Saliva as a non-invasive diagnostic tool: COVID-19 and T2DM as case-study

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CHAVE MÓVEL

Dedicatory

To my family

To both my princesses

To SalivaTec colleagues

To all that give up the “work” and embrace the “art” of challenging yourselves in the non-protocoled science

Acknowledgments

First of all, to my esteemed advisor Professor Nuno Rosa, a person I hold in high regard for his intellectual and scientific abilities associated with his simplicity. To Professor Ana Cristina Esteves to whom I respect for her honesty and scientific quality and who played a fundamental role in encouraging me to proceed my interests. My thanks to them for their availability and commitment to monitoring me on this project, with the ease and difficulties inherent to long journey on different projects. To Professor Liliana Bernardino who supported on unbalanced moments and believe on what is my work. I must thank Prof. Marlene Barros for her friendly support and for the opportunity to develop all my project at SalivaTec research lab. I extend my thanks to Professor Maria José with whom I am connected to by bonds of friendship, gave me the moments to ease and clear my mind, with highly instructed overviews on scientific perspectives.

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Finally, I thank my family, for their unconditional support and to whom I penalized with many absences for multiple reasons. To all, my sincere thanks.

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List of Publications

Esteves E, Mendes AK, Barros M, Figueiredo C, Andrade J, Capelo J, et al. (2022). Population Wide Testing Pooling Strategy For SARS-CoV-2 Detection Using Saliva. PLoS ONE 17(1): e0263033. https://doi.org/10.1371/journal.pone.0263033

Esteves, E, Mendes, VM, Manadas, B, Lopes, R, Bernardino, L, Correia, MJ, Barros, M, Esteves, AC, Rosa, N. (2022). COVID-19 Salivary Protein Profile: Unravelling Molecular Aspects of SARS-CoV-2 Infection. J. Clin. Med. 2022, 11, 5571. https://doi.org/10.3390/jcm11195571

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Resumo

A Saliva tem atualmente um vasto background de investigação sobre como pode espelhar o estado de saúde do corpo. Já foram identificados biomarcadores salivares específicos para múltiplas doenças e são particularmente úteis para a monitorização de medicamentos. A saliva é um tipo de amostra particularmente favorável para a deteção de doenças infeciosas. Trata-se de um fluído não-invasivo, indolor e passível de auto recolha, ideal para estudar diferentes aspetos de duas doenças pandémicas conhecidas: COVID-19 e Diabetes Mellitus Tipo 2.

A COVID-19 é uma das pandemias mais impactante de todos os tempos, exigindo testes frequentes às populações. A necessidade de identificar estratégias rentáveis para a deteção do surto de SARS-CoV-2 tornou-se uma prioridade global. As amostras nasofaríngeas foram consideradas como o padrão de amostragem, apesar de requerem um profissional de saúde qualificado para efetuar a colheita além de causar desconforto e dor ao doente. Uma vez que a saliva demonstrou ser bem-sucedida na deteção da SARS-CoV-2, o próximo passo seria criar uma estratégia de pools de amostras com vista a diminuir o número de testes individuais e de resíduos de materiais perigosos, o que também seria benéfico para o ambiente. Esta estratégia foi testada em duzentas e setenta e nove amostras de saliva com pools de 10 e 20 amostras aleatórias. O Cycle-Threshold médio dos genes detetados foi de 29,7. Reações de RT-PCT consecutivas em amostras positivas mostraram reprodutibilidade. A análise de comparação de amostras positivas individualizadas mostrou uma carga viral mediana mais elevada em amostras de saliva comparativamente a amostras nasofaríngeas. Concluímos que as amostras de saliva-pool permitiram um rastreio eficaz da SARS-CoV-2 em pools de 10 e de 20 amostras. Esta estratégia foi aplicada com sucesso em testes populacionais de mais de 2000 indivíduos, mostrando que é possível utilizar saliva em pool como líquido de diagnóstico para a infeção pelo SARS-CoV-2.

Está demonstrado que a deteção de SARS-CoV-2 está bem estabelecida e com metodologias fiáveis, incluindo a saliva como fluido de deteção. No entanto as alterações moleculares induzidas por este vírus continuam por desvendar. Desenvolvemos uma estratégia proteómica híbrida para estabelecer um perfil proteico salivar COVID-19. As proteínas que mostram uma maior distinção entre amostras saudáveis e COVID-19 foram definidas através da análise PLS-DA e de enriquecimento funcional. Paralelamente, foram previstas as interações proteína-proteína entre o vírus e o hospedeiro. Foram identificados cinco processos biológicos desregulados no perfil do proteoma COVID-19: Apoptose, Vias de Energia, Resposta Imune, Metabolismo de Proteínas, e Transporte. Foram identificadas 10 proteínas (KLK 11, IMPA2, ANXA7, PLP2, IGLV2-11, IGHV3-43D, IGKV2-24, TMEM165, VSIG10 e PHB2) que nunca tinham sido associadas à infeção por SARS-CoV-2, representando novas provas do perfil molecular por detrás da COVID-19. A análise de interactómica mostrou influência do vírus na resposta

imunitária do hospedeiro, principalmente através da interação com a desgranulação dos neutrófilos. A partir destes resultados, é possível concluir que o vírus altera não só a resposta imune, mas também o metabolismo energético do hospedeiro e interfere com os mecanismos de apoptose.

A Diabetes Mellitus Tipo 2 é uma doença metabólica crónica e constitui um risco de saúde importante devido às suas complicações características a longo prazo. Estima-se que cerca de 537 milhões de pessoas vivem com diabetes em todo o mundo e continuarão a aumentar. O diagnóstico e a monitorização da glicose na diabetes estão atualmente bem estabelecidos. Contudo, a monitorização de muitas das complicações da diabetes continua a ser um desafio, comprometendo o prognóstico e a qualidade de vida dos pacientes. Tivemos como objetivo estabelecer uma estratégia híbrida que identificou os marcadores salivares do T2DM e as suas complicações. Da análise funcional destacamos processos metabólicos, resposta a estímulos, processos do sistema imunitário e sinalização como processos biológicos perturbados pela relação conhecida com o T2DM. A análise de enriquecimento identificou 11 processos biológicos desregulamentados em que demos destaque a 20 proteínas diretamente relacionadas com complicações na diabetes. A retinopatia diabética, síndrome metabólica, resistência à insulina, impacto molecular da glicose e desregulação da homeostase da insulina, aterosclerose, úlcera do pé diabético, catabolismo proteico e função da glândula salivar são complicações diabéticas capazes de serem monitorizadas usando saliva. Concluímos que a saliva tem o potencial de identificar proteínas alteradas em doentes diabéticos em comparação com não diabéticos e que podem ser biomarcadores não só da diabetes, mas também das diferentes complicações desta doença.

Em conclusão, com esta investigação confirmamos o potencial da saliva como fluido de interesse tanto no diagnóstico como na descoberta de novos insights sobre doenças. A saliva foi crucial para demonstrar a flexibilidade e capacidade de seu uso demonstrada pelo desenvolvimento de uma deteção confiável de SARS-CoV-2, a identificação e discussão de aspetos moleculares da infeção viral no hospedeiro e com o hospedeiro, bem como a descoberta de novos marcadores no diagnóstico e monitorização da Diabetes Mellitus tipo 2.

Palavras-chave

Saliva, SARS-CoV-2, Diabetes Mellitus Tipo 2, Mecanismos moleculares, Biomarcadores, Proteómica.

Abstract

Saliva has nowadays a vast research background of how it can mirror the body's health status. Specific salivary biomarkers have been already suggested for multiple diseases and is particularly useful for detecting infectious diseases. We focused on the importance of having a non-invasive, painless and self-collected fluid to study different aspects of two known pandemic diseases: COVID-19 and Type 2 Diabetes Mellitus.

COVID-19 is the most impacting global pandemic of all time requiring frequent testing of populations. The necessity to identify cost-effective strategies for the detection of SARS-CoV-2 outbreak became a priority. Nasopharyngeal samples were considered the sampling golden standard but require a healthcare professional to collect the sample causing discomfort and pain to the patient. As saliva has proved successful in SARS-CoV-2 detection, a pooling strategy could be a good approach to decrease the number of individual tests and hazardous material waste which is also beneficial for the environment. We have tested this strategy on two hundred and seventy-nine saliva samples with pools of 10 and 20 randomized samples through RT-PCR. Cycle Threshold of the genes detected was 29.7. Consecutive reactions analysis of positive samples showed an equivalent cycle threshold average ($p < 0.05$). Our individual positive sample comparison analysis showed a higher median viral load (32.6) in saliva samples versus nasopharyngeal samples (28.9), with no significant differences detected ($p > 0.05$). We concluded that saliva-pool samples allowed effective SARS-CoV-2 screening on 10-sample and 20-sample pools. Our strategy was successfully applied in population-wide testing of more than 2000 individuals, showing that it is possible to use pooled saliva as diagnostic fluid for SARS-CoV-2 infection.

