal bacteria can contribute to atherogenesis by destabilization of the atherosclerotic plaque. Prevotella gingivalis infection is associated with the proliferation of smooth muscle cell tissue in developing aneurysms, especially aortic aneurysms [21].

The concentrations of IL-18 and IFN-γ are reduced by 90% at 12 months post periodontal therapy. This finding is of special importance as levels of IL-18 are used to predict (1) acute myocardial infarction in coronary artery disease patients, (2) major cardiovascular events seen 6 months after hospitalization in individuals with acute coronary syndrome and (3) IL-18 independently predicts congestive heart failure, myocardial infarction, cardiovascular death, as well as all-cause non-CVD death [22].

Status of lung microbiome in coronavirus disease 2019 and relationship to oral microbiome: oral-lung axis

The pathway to which microbes reach the lungs is believed to originate from the oral cavity, through microaspiration as taxonomic profiles showed identical organisms. The use of culture-independent techniques concluded that the mouth and lung microbiota are similar, which suggests that saliva does not only reflect low-grade systemic inflammation but gives an idea about the lung environment as well. One of the major periodontopathic strains, Prevotella, has been associated with negative outcomes in the lungs [23–25].

Shen suggests that there is an association between microbiota, COVID-19 and some opportunistic infectious agents. *Prevotella* significantly coexists with COVID-19, alongside Capnocytophaga and Veillonella [26,27].

These findings were confirmed by Chakraborty *et al.* [6] by identifying the interplay between Prevotella and

SARS-CoV-2 in the lung microenvironment, leading to the development of severe acute symptoms and the persistence of chronic lung injury in affected patients. Lung epithelium and proximal and distal airways play a pivotal role in maintaining immune homeostasis, mainly as a first line of defense against inhaled bacterial, fungal and viral pathogens. Culture supernatant fluids of P intermedia are cytotoxic to airway epithelial cells, resulting in their destruction and subsequently the loss of the barrier that protects against viral infections, which may explain the link between Prevotella and SARS-CoV-2 in the Shen and Chakraborty series [28].

A major function of lung microbiota is to mitigate the inflammatory response located within the lungs (alternatively termed 'tolerance'). The presence of high bacterial load and predominant taxa derived from the upper respiratory tract has been associated with an attenuated immune response of alveolar macrophages to lipopolysaccharide. This suggests the role of microbiota in regulating inflammatory responses at the pulmonary mucosal surface. The presence of members of the Bacteroidetes phylum decreases lung inflammation, while Prevotella spp. and Veillonella spp. increase Th-17 cell-mediated lung inflammation, as demonstrated by other human studies [29,30].

Prevotella and Veillonella species are associated with low-grade systemic inflammation, defective lung epithelial barriers and increased likelihood of exaggerated pulmonary inflammation. Evidently, these two taxa gain access to the lungs through the oral cavity, as they are predominant periodontopathic strains. Table 1 summarizes the above-mentioned evidence of Prevotella and its reported systemic and pulmonary proinflammatory roles.

Salivary markers: a mirror of systemic and pulmonary proinflammation

Mechanism of secretion of salivary markers: saliva is a mirror of the serum markers

One of the most important diagnostic tools for detecting salivary pathologies is salivary analysis. It is simple, non-invasive, inexpensive and easy to store. Salivary analysis

Table 1 Examples of the oral, systemic and pulmonary pathogenic roles of Prevotella species

Oral dysbiosis		Low-grade systemic inflammation
Prevotella is a major pathogen of periodontitis reflecting oral dysbiosis [23]		Prevotella has been fund as a component of atherosclerotic plaques [20]
Pulmonary effects		Prevotella is linked to higher obesity risk, and higher rates of obesity related complications [15]
Prevotella is linked to lung disorders associated with increased leukotrienes such as asthma [30]		
Prevotella in BAL has been linked to severe lung injury in influenza patients [25]	Prevotella in nasopharyngeal samples and BAL has been linked to severity of COVID-19 [6,26]	

BAL, bronchoalveolar lavage; COVID-19, coronavirus disease 2019.

has become a very popular laboratory investigation for the diagnosis of oral and systemic diseases.

The use of saliva has been appreciated as it can determine the normal internal characteristics and disease state of an individual 7, due to the fact that it is exchanged with different substances that are components of plasmatic liquid. This is as a result of the presence of a thin layer of epithelial cells, which separates the salivary ducts from the systemic circulation. Substances could be transferred to the saliva through (1) active carriage, (2) diffusing through the membrane (ultrafiltration) or (3) through passive diffusion via a concentration gradient [31].

The oral cavity can host many types of oral microbiota, which are inducers of inflammatory mediators such as cytokines. Additionally, there are other forms of microbiota that can gain access to the serum via inflamed and disrupted periodontal pocket.