The SARS-CoV-2 detection is well established with reliable methodologies including saliva as a detection fluid. In the opposite direction are the molecular alterations induced by this infectious virus which remain elusive. We developed a hybrid proteomics and in silico interactomics strategy to establish a COVID-19 salivary protein profile. The most distinctive proteins between healthy and COVID-19 samples were defined with the Partial Least-Squares Discriminant Analysis and the enrichment analysis was performed with FunRich software. In parallel, Protein-Protein virus-host interactome was identified with OralInt algorithm. Five dysregulated biological processes were identified in the COVID-19 proteome profile: Apoptosis, Energy Pathways, Immune Response, Protein Metabolism, and Transport. We identified 10 proteins (KLK 11, IMPA2, ANXA7, PLP2, IGLV2-11, IGHV3-43D, IGKV2-24, TMEM165, VSIG10, and PHB2) that had never been associated with SARS-CoV-2 infection, representing new evidence for the molecular profile behind COVID-19. Interactomics analysis showed viral influence on the host immune response, mainly through interaction with the degranulation of neutrophils. From

our results, we can conclude that the virus also alters the host's energy metabolism and interferes with apoptosis mechanisms.

Type 2 Diabetes Mellitus is a chronic metabolic disease and is a major health risk due to its characteristic long-term complications. It is estimated that about 537 million people live with diabetes worldwide and will continue to increase. Diagnosis and glucose monitoring in diabetes are well established. However, monitoring the many of diabetes complications remains a challenge, compromising patients' prognosis and quality of life. We established a hybrid strategy that identified salivary markers of T2DM and its complications. From the functional analysis we highlight metabolic processes, response to stimulus, immune system processes and signalling as disrupted biological processes by the known relation with T2DM. The enrichment analysis identified 11 deregulated biological processes emphasizing 20 proteins directly related to complications in diabetes. Diabetic retinopathy, metabolic syndrome, insulin resistance, molecular impact of glucose and insulin homeostasis dysregulation, atherosclerosis, diabetic foot ulcer, protein catabolism and salivary gland function are diabetic complication capable of being monitored using saliva. We conclude that saliva has the potential to identify several molecules altered in diabetic patients compared to non-diabetic patients and that may be biomarkers not only of diabetes but also of the different complications of this disease.

In conclusion, with this research we have confirmed the potential of saliva as a fluid of interest in both diagnosing and discovering new insights into diseases. Saliva was crucial in demonstrating the flexibility and capacity of its use demonstrated by the development of a reliable detection of SARS-CoV-2, the identification and discussion of molecular aspects of viral infection in and with the host, and the discovery of new markers in the diagnosis and monitoring of Type 2 Diabetes Mellitus.

Keywords

Saliva, SARS-CoV-2, Type 2 Diabetes Mellitus, Molecular mechanisms, Biomarkers, Proteomic.

Thesis Outline

This research aimed to propose innovative strategies for the clarification of underlying molecular changes in COVID-19 and Type 2 Diabetes Mellitus, using saliva. Different approaches were used, being the thesis organized into 6 chapters.

Chapter I corresponds to a General Introduction addressing the main topics of the work. The aims of the thesis are defined in chapter II.

Chapter III presents an innovative strategy for SARS-CoV-2 detection and massive testing using saliva as sampling fluid. A down-stream approach was applied to identify the molecular changes upon SARS-CoV-2 infection, described in chapter IV.

Biomarkers for Type 2 Diabetes Mellitus and its complications, representing the main molecular changes detected in saliva, are presented in chapter V.

In chapter VI, the general discussion, as well as relevant directions to follow in the future are presented.

At the end of the dissertation (annexes), all raw data used are presented, allowing the reader to search for detailed and complementary information.

The thesis is presented in 'article format'. Chapters III and IV were published and Chapter V is in preparation for submission:

Esteves E, Mendes AK, Barros M, Figueiredo C, Andrade J, Capelo J, et al. (2022) Population wide testing pooling strategy for SARS-CoV-2 detection using saliva. PLoS ONE 17(1): e0263033. doi: 10.1371/journal.pone.0263033

Esteves E, Mendes, VM, Manadas B, Lopes R, Bernardino L, Correia MJ, Barros M, Esteves AC, Rosa N. COVID-19 salivary protein profile: unravelling molecular aspects of SARS-CoV-2 Infection. J. Clin. Med. 2022, 11, 5571. doi: 10.3390/jcm11195571

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List of Abbreviations

SIgA	Secretory IgA
miRNA	microRNA
2-DE	Two-dimensional gel electrophoresis
qMS	Quantitative mass spectrometry
ELISA	Enzyme-linked immunosorbent assay
SELDI-TOF-MS	Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry
RS	Raman spectroscopy
GPMDB	Global Proteome Machine Database
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
RT-PCR	Reverse transcription polymerase chain reaction
COVID-19	Coronavirus disease of 2019
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
AEC2	Alveolar epithelial type 2
ACE2	Angiotensin-converting enzyme 2
Ang II	Angiotensin II
AT1R	Ang II type 1 receptor
T2DM	Type 2 diabetes mellitus
ROS	Reactive oxygen species
PCR	Polymerase chain reaction
SAL	Saliva
NPS	Nasopharyngeal Swab
CT	Cycle threshold
K	Kappa
PPV	Positive predictive value
NPV	Negative predictive value
CI	Confidence interval
MS	Mass spectrometry
DIA	Data-independent acquisition
ORF	Open reading frame

Chapter I - General Introduction

1. Saliva as a diagnostic fluid

Saliva has long been recognised as a valuable tool in scientific research. It is secreted by salivary glands and aids in oral tissue moisturization and digestion, as well as in maintaining oral cavity homeostasis. Saliva has been suggested as an excellent non-invasive diagnostic material, since various significant inorganic and organic compounds (e.g., proteins) are released into it. Saliva can be employed in the early detection and monitoring of numerous diseases, such as COVID-19, (1,2), cancer (3) or metabolic disorders (4), and also for pharmacokinetic investigations, and therapeutic medication monitoring. Furthermore, salivary collection is painless, simple, affordable, and fully safe for both patients and medical professionals. As a result, examination of the concentrations of numerous salivary components is becoming increasingly relevant for the diagnosis and monitoring of many oral and systemic illnesses (5,6).

1.1 The salivary glands and the secretion of saliva

Whole or mixed saliva refers to the saliva that is continually present in the oral cavity containing gingival crevicular fluid, bacteria, desquamated epithelial cells, and food debris in varying proportions and covers the teeth and oral mucosa. It is a complex mixture of fluids secreted by three pairs of major salivary glands, the parotid, submandibular, and sublingual glands, as well as countless salivary minor glands situated in the labial, buccal, palatal, lingual, and retromolar regions of the oral mucosa (7–10).

The parotid gland is the largest (15-30 g), usually single, salivary alveolar gland with a typical serous nature, positioned on the lateral side of the oral cavity (10). The glossopharyngeal nerve innervates the parotid gland parasympathetically, and the spinal nerve innervates it sympathetically (10).

The submandibular tubular-alveolar gland is located at the bottom of the submandibular triangle and weighs substantially less (7-16 g) than the parotid gland (10). The submandibular gland has a firmer consistency than the parotid gland and secretes a mixed (serous-mucous) secretion. The facial nerve innervates the submandibular gland parasympathetically and the spinal nerve sympathetically (10).

Sublingual glands (3-5 g) are the smallest of the major salivary glands (placed at the bottom of the oral cavity and protected by a mucous membrane). Sublingual glands are made up of 5-20 small, distinct glands (10). Sublingual glands are mixed salivary glands that release primarily mucus and have a shape similar to the submandibular gland (10).

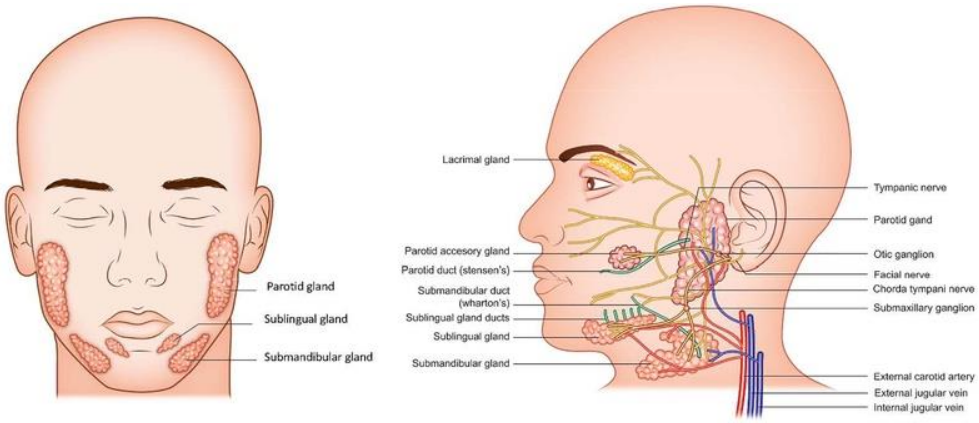

Figure 1. Human salivary gland anatomy. There are three pairs of main salivary glands (the parotid, sublingual, and submandibular glands), as well as hundreds of tiny salivary glands (not illustrated). Each major gland drains its contents into a unique duct that ends in the oral cavity. Although the primary salivary glands receive blood and neural impulses from intricate networks of arteries and nerves, only parasympathetic innervation is seen. Nerves (yellow), arteries and veins (red and blue, respectively), and ducts are depicted (green) (11).

The parotid gland produces more alpha amylase but less calcium than the submandibular gland. Mucins are mostly produced by the submandibular and sublingual glands, while proline- and histatin-rich proteins are primarily produced by the parotid and submandibular glands. Minor salivary glands are mostly mucus (7).

Daily secretion of saliva rates ranges from 500 to 700 mL, with an average volume in the mouth of 1.1 mL. The autonomous nervous system regulates saliva production. At rest, secretion ranges from 0.25 to 0.35 mL/min, with the submandibular and sublingual glands producing most of it. Sensory, electrical, or mechanical stimuli can increase the secretion rate between 1 to 4 mL/min. Saliva has a secretion peak at roughly 12 a.m. decreasing significantly at night, when sleeping. The major salivary glands produce around 90% of the fluid secretion while the minor glands produce less than 10%. However, small salivary glands secrete a sizable portion of the salivary mucins, which lubricate the oral surfaces. Table 1 shows the contribution of each gland to total saliva volume under both unstimulated and stimulated situations (7,12,13).

Table 1. Characterization of saliva secretory components and their contribution to entire saliva under unstimulated and stimulated conditions (7).

		Secretory product	**Contribution to saliva volume (%)**
Major salivary glands	Parotid	Watery, amylase-rich	Resting: 25% Stimulated: 50%
	Submandibular	Viscous, mucin-rich	Resting: 60% Stimulated: 35%
	Sublingual	Viscous, mucin-rich	Resting: 7%-8% Stimulated: 7%-8%
Minor salivary glands	Buccal	Mucin-rich	Resting: 8% Stimulated: 8%
	Labial	Mucin-rich	
	Lingual	Watery, lipase-rich	
	Palatinal	Mucin-rich	
	Retromolar	Viscous, mucin-rich	

The formation of saliva occurs in two phases. An initial secretion with plasma-like electrolyte content is stimulated. This primary isotonic saliva is then changed by reabsorbing sodium and chloride ions and adding bicarbonate and potassium ions without changing the water content due to the low water permeability. As a result, the final saliva that reaches the mouth cavity is hypotonic and contains less sodium than plasma (7).

Most organic compounds in saliva are created locally in the salivary glands, however certain molecules enter saliva from the bloodstream. Molecular transfer from blood to saliva is possible via several intracellular and extracellular mechanisms, including passive diffusion of lipophilic substances (such as steroid hormones) or active transport of proteins via ligand-receptor binding transfer biomolecules into saliva (14).

Unaided or passive diffusion is the most prevalent method for chemicals to transfer from blood to saliva (Figure 2). Many tiny chemicals can pass through the capillaries around the salivary glands. A serum molecule that diffuses into saliva must pass through five barriers: the capillary wall, the interstitial space, the acinus cell or duct cell's basal cell membrane, the cytoplasm of the acinus cell or duct cell, and the luminal cell membrane (14).

A molecule's capacity to diffuse passively through cell membranes is determined in part by its size and in part by the electrical charge and hydrophobicity. If a chemical is polar or splits into charged ions in solution, it will have difficulty passing through the ductal cell membranes, which are comprised of phospholipids. Steroid hormones, for example, are quite small and are mostly composed of fatty acids, therefore they tend to flow relatively quickly by diffusion. Other compounds attached to large carrier proteins, such as serum albumin, are too large to enter through this pathway (14).

Active transport through the secretory cells of the glands, which secretory IgA uses, is a second method for molecule entrance into saliva (SIgA). Polymeric IgA, for example, which is secreted by B-lymphocyte cells near salivary cells, is then bound by IgA receptors on acinus cells and released into saliva. It has been demonstrated that neuronal stimulation of the salivary glands increases SIgA production, but the precise mechanism by which the transport is expedited is unknown. Because SIgA concentrations in saliva are known to drop when saliva flow is encouraged, there must be a speed limit for transfer (14).

The process of filtration through the gaps between acinus and ductal cells is known as ultrafiltration (an extracellular mechanism, Figure 2). Molecules must be fairly small to follow this type of transfer into saliva. Sulfated steroids and estriol sulfates, which are electrically charged and cannot pass through the phospholipid bilayer of cell membranes, are thought to enter primarily by this pathway (14).

Furthermore, serum components may enter the saliva via the crevicular fluid (produced by the sulcular epithelium of the oral mucosa). Another route by which molecules are transferred to saliva is transudation, either from crevicular fluid or directly from the oral mucosa. The presence of several typical plasmatic components (such as plasma albumin) in the saliva is mostly determined by this process (14).

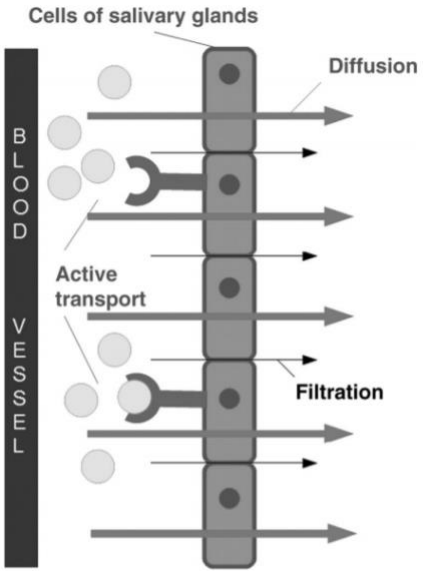

Figure 2. Representation of biomolecule's transport from blood capillaries into saliva (14).

1.2 Saliva – a complex biofluid

The composition of saliva differs between individuals at different times of the day (12). It is mainly composed of water (94–99%), but it also contains organic and inorganic compounds. The concentration of various components determines chemical and physical qualities such as the pH (6.2-7.6 with 6.7 being the average pH in fresh saliva). Changes in these concentrations have an impact on the oral cavity's homeostasis (6,15–18).

Saliva is sterile as it leaves the salivary glands, but it becomes contaminated when it comes into contact with crevicular fluid, food remnants, germs, desquamated oral mucous cells, and other substances (13). Urea, ammonia, uric acid, glucose, cholesterol, fatty acid, triglycerides, neutral lipid, glycolipid, amino acid, steroid hormones, mucin, amylase, lectin, glycoprotein, lysozyme, peroxidase, and lactoferrin are all part of the complex composition. It also has significant quantities of inorganic cations like Na^+, K^+, Mg^{2+}, Ca^{2+}, and anions like Cl^-, F^-, and I^- from the serum. These ions are involved in the transfer of active substances via cellular membranes, the development of dental structures, and the activation of enzymes as hydrolases, phosphatases, dehydrogenases, and peroxidases. Furthermore, saliva contains over 700 bacteria linked to oral and systemic disorders (6,15–18).

Over 4000 proteins are identified in human saliva (19). Mucins are the most abundant salivary proteins. These glycoproteins account for 20-30% of total proteins. They oversee saliva's viscosity and density. The primary function of glycoproteins is to ensure lubricity, participate in the mineralization of teeth, and defend the immune system by binding to pathogens (15,17). Overexpression of mucins in the oral cavity has been associated with cancer (12,20). The remaining proteins in saliva are cystatins and immunoglobulins, involved in inflammatory processes, while lysozyme and a-amylase are involved in bacterium digestion. Saliva contains lipids (10-100 g/mL) in addition to proteins, which may play an essential role in the production of dental plaque and caries. Finally, chemicals found in the blood and tissues, such as DNA and RNA, can be found also in saliva (15,17).

1.2.1 Factors influencing saliva composition

The composition of saliva depends on several factors that should be considered when using saliva as a diagnostic fluid. Saliva changes can be quantitative (flow rate) or qualitative (composition, rheology, lubrication).

As explained in chapter 1.1, nerve impulses from the autonomic nervous system and the central nervous system influence physiological salivary secretion (17). Salivary composition and secretion depend also on the type of the gland that secretes saliva, as well as a patient's age, gender, number of teeth, body weight, and the type of stimulating factor (6,7). Salivary secretion is also influenced by the body's degree of hydration (7). The stimuli and saliva components (described in section 1.2) can change the volume, flow, and content.

Drugs can have a variety of effects on the salivary secretory systems. The most prevalent cause of salivary gland hypofunction and xerostomia is pharmaceutical use, including antidepressants, anxiolytics, opiates, antihypertensives, diuretics, and antihistamines (7).