Therefore, the link between saliva and blood appears bidirectional, whereby the composition of both these body compartments affect and reflect each other.

Salivary markers and lung inflammation

Saliva reflects the systemic markers of inflammation. There is increasing evidence that suggests a continuous migration of pulmonary inflammatory markers to the oral cavity. One of the most important markers of decreased epithelial integrity and lung inflammation is leukotrienes. Ono *et al.* [32] proved that salivary leukotrienes are can be relied upon as sensitive indicators of airway inflammation in asthma. The mechanism by which the lung microenvironment confers its markers to the oral cavity is not completely understood. However, one of the suggested mechanisms is the exchange of exosome-like vesicles between different organs. The infrastructure of extracellular vehicles has been extensively investigated. Extracellular vehicles carry bioactive compounds (proteins, nucleic acids, lipids and metabolites like leukotriene) within their inner core or in their lipid bilayer, which may suggest that the source of salivary leukotrienes is partly conveyed by such vesicles [7].

Implication: use of salivary biomarkers for risk stratification of coronavirus disease 2019

The detection of certain bacterial taxa in the oral cavity is associated with low-grade systemic and pulmonary inflammation in the context of COVID-19, as discussed in section II. These periodontopathic taxa, namely Prevotella and Veillonella, have been found to overpopulate the lung microenvironment.

A major prognostic predictor for COVID-19 associated complications is the detection of such strains in the oral cavity. The use of salivary biomarkers may serve as an alternative prognostic tool for COVID-19 disease.

Research has yet to point out an association of these bacterial taxa in the oral cavity with the elevation of particular biomarkers, but several studies have compared the incidence of periodontitis (associated with the same strains) with the elevation of salivary biomarkers.

Salivary C-reactive protein

Salivary testing has recently gained much interest, as it is noninvasive, inexpensive and can be self-sampled as compared to serum markers. This interest has extended the use of saliva-based screening for COVID-19 in place of the traditional nasopharyngeal swab; saliva was equal to swab testing in the diagnostic accuracy of COVID-19 [33].

Salivary CRP, initially produced by the liver, enters the saliva via gingival crevicular fluid as one of its components or through the salivary glands. According to the study done by Savitha and Shasmitha, salivary CRP concentrations were higher in periodontitis patients compared to healthy individuals, which suggests that salivary CRP is increased in inflammatory conditions of the oral cavity [34]. Salivary inflammatory markers were also identified by Labat *et al.* [35] to aid in cardiovascular disease and risk factors prediction. High salivary CRP levels correlating to plasma CRP levels were associated with inflammatory conditions such as arterial stiffness, atherosclerosis and metabolic syndrome. The suggestion arises that salivary CRP is used as a prognostic marker during the earliest encounter with the COVID-19 patient.

Salivary leukotrienes (LBT4)

Leukotrienes are lipid inflammatory mediators that are suggested markers of airway hypersensitivity. Multiple extrapulmonary negative outcomes have been tied to Leukotrienes. LTB4 is highly linked to periodontitis and was proven by Emingil *et al.* [36] to be a periodontitis marker.

Leukotrienes were shown to be involved in the arterial stiffness pathogenesis. In atherosclerosis, some inflammatory alterations involve the endothelial monolayer, which cannot resist leucocyte contact compared to the normal state. In subclinical atherosclerosis, leukotrienes have been proposed to be mediators, biomarkers and therapeutic targets. Their role in the pathogenesis of COVID-19-related complications was initially discarded until Funk *et al.* [37] pointed out the principal role of leukotrienes in the pathogenesis of acute respiratory distress syndrome (ARDS) in COVID-19. Leukotrienes were not previously considered due to undetectable levels in serum. Since the comparison of bronchoalveolar lavage (BAL) leukotrienes levels across patients with variable severity is nearly inapplicable, it became difficult to define their exact role in COVID-19. However, oral samples have been shown to include readily measurable LTB4 concentrations, which then decreased after oral