Salivary flow can also be affected by pathological conditions. Some systemic disorders, such as Sjögren's syndrome, cause progressive destruction of the salivary glands, whereas others, such as hypertension, depression, malnutrition, dehydration, diabetes, and others, cause vascular or neurological changes that have transitory and reversible effects on saliva production (7,13). Changes in salivary flow, on the other hand, modify the composition, which has a direct impact on the quantity, quality, and buffer effect (pH) to teeth protection. Due to the low flow rate and higher pH, which contribute to dental erosion, dental problems may be linked to other diseases such as xerostomia or gastroesophageal reflux (21–23).

1.2.1.2.1 Circadian rhythm

The circadian rhythm controls the flow rate cycle, pH and mouth temperature. Salivary protein concentrations follow a similar pattern, although salivary sodium and chloride concentrations follow the opposite rhythm, peaking in the early morning (7,13). Saliva flow and mouth temperature exhibit varied circadian cycles when stimulated versus unstimulated. It has been proven that salivary component secretion levels follow a circadian pattern (24).

The circadian rhythm and sleep have been connected to salivary pH. Salivary flow changes have been linked to dental erosion, which is the chemical-mediated deterioration of teeth caused by low pH in the oral cavity. This is most common in those who have a decrease in salivary flow. Other symptoms include dry mouth, gastric reflux disease, and sleep problems (21–23,25).

Intraoral temperature fluctuates along with the circadian rhythm and that influences the saliva composition during the day. It is thought to have a close link with pH. High temperature and sleeping with an open mouth have been linked to low saliva volume and acidic pH. When the temperature is lowered, such as by opening the mouth during sleep, the saliva evaporates, leaving less saliva to maintain the pH (21–23,25).

Protein expression and circadian rhythms are not equal for all salivary glands, with a significant difference in the total protein secreted by the parotid during the day, which has a strong influence on the concentration and composition of this type of saliva, and a minor variation for whole saliva. These variations reflect the stability of

protein synthesis in the submandibular and sublingual glands. Variations within subjects for numerous specific salivary proteins and total protein concentrations during the day show that these proteins are sensitive to short-term variation at the time of collection (17).

1.2.1.2.2 Age and gender

One of the elements determining salivary flow rate is age. Although some studies relate flow differences with age, this relationship is not consensual. Some studies indicate that the differences may be due not to age but to other factors such as medication, people's health status, collection and analysis methods, or the use of multiple medications (7,17,26). Hyposalivation is a common occurrence in the elderly because of this polypharmacy for age-related chronic disorders. Furthermore, like with most physiological activities, immunological activity declines with age, as demonstrated by a reduction in salivary immunoglobulin concentrations (17).

Salivary components, enzymes, hormones, electrolytes, and inorganic compounds can fluctuate dramatically from infancy through puberty, and their concentration reflects both the age and developmental stage. More specifically, salivary - amylase levels rise with age, peaking in adulthood, while calcium and magnesium levels fall, possibly due to the physiological growth of salivary glands. As a result, salivary cortisol levels are largely dependent on the circadian rhythm, which develops throughout the first three years of life (18).

Interestingly, studies on changes in salivary composition in healthy elderly individuals are relatively scarce (Table 2). Age has a particularly infamous effect on the salivary proteome pattern in humans, which is mainly translated as a general decline in the expression of several proteins. For example, there is a considerable age-related decline in histatin concentration in parotid saliva as well as submandibular/sublingual saliva (17). There is a general agreement that mucin concentrations decline with age. Salivary mucins (a family of glycoproteins found in saliva that cover the oral mucosa) produce an immobile pellicle that is kept on epithelial cells (membrane-associated mucins: MUC1, MUC3, MUC4, MUC12) and a mobile salivary film (secreted soluble mucins: MUC2, MUC5A, MUC5B, MUC6, MUC7). MUC1 levels were found to be lower in elderly participants, promoting the development of oral mucosal illnesses in the elderly population. Furthermore, MUC1 and MUC2 levels in unstimulated saliva are considerably lower in the healthy aged group compared to young individuals. Dry mouth’s patients have altered saliva rheological characteristics and decreased mucosal hydration, indicating functionally compromised saliva, which is mostly attributed to MUC5B and MUC7 deficiency (26). Other saliva proteins, however, change their expression with age, such as amylase, whose levels rise until middle age (the 40s) and then fall. Surprisingly, the quantity of salivary N-glycoproteins appears to rise with age (particularly so in males), primarily the acidic and low molecular weight glycoproteins. Variations in saliva’s viscosity and volume have been recorded with aging (17).

Table 2. Significant changes in human unstimulated saliva in elderly people (26).

Saliva components	Reported increased concentration	Reported decreased concentration
Inorganic	Potassium Chloride Phosphate Uric acid	Calcium (Ca^{2+})
Organic	Lysozyme Amylase IgA	MUC1, MUC2 Lactoferrin, Transferrin Reduced and oxidized Glutathion Peroxidase activity

When comparing the elder to the young population, the concentration of inorganic components such as potassium, chloride, phosphate, and uric acid increased significantly. This rise in salivary ionic concentration was linked to decreased salivary volume. With a reduced salivary flow rate, the water secretion route is disrupted, resulting in concentration effects on the ions. However, a drop in calcium was recently detected (7,26).

Studies on basal salivary amylase activity show undetectable levels in neonates and levels comparable to adult concentrations during adolescence, which could be related to the physiological development of salivary glands. Divergent outcomes on salivary amylase levels after various forms of stresses are reported across different age groups (27,28).

Microbial colonisation also changes the salivary composition and is impacted by the style of delivery (caesarean or vaginal), the start of feeding, and the kind of nutrition (breast-feeding or formula). Teeth eruption, the presence of crevicular fluid, and the introduction of solid foods result in additional variations in oral microbiota and salivary profile differentiation between 3 and 6 months of age (29,30).

The unstimulated salivary proteome shows gender variations in immunological function, metabolism, and inflammation. Male and female bodies have various physiologies, and salivary gland discharge differs by gender. Females exhibit lower salivary pH, buffering capacity, protein content, and mean salivary flow rate in unstimulated saliva (6,17). In fact, total protein concentration was found different between genders (31).

1.2.2 Salivary omics

Researchers have introduced the concept of salivaomics due to the significant development made in salivary studies. Salivaomics is a broad term that includes the study of genomes, transcriptome, proteome, metabolome, and even microRNA (miRNA) (5,6,8,32).

Proteomics - the study of protein profiles - is under the focus of this dissertation and will be discussed below.

SDS-PAGE, two-dimensional gel electrophoresis (2-DE), quantitative mass spectrometry (qMS), Western blotting, enzyme-linked immunosorbent assay (ELISA), surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS), or Raman spectroscopy (RS) are some of the most common proteomic technologies (6,8,12,32) applied to the study of saliva. From a biochemical/metabolic

perspective, proteins are the most important constituents of saliva. The main target of proteomics is the identification and characterization of peptides and proteins that display biological activity at the glandular level and/or under various pathological conditions (14,33,34).

Typical proteomics workflows are divided into bottom-up and top-down platforms. Top-down proteomics' approaches look at each protein, avoiding sample modifications as much as possible. Bottom-up proteomics involves pre-digesting the sample (usually with trypsin) and then analysing peptide fragments using high-throughput analytical methods. The discovery of one or more of a protein's particular (proteolytic) fragments in the sample implies bi-univocal correspondence between the parent protein and its fragments. The bottom-up strategy's minimalistic approach may result in the loss of essential chemical information. As a result, numerous fragments might not be linked to a single parent protein. Despite this, bottom-up platforms have demonstrated the highest throughput in terms of the number of detected components. The sensitivity of next generation mass spectrometers is sufficient to detect thousands of peptides in a single analysis, but the main issue is the increase in time required for the various separation stages. As a result, advancements in the separation platform used in shot-gun proteomics result in simpler peptide detection. Shot-gun proteomics covers the broadest range of detectable components, regardless of mass, because proteolytic breakdown of large proteins almost invariably generates proteolytic peptides that reveal the existence of the parent protein in a complex mixture (33).

Top-down platforms are inherently constrained by the sample treatments required for mass spectrometry coupling (usually formic acid or trifluoroacetic acid treatment), which unavoidably excludes proteins that are insoluble in acidic solution. Furthermore, even high-level MS apparatus cannot access intact high-molecular-weight proteins or heterogeneous glycosylated proteins in their naturally occurring forms. As a result, every proteomic platform has advantages and disadvantages. For all of the reasons stated above, the ideal strategy to do a robust biomarker identification is to analyse an adequate number of samples using several proteome techniques, even though this option is not available to the majority of laboratories (33).

MS-based proteomics deposition in publicly available databases still underdeveloped comparing to other data-intensive fields like genomics. Among other factors, are the intrinsic complexity of the data, the range of data formats, and experimental processes. Several publicly accessible repositories for MS proteomics studies have been created in response to this demand, each serving a particular function. The general results from these MS-based proteomic approaches are stored most of the time as raw results from the experiences, without a finest curation. The Global Proteome Machine Database (GPMDB), PeptideAtlas, and the PRIDE database are the most known sources (35).

These complex but not curated databases open a gap to databases like the Saliva Proteome Knowledge Base (http:// www.skb.ucla.edu) and SalivaTecDB (http://salivatec.viseu.ucp.pt/salivatec-db) that centralize proteomic data, and provide curated MS-based, annotated saliva proteins (19,36).

SalivaTecDB has information on 4231 human proteins, 15126 microbial proteins, 6121 mi-RNAs, and 2056 distinct oral cavity taxa (saliva, crevicular fluid, mucosa, oral biofilms, http://salivatec.viseu.ucp.pt/salivatec-db/, accessed on July 2022). These proteins were retrieved from peer-reviewed, indexed articles describing proteomics

data from the oral cavity. SalivaTecDB comprises oral protein data from healthy people as well as patients with one of 24 pathologies/conditions (Table 3): 10 oral and 14 others.

Table 3. Oral and systemic disorders related to human oral proteins accumulated in SalivaTecDB. Adapted from (36).

	Condition	**Nº of Proteins**
Health	Health	3033
Oral diseases	Aggressive periodontitis	111
	Chronic periodontitis	1235
	Dental caries	886
	Gingivitis	396
	Head and neck neoplasms	8
	Mouth neoplasms	228
	Leukoplakia, oral	2
	Lichen planus, oral	2
	Peri-implantitis	38
	Precancerous conditions	62
Other diseases	Alzheimer disease	1
	Breast neoplasms	178
	Cardiovascular diseases	25
	Diabetes mellitus, type 1	34
	Diabetes mellitus, type 2	497
	Liver neoplasms	1
	Lung neoplasms	2
	Ovarian neoplasms	2
	Pancreatic neoplasms	1
	Parkinson disease	3
	Prostatic neoplasms	2
	Scleroderma, diffuse	9
	Sjogren's syndrome	458
	Skin neoplasms	1

Protein analytic tools have advanced significantly, resulting in a new revolution in salivary proteomics when paired with bioinformatics. A comprehensive study of the salivary proteome is required to fully understand its diagnostic potential (34).

1.3 Proprieties and functions

Saliva serves multiple functions important for the maintenance of oral and general health (37). Saliva lubricates and cleanses the teeth and oral mucosa, maintains neutral pH through its buffering capacity, prevents tooth demineralization, has antimicrobial properties, aids in taste and bolus formation, initiates enzymatic starch digestion, and is required for mastication, swallowing, and speech articulation. It is also involved in the production of the acquired enamel pellicle and the mucosal pellicle, which, in addition to protecting the teeth, determine the initial adhesion and colonization of microorganisms as well as the composition of the resident oral microbiota (6,7,12,14,37).

Saliva contains a variety of proteins and peptides with distinct biological roles, some of which are microbial in origin. There is a diverse panel of host salivary proteins and peptides, as well as a core set of proteins (

Table 4). Salivary proteins perform a variety of activities; for example, acidic proline-rich proteins, histatins, cystatins, and statherins bind to calcium and have a high affinity for hydroxyapatite. Statherin inhibits the formation of calcium phosphate salts from saliva, playing an important role in tooth integrity. Histatins, a group of basic proteins that play a role in wound healing, have antibacterial activity, and bind to enamel, are examples of more prevalent salivary proteins that have multifunctionality and act in synergy (6,7,12,14).

The saliva components phosphate, bicarbonate, urea, and enzymes provide the clearance activity. Saliva contains cystatins, which suppress inflammation, and mucins, which protect the mucous membrane from dietary toxins. Additionally, the moisturising effect guards against dryness and premature cell death. Magnesium and calcium ions stimulate wound healing by inhibiting demineralization (13,15).

Salivary fluid employs two infection-prevention techniques. One is based on nonspecific antibacterial and antifungal components such as salivary peroxidase, histatin, and lysozyme. Saliva, on the other hand, uses specialised immune components such as immunoglobulins like IgA, IgM, and IgG, which are responsible for phagocytosis. Additionally, saliva contains several antioxidants that inhibit cellular oxidation. Saliva, due to its rich composition, is functionally equivalent to blood in reflecting the physiological status of the body, and so it may provide information about the organism's hormonal, nutritional, and immunological functions (13,15).

Table 4. Saliva functions are related to its components and their mode of action. Adapted from (7,13,15).

Function		Component	Mode of action
Maintenance of oral health	Lubrication of oral surfaces	Mucins	Mucins are large, highly glycosylated proteins that form a hydrophilic network. MUC5B is the primary gel-forming mucin, MUC7 is less efficient as lubricant. Moisten and lubricate oral surfaces, give saliva its texture and viscosity
		Glycosylated proline-rich proteins	
		Water	
	Oral clearance	Water	Elimination of microorganisms, dietary sugars and acids by dilution and swallowing
	Buffer capacity	Bicarbonate	Buffer acids from dietary intake and acids produced by bacterial fermentation of sugars, thereby maintaining pH in the neutral range, decreasing the tooth demineralisation rate and promoting/maintaining a balanced oral microbiota
		Phosphate	
		Proteins	
	Salivary pellicle formation	Salivary proteins	Salivary proteins, *e.g.* mucins, proline-rich proteins, α-amylase, cystatins, statherins, lysozyme, lactoferrin, sIgA a.o. interact with dental and mucosal surfaces, each other, and oral microorganisms, thereby altering their properties and ability to modulate the microbial colonisation in the oral cavity. MUC1 and MUC4, which play a role in cell signalling, also interact with other salivary proteins
	Tooth mineralisation	Proline-rich proteins	High affinity to hydroxyapatite, bind to calcium, inhibit spontaneous precipitation of calcium phosphate salts from the dental surfaces, important for the integrity of the teeth
		Cystatins	
		Statherins	
	Antimicrobial actions	Mucins	Mucins, promote aggregation of microorganisms, especially MUC7; antibacterial, antifungal and antiviral
		Histatins	Antifungal, moderate antibacterial
		Cystatins	Antibacterial, antifungal and antiviral
		Statherins	Antibacterial, antifungal and antiviral
		Proline-rich proteins	Antibacterial (Gram-negative), antiviral
		Peroxidases	Catalyse oxidation of thiocyanate to hypothiocyanite by hydrogen peroxide; antibacterial and antifungal
		α-amylase	Antibacterial, provide nutrition for certain bacteria via hydrolysis of starch
		Lysozyme	Hydrolysis of the polysaccharide layer of the gram-positive bacterial cell wall; antibacterial, antifungal and antiviral
		Lactoferrin	Binding and sequestering of iron, depriving microorganisms of iron; antibacterial, antifungal and antiviral
		Immunoglobulins, mainly sIgA	Inhibit microbial adhesion, enhance phagocytosis, aggregate microorganisms in interactions with other proteins. Antibacterial, antifungal and antiviral
		Defensins	Antimicrobial peptides
	Tissue repair	Growth factors	Epidermal growth factor (EGF) promotes proliferation and migration of oral epithelial cells for wound healing; fibroblast growth factor (FGF) promotes wound healing and tissue repair
		Water, mucins	Protects oro-oesopharyngeal mucosa from injury

Digestive functions	Taste	Water, mucins	Dissolution and transport of taste substances to taste buds
		Gustin	Growth and development of taste buds, integrity of taste sensitivity
		Salivary proteins	Salivary composition influences the perception of fat, saltiness, bitterness, and the perception of texture
		Electrolytes	non attributable
		Proline-rich proteins	Precipitate tannins and thus contribute to the sensation of astringency
	Initial digestion	α-amylase, lipase	α-amylase cleaves the α-1,4- glycosidic linkages of starch into maltose, maltotriose and dextrins
	Mastication		Hydrolyses triglycerides into partial glycerides and free fatty acids
	Food bolus formation, swallowing	Water, mucins	Promotes and facilitates bolus formation and swallowing
Articulation of speech		Water, mucins	Facilitates articulation of speech

1.4 Collection, processing, and storage of saliva samples

Researchers collect saliva with unstimulated collection methods. Saliva is allowed to accumulate on the tongue's floor, and participants spit into a sterile tube for a period (usually 5 min). This is known as 'unstimulated saliva, and it occurs when salivary flow is not impacted by external stimuli. A tube is used for 'stimulated saliva' collection, where participants actively spit a chemical or mechanical stimulant, such as citric acid solution, or chew a piece of paraffin-wax, gauze, or other innocuous chewable substance. Unstimulated saliva is mainly secreted from the sublingual and submandibular glands, while stimulated saliva is secreted mostly by the parotid gland. Stimulated saliva contains lower quantities of protein (e.g. glycosylated mucin) and has a lower viscosity than that of the unstimulated counterpart (5,6,14,18,26).