Fig. 1
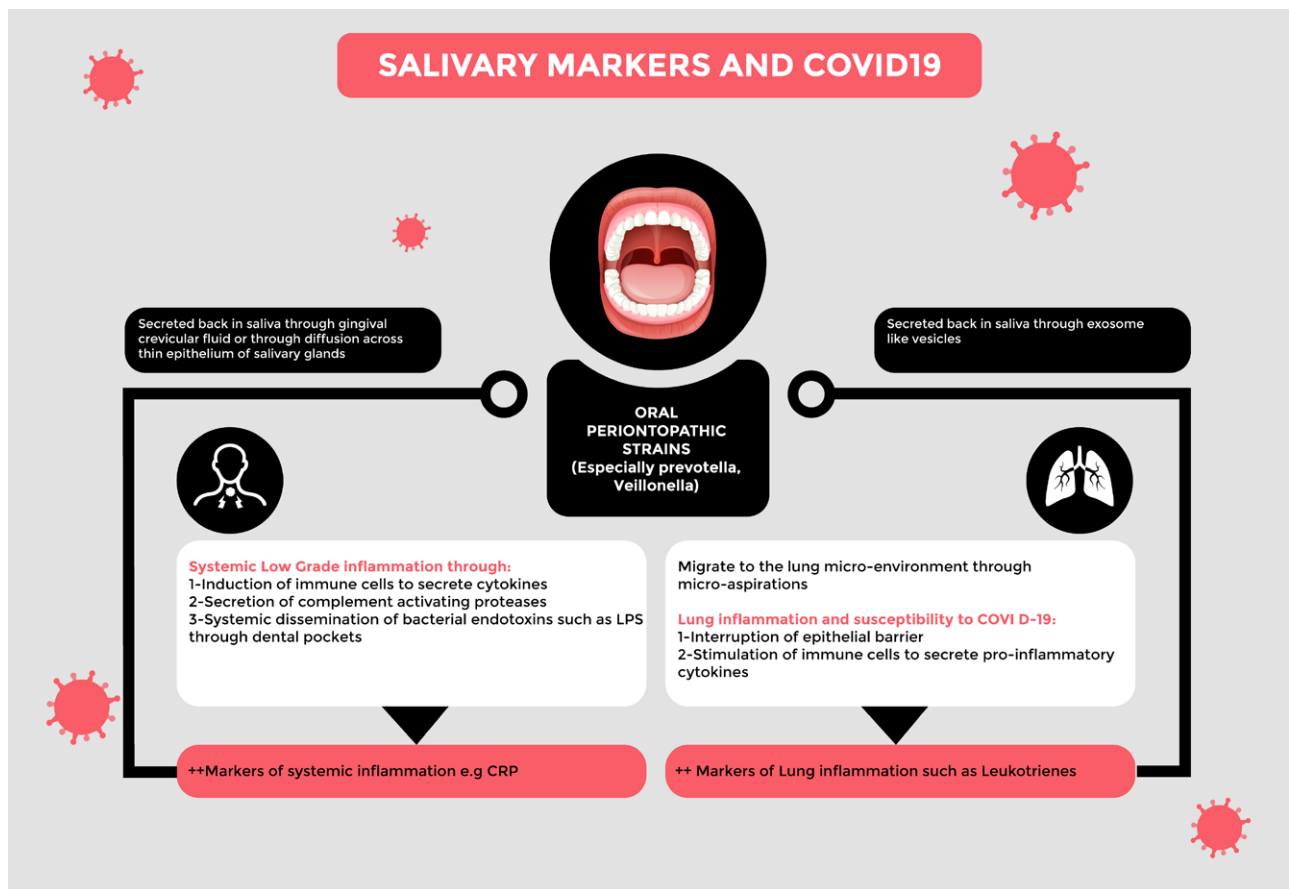
Salivary markers and COVID-19. COVID-19, coronavirus disease 2019; CRP, C-reactive protein.

treatment with an antiasthmatic leukotriene synthesis inhibitor. Widegren *et al*. [38] proved that LTB4 utilizes specific neutrophil chemoattractant properties and virucidal effects against Coronaviridae.

More studies are needed to confirm the association of salivary markers with the predominance of Prevotella and Veillonella in the oral and lung microenvironment.

Figure 1 summarizes the connection between oral microbiome, LGI and lung damage, as well as the implications for the use of salivary makers in risk stratification of COVID-19 cases.

Conclusion
Periodontopathic strains, namely Prevotella and Veillonella, have the potential to induce low-grade systemic inflammation, increasing the likelihood of vascular degeneration and metabolic complications such as obesity. They can also compromise the lung mucosal barrier, thus facilitating SARS-CoV-2 entry. These taxa have been persistently found in BAL and nasopharyngeal samples of COVID-19 patients, and their retrieval has been associated with a worse prognosis. The use of

next-generation sequencing to detect the presence of these species in the oral cavity would be a useful but a costly strategy for prediction of vascular complications of COVID-19. Alternatively, salivary biomarkers can predict the presence of these periodontopathic strains and are sensitive markers of vascular degeneration as well as pulmonary inflammation. Their use is superior to serum sampling due to the possibility of self-sampling, thus minimizing contact with infected patients. Their use can also better reflect the ongoing damage in the lungs as sensitive markers of lung damage (such as leukotrienes) are retrievable from the saliva while being almost undetectable in the blood. Prospective studies should be conducted by working groups globally to determine the exact salivary signature of COVID-19 and to test its feasibility as prognostic tool for the risk stratification of patients.

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