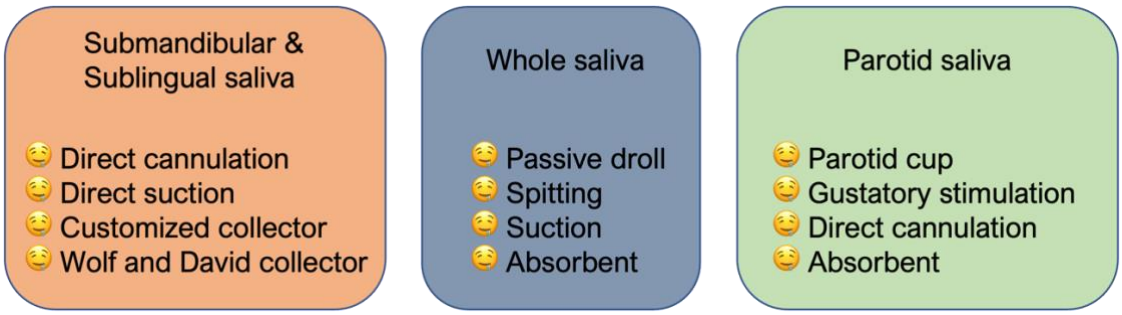

Figure 3. Main saliva collecting methods for the submandibular/sublingual glands, whole saliva, and parotid saliva (16).

Draining (passive drool), Spitting, Suction, and Absorbent are the unstimulated saliva collecting procedures. To be collected, saliva is allowed to accumulate in the floor of the mouth with a forward tilted head, and saliva is allowed to drop continuously off the lower lip into a collecting tube (a funnel may help), with any oral movement restricted. In principle, this is the optimum way because it eliminates any bias such as reflex stimulation or differing contributions from salivary glands. Spitting is a form of draining that requires the individual to spit saliva into a collection tube

after the saliva has accumulated in the mouth. Higher bacterial contamination of the sample is expected with this approach. The suction method draws saliva from the floor of the mouth using a small aspirator device. The absorbent method is inserting a cotton roll, swab, or foam substance into the mouth, withdrawing it from the oral cavity, and centrifuging the adsorbed material to produce the final sample. To reduce operator errors in large studies with different personnel involved in collecting, the use of absorbent devices may be recommended. Furthermore, their use is required in the event of small toddlers or adults with motor or sensitivity problems who struggle with the passive drool approach. The location of the absorbent in the mouth is critical in this approach because each gland may contribute differently. Nonetheless, the results from the saliva of the swab put beneath the tongue should be comparable to those obtained from complete saliva collected by passive drool (17).

After gustatory, masticatory, pharmacologic, or mechanical stimulation, all forms of saliva can be collected with enhanced excretion rates. Gustatory stimulation with acidic solutions (e.g., citric acid) and mechanical stimulation by chewing are the two most employed treatments. The varied proportions of saliva released from each gland based on the technique of stimulation cause variation in saliva's rheology. Other differences are related to differences in subject characteristics such as oral buffer capacity, latency time, or blood perfusion circumstances. When salivary glands are stimulated, the first fluid should be removed to allow the latency time to stabilise secretion (at least during the first minute). Many salivary proteins are released in response to acid stimulation, affecting the salivary proteome composition, which should not be overlooked. In practise, stimulation is useful in two situations: when not enough saliva can be collected without their use (*e.g.*, xerostomic patients) or when collecting saliva from parotid glands, that have low saliva flow rates at rest. There are several additional benefits to collecting stimulated saliva, such as better standardisation of salivary flow in a heterogeneous group of patients, faster collection, and more subject convenience. Stimulants should be used sparingly and consistently during the experiment since they can aggravate interindividual variance and changes in saliva content (17).

Although salivary collection procedures may have a major impact on the precision and determination of biomarkers, there are currently no recognized uniform guidelines for the collection of human saliva (6). Nonetheless, there are some recommendations that should be followed:

- Saliva should be collected between 8 and 10 a.m. to minimize the effect of circadian rhythms on the outcome of salivary biochemical assays.
- For at least two hours before salivary collection, patients should not eat or drink (except pure water) and should not do any hygienic operations inside the oral cavity (*e.g.*, teeth cleaning).
- Because many medicines influence salivary secretion, patients should avoid taking them at least 8 hours before salivary collection.
- After 5 minutes of acclimation to the environment, saliva should be collected in a separate room from a sitting, comfortable patient with their head slightly bowed down and minimum movement of the face and lips (5,6).

1.5 Diagnosis using saliva

Whole saliva has recently gained interest as a potentially valuable fluid in the finding of biomarkers for illness diagnosis and monitoring (8). It is a very appealing body fluid for disease diagnosis for many reasons: i) collection of saliva is usually inexpensive, safe and easy, and can be performed without the assistance of healthcare workers (it allows for home-based sampling); ii) it is considered an acceptable and non-invasive process by patients since it does not provoke any pain (and can be easily collected by paediatric patients), alleviating patients' discomfort and ensuring compliance (6,18,26). Saliva is a good diagnostic medium and has frequently been utilized for investigations in different populations; it is a feasible biofluid to get in community settings, and it is especially suitable for large epidemiological cohort studies. Furthermore, saliva allows for repeated sampling, is easy to handle, and does not require specialized equipment (18). Nonetheless, the low concentration of analytes in saliva compared to blood has been a drawback of salivary diagnostics.

Proteomic has been applied to the study of human saliva, identifying and characterizing over 4000 differentially expressed proteins and peptides [data available at SalivaTecDB (19,36)]. It is expected that the comprehensive analysis of the massive amount of data currently available will allow for the tailoring of therapeutic interventions, in the near future (6,18,33). The level of evidence supporting the validity of the salivary biomarkers varies greatly (18). The concept of testing saliva for infection or disease may appear simple; yet, implementing it in clinical practise has proven problematic. Some biomarkers are more reliable than others; for example, salivary cortisol is a well-studied stress biomarker that is recognised as a "gold standard" in clinical trials, as opposed to salivary amylase, for which the literature is still inconsistent (6,18).

The identification and analysis of biomolecules found in saliva can provide information on the function of various organs in the body (8). A range of oral and other disorders, such as Alzheimer's disease, diabetes, cystic fibrosis, and oncological diseases (especially head and neck tumours), can produce quantitative and qualitative changes in saliva (6). Advances in molecular biology and techniques such as mass spectrometry and RT-PCR now allow for the examination of qualitative and quantitative variations in these biomolecules in the dynamic health-disease processes. These advances enable the identification of prospective biomarker candidates for detecting physiological changes before clinical symptoms appeared (8).

Saliva protein profiles are indicative of an individual's health and well-being status at the time of collection: saliva can be considered a "real-time" fluid. Whole saliva is the most used method for diagnosing systemic disorders since it is easily obtained and, more importantly, contains serum components (38). While proteome constituents are a natural first choice for salivary diagnostic analytes, genomic targets have emerged as extremely informative and discriminatory indicators (39). To improve diagnostic accuracy and specificity, saliva diagnostics should rely on combinations of biomarker panels employed as screening tools in the future. One biomarker may not be sufficient as a credible source for researchers to define the pathophysiology of the underlying disease. The use of biomarker combinations may give additive and potent diagnostic information (14).

1.5.1 Oral diseases

Oral health is an important component of general health and well-being since it allows for basic daily functions. Dental caries (tooth decay), periodontal (gum) disease, and oral malignancies are examples of chronic clinical illnesses that affect the teeth and mouth (40).

Despite being largely preventable, oral diseases are extremely widespread, impacting over 35 billion people worldwide; dental caries is the most frequent disease globally, with rising prevalence in many low- and middle-income nations (40,41). Many studies have shown that *S. mutans* has a function in the initiation of dental caries, whilst Lactobacilli play a role in the progression of carious lesions. High salivary levels of these pathogens, measured by a commercially available test (CRT bacteria®, Ivoclar-Vivadent Inc., Amherst, U.S.A.), have been linked to the prevalence of caries in both children and adults (42). Changes in the quantity and composition of saliva can also be used to detect and monitor caries. However, no single salivary test has consistently demonstrated consistent accuracy in diagnosing caries. Rather, a combination of recognised risk variables has been proposed to predict persons at risk for caries. To yet, however, none of the risk assessment programmes presented have demonstrated consistent validity (18,43).

The prevalence of periodontal disease was recently estimated to range from 20% to 50% around the world (38). Several salivary biomarkers have been examined for periodontal disease diagnosis and prognosis. More particular, the presence of matrix metalloproteinase-8 (MMP-8, an enzyme responsible for tissue degradation) in GCF has been linked to the advancement of periodontitis. Salivary soluble toll-like receptor-2 and interleukin-4 have recently been linked to the progression of periodontal disease (18,43).

Because periodontitis is a complex illness, it has been advocated that not only host-derived variables, but also oral infections should be examined in saliva to predict it. Most periodontal infections can be detected with a salivary test (MyPerioPath®, OralDNA® Labs) (43). Periodontal disorders can be caused by abnormalities in more than 70 genes (43).

1.5.2 Systemic diseases

It has been suggested that saliva can be viewed as “the mirror of the body”, reflecting the body’s general state of health (16).

Some autoimmune disorders can already be detected using saliva (43). Salivary proteomics were suggested as useful method for diagnosing Sjogren's syndrome (44). It is based on the identification of numerous biomarkers that are impacted by the disease at the same time. A panel of putative salivary biomarkers of Sjogren's syndrome was recently studied (43).

C-reactive protein levels in saliva correspond with plasma C-reactive protein levels in a population at risk for cardiovascular problems (45). It is also feasible to detect cardiac troponin, a biomarker for acute myocardial infarction that is released in response to cardiac cell necrosis, in saliva (46). In patients suffering from an acute myocardial infarction, salivary C-reactive protein levels were demonstrated to be a monitoring/diagnosis tool as sensitive as blood levels (43).

The Food and Drug Administration approved two commercial kits to detect HIV using saliva. The results were then compared to serum. The results showed a predicted positive value above 98% in high-risk populations and 88% on low-risk populations (43).

COVID-19 diagnosis is primarily based on reverse transcription polymerase chain reaction (RT-PCR) detection of SARS-CoV-2. Although SARS-CoV-2 RNA detection in nasopharyngeal swabs has been reported as the gold standard method for COVID-19 diagnosis, sample collection by this method needs close contact with infected patients or suspected COVID-19 cases (47). The detection of SARS-CoV-2 in saliva allowed the diagnosis of COVID-19, and can be as reliable as the oropharyngeal swabs(47,48).

There has been very little investigation on salivary testing for diabetes diagnosis. This is most likely due to the availability of simple pinprick assays to check glucose blood levels on the market. Salivary proteomics, on the other hand, is an appellative option for individuals who prefer a less invasive approach to screening. As a result, protein profiling in saliva could be an interesting future option for diagnosing and monitoring diabetes (43).

2. COVID-19 and Diabetes – modern world major challenges

2.1 Emergent pandemic diseases

Due to the sharp demographic growth, the ease of contact between people from distant countries due to improvement of transport, among other factors, diseases with a profound and widespread impact (pandemic diseases) have emerged.

Two of the most widespread and most worrying diseases today are Diabetes, for its "silent" growth over the last few decades, and Coronavirus disease of 2019 (COVID-19) for its extraordinary efficiency in transmitting from person to person and its relatively high level of morbidity and mortality, especially among elderly and those with underlying co-morbidities (49,50).

Presently, diabetes and COVID-19 are considered major global health concerns (49).

2.1.1 COVID-19

Because of the global COVID-19 outbreak, health services around the world have been in an unprecedented state. Urgent collaborative research efforts on the development of SARS-CoV-2 rapid tests, accurate diagnosis, particularly early recognition, and effective treatment of life-threatening complications were highly desirable for humanity and the medical workforce all over the world (51). This pandemic infected about 630 million individuals and killed over 6,5 million (52). Regular testing was required to restore normalcy to life.

2.1.1.1 Molecular Mechanisms and health impact

COVID-19 is a highly contagious respiratory infection that is caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). SARS-CoV-2 infects mainly alveolar epithelial type 2 (AEC2) cells through the angiotensin-converting enzyme 2 (ACE2) receptor. Upon the occupancy of ACE2 by SARS-CoV-2, the increased serum level of free Angiotensin II (Ang II) due to a reduction of ACE2-mediated degradation promotes activation of the NF-kappa B pathway via Ang II type 1 receptor (AT1R), followed by interleukin-6 (IL-6) production. SARS-CoV-2 also activates the innate immune system; macrophage stimulation triggers the overproduction of pro-inflammatory cytokines, including IL-6, and the "cytokine storm", which results in systemic inflammatory response syndrome and multiple organ failure. The combined effects of complement activation, dysregulated neutrophilia, endothelial injury, and hypercoagulability appear to be intertwined to drive the severe features of COVID-19 (53–55).

Most patients endorsed at least one symptom 6 months after symptom onset: fatigue or muscle weakness, sleep difficulties, and worry or sadness. Patients who were critically sick were at a higher risk of lung diffusion anomaly, weariness or muscle weakness, and anxiety or depression. The neutralising antibody seropositivity and titres were much lower than in the acute period (56).

COVID-19 epidemic posed significant challenges to healthcare systems around the world (57). One of the main vulnerabilities of healthcare systems worldwide is the risk to healthcare employees. Given that most healthcare employees cannot work remotely, methods such as the early deployment of viral testing for asymptomatic and/or frontline healthcare staff were critical. High healthcare expenditures, a lack of protective equipment such as N95 face masks, and a scarcity of ICU beds and

ventilators have all exposed flaws in patient care delivery (58). This created a need for self-sample collection to reduce professional health-worker viral transmission and the overwhelming workload of these workers on these duties.

2.1.1.2 Economic impact

Social isolation, self-isolation, and travel limitations have resulted in a diminished workforce across all economic sectors, resulting in the loss of numerous employments. Schools have closed, and the demand for commodities and manufactured goods has dwindled. In contrast, the need for medical supplies has skyrocketed. The food industry was also seeing higher demand as a result of panic buying and hoarding of food supplies (58).

According to a recent study conducted in the United States by Bartsch et al., the average direct medical costs per individual throughout the illness were estimated to be $3045 US Dollars (59).

The development of the disease resulted in various indirect expenses, including potential production loss owing to job absenteeism during hospitalisation and recovery at home, as well as production loss due to early mortality. The productivity loss of COVID-19 patients was approximately twice as high as the direct medical costs per patient on average (59).

2.1.1.3 Diagnosis and early detection

COVID-19 outbreak in China was linked to virus spreading because of asymptomatic illness characteristics and late onset of symptoms, due to a long incubation period. A longer incubation period offers more opportunities to prepare and take early action against COVID-19. Asymptomatic patients can be diagnosed at the outset of the disease, and early symptoms arising over the course of the disease provide a credible possibility to establish an earlier diagnosis than typical (60).

COVID-19 is currently detectable via RT-PCR, which detects the presence of genetic fragments of SARS-CoV-2 inside nasal and pharyngeal epithelial mucus membrane secretions. The RT-PCR and immunoglobulin presence detection methods used have their own limits in terms of detection within a specified period. Without regular monitoring, the unawareness of people being infected with COVID-19 result in an increase in transmission rate (60).

2.1.2 Type 2 Diabetes Mellitus

Diabetes Mellitus is a rapidly spreading metabolic illness that is estimated to affect 9.3% (463 million people), rising to 10.2% (578 million) by 2030 and 10.9% (700 million) by 2045 (61). American Diabetes Association has placed the cost of diagnosed diabetes in 2017 at $327.2 billion (62). Due to the long-term asymptomatic clinical manifestation of diabetes mellitus, identifying biomarkers as a practical guideline with high specificity and sensitivity for diagnosis, prognosis, and clinical management of Diabetes Mellitus is one of the issues of significant interest among Diabetes Mellitus researchers (63).

2.1.2.1 Molecular mechanisms and health impact

Type 2 Diabetes Mellitus (T2DM) is characterized by chronic hyperglycemia due to insulin resistance of peripheral tissues (skeletal muscle, liver, adipose tissue) and insufficient compensatory insulin secretion by pancreatic beta cells. Both insulin

resistance and beta cell dysfunction are thought to result from the complex interplay of many different pathways under the combined control of environmental and genetic factors The inhibition of ITS1 funtions promotes the insulin resistance through the FFA, TNFalpha and cellular stress factors. Serine/threonine phosphorylation, interaction with SOCS, regulation of the expression, modification of the cellular localization, and degradation represent the molecular mechanisms stimulated by them. The development of type II diabetes requires impaired beta-cell function. Chronic hyperglycemia has been shown to induce multiple defects in beta-cells. Hyperglycemia has been proposed to lead to large amounts of reactive oxygen species (ROS) in beta-cells, with subsequent damage to cellular components including PDX-1. Loss of PDX-1, a critical regulator of insulin promoter activity, has also been proposed as an important mechanism leading to beta-cell dysfunction (53–55).

Diabetes can cause damage to the heart, blood vessels, eyes, kidneys, and nerves over time. Diabetes increases the risk of heart attack and stroke by two to three times in adults (64). Diabetes is linked to the development of complications, which can impair health-related quality of life and increase mortality risk. These include both macrovascular (e.g., coronary artery disease, stroke, and peripheral vascular disease) and microvascular consequences (e.g., retinopathy, nephropathy, and neuropathy) (65).

Diabetes-related distress is a phenomenon that occurs because of the progression and diagnosis of this disease. This refers to unfavourable emotional reactions to a diabetes diagnosis, self-management demands, complications risk, insufficient provider care, and unsupportive interpersonal interactions (65).

2.1.2.2 Economic impact

Diabetes is one of the most expensive chronic diseases, accounting for $327.2 billion in economic expenditures in 2017 (62). Diabetes is the fifth cause of death in Portugal, with 1 million people estimated to have the disease and 1.7 estimated to be at risk. The direct costs including healthcare and treatments reach 1,3 to 1,5 billion Euros and the indirect costs can reach 1 to 1,2 billion Euros (66).

In Portugal, approximately one-third of Type 2 Diabetes Mellitus (T2DM) patients are supervised by caregivers and get treatment at home. However, this condition is more common in individuals with co-morbidities and greater illness severity, leading to an heath system overburden and societal expenditures (60). The average total direct cost of a hypoglycaemic episode was estimated to be 2323.73€. In terms of treatment groupings, insulin-treated patients have a higher average total direct cost (2673.02€) (67).

In 2019, an estimated USD 161.4 billion was spent on diabetes in Europe, with approximately one-third of that corresponding to indirect expenditures, primarily owing to early death. This economic impact is mostly caused by two factors: 31.4% of T2DM deaths occur before the age of 60 (that is, in patients in the economically active age group); 2) individuals with severe hypoglycemia are more likely to die, independently of the therapeutic strategy for glycemic control (intensive or conventional) (67).

2.1.2.3 Risk factors

Though there is a significant hereditary component, most occurrences are associated to risk factors such as age, overweight and obesity, and physical inactivity.

Although smoking has been demonstrated to raise the risk of diabetes, increasing body fat is by far the most significant risk factor, which may influence the development of insulin resistance and disease progression (68). A high sugar and fat intake, for example, has also been related to an increased risk of type 2 diabetes. Not only are the risk factors for gestational diabetes comparable to those for type 2 diabetes - family history, age, overweight and obesity, physical inactivity - but they also include excessive weight gain during pregnancy (69–71).

Pre-diabetes may be associated with an elevated risk of cardiovascular disease in addition to an increased risk of developing T2DM. Although there is evidence that pre-diabetes increases the risk of CVD by 20%, it is unclear whether pre-diabetes causes cardiovascular disease directly or through established cardiovascular disease risk factors such as dyslipidaemia and hypertension (69).

2.1.2.4 Diagnosis and early detection

Even in high-income countries, a significant number of T2DM cases are undetected due to a lack of obvious symptoms. Diabetes is diagnosed by detecting glucose levels in a fasting blood sample or 2 hours after a 75 g oral glucose load. Glycated haemoglobin A1c (HbA1c) testing can also be used to identify diabetes, but it is more expensive than blood glucose testing. In primary care settings, basic diagnostic testing should be offered (64).

Previous research aimed at identifying biomarkers for the risk of T2DM had flaws. Even though novel biomarkers for T2DM have been identified, it is still unknown what pathways contribute to the conversion to T2DM (72). The continuous glucose monitoring has been purposed, but it has only a few potential advantages and it cannot predict typical diabetes complications. Not to mention the ability to get in-the-moment glucose readings without a finger stick may be appealing to patients (73). The identification of prognostic biomarkers for Diabetes Mellitus type 2 would allow the symptoms evolution monitoring and complications prediction.

Chapter II - Aims

Whether caring for a single patient or responding to a worldwide pandemic, the rapid and accurate establishment of a cause is fundamental to quality care. Despite the technological advances, many patients do not access the appropriate therapy, due to a lack of an efficient diagnostic strategy. In the case of pandemics, there is a need for rapid, efficient, and massive testing allowing that a higher number of persons can be tested, at a lower cost, which ultimately improves patients' quality of life and leads to less lives lost.

The ongoing outbreak of COVID-19 has posed an extraordinary threat to global public health. This pandemic disease infected about 630 million individuals and killed over 6,5 million (52). Regular testing was a mandatory procedure to control the infection rates, requiring alternative and innovative diagnosis strategies. Furthermore, much remains unclear regarding the molecular consequences of infection in the host.

Diabetes is one of the largest global public health concerns, imposing a heavy global burden on public health with a large socio-economic impact. To date, the International Diabetes Federation (IDF) has estimated that 537 million adults live with diabetes worldwide (74), being the total number of people living with diabetes projected to rise to 643 million by 2030 and 783 million by 2045. Although the diagnosis is well established, there is a large gap in monitoring the many complications of Diabetes.

The main objective of this work was to validate saliva as a diagnostic fluid for COVID-19 and Diabetes Mellitus type 2.

The specific objectives were:

1. Develop a strategy to detect SARS-CoV-2 in saliva.
2. Develop a strategy for wide population testing of COVID-19.
3. Identify the molecular mechanisms altered upon SARS-CoV-2 infection, using saliva.
4. Identify potential prognostic biomarkers for Diabetes Mellitus type 2 and its complications.

Chapter III - Population wide testing pooling strategy for SARS-CoV-2 detection using saliva

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Chapter III

1. Abstract

SARS-CoV-2 pandemic has forced frequent testing of populations. It is necessary to identify the most cost-effective strategies for the detection of COVID-19 outbreaks. Nasopharyngeal samples have been used for SARS-CoV-2 detection but require a healthcare professional to collect the sample and cause discomfort and pain to the individual. Saliva has been suggested as an appropriate fluid for the diagnosis of COVID-19. We have investigated the possibility of using pools of saliva samples to detect SARS-CoV-2 in symptomatic and asymptomatic patients. Two hundred and seventy-nine saliva samples were analyzed through RT-PCR of Envelope, Nucleocapsid and Open Reading Frame 1ab genes. Reproducibility assays showed an almost perfect agreement as well as high sensitivity (96.6%), specificity (96.8%), positive predicted value (96.6%), and negative predicted value (96.8%). The average Cycle Threshold of the genes detected was 29.7. No significant differences ($p > 0.05$) were detected when comparing the cycle threshold average of two consecutive reactions on the same positive saliva samples. Saliva samples have a higher median viral load (32.6) than in nasopharyngeal samples (28.9), although no significant differences were detected ($p > 0.05$). Saliva-pool samples allowed effective SARS-CoV-2 screening, with a higher sensibility (96.9%) on 10-sample pools than in 20-sample pools (87.5%). Regardless of pools size specificity was high (99.9%) and an almost perfect agreement was observed. Our strategy was successfully applied in population wide testing of more than 2000 individuals, showing that it is possible to use pooled saliva as diagnostic fluid for SARS-CoV-2 infection.

Keywords: Saliva, SARS-CoV-2, Virus testing, Gene pool, Virus load, COVID-19, Reverse Transcriptase PCR, RNA extraction

2. State of the art

The COVID-19 pandemic, caused by SARS-CoV-2, has created challenges at a global scale (1–11). Currently, the standard diagnostic method for COVID-19 is to perform a real-time polymerase chain reaction (RT-PCR) of a sample taken by smear (swab) from the naso and oropharynx. The replacement of the nasopharynx swabs with less invasive collection techniques is desirable and has been moderately explored (8, 12–18). Saliva is commonly used to diagnose viruses similar to SARS-CoV-2 and has been suggested as appropriate for the diagnosis of COVID-19 (6, 12, 15, 17, 19–21) but the detection method still needs improvement. Until now, saliva testing was less sensitive than nasopharyngeal swab (22), however recent studies have reported similar results for saliva and nasopharyngeal swabs (23). The collection of saliva is non-invasive and can be performed by the patient him/herself, reducing the risk of cross-contamination and the need of specialized healthcare workers. Saliva is thus more amenable to frequent testing since it is safer (to the patient and to the healthcare worker), cheaper and imposes less discomfort in the individual being tested (6, 19, 24).

Institutions and authorities are forced to choose carefully which are the most cost-effective strategies to avoid spread and outbreaks. One of the most effective ways to prevent virus spreading includes the routine testing of individuals using a highly sensitive method to detect asymptomatic or pauci-symptomatic and mild cases (25). On the other hand, mass testing, using the current nasopharynx swabs/PCR tests, represents a huge economic cost that most institutions cannot support (16–19). The burden of mass testing can be alleviated by means of pool-based strategies (26–29). The rational is that if a pool of samples is positive then, and only then, individual samples of the pool are subsequently tested, which can markedly reduce the number of tests being carried out. But cost is not the only factor, indeed pooling or group testing of specimens is faster than individual testing and saves resources, by expanding the detection capacity while limiting the risk of reagent shortage (30), as well as, reducing the environmental impact of all the residues produced by mass testing (31, 32).

For nasopharyngeal samples it has been established that an individual positive sample can still be detected in pools of up to 32 samples, and even 64 samples, provided that additional polymerase chain reaction (PCR) amplification cycles are conducted with a sensitivity of 96% (6, 8, 22). Currently, several studies on saliva pool-testing for SARS-CoV-2 have been conducted (13, 27–29). However complex protocols, prone to error, are frequently followed (33) and the maximum number of samples that should be present in a pool without losing sensibility is not clearly established, although some studies report small pool samples of less than 20 samples per pool. In fact, there is no consolidated information about the use of saliva in pool testing (13, 27–29).

Saliva sample pooling has the advantages of sample pooling described above as well as the added advantage of a simpler and more comfortable collection for the patient. Children and senior patients will benefit tremendously with this strategy. Therefore, the aim of this study is to provide evidence on the use of saliva sample pools for SARS-CoV-2 detection using large saliva pools of 20 and 10 samples.

3. Material and methods

3.1 Sampling process, sample and research participants

Saliva (SAL) and Nasopharyngeal Swab (NPS) samples were collected at Centro Hospital Tondela Viseu (Viseu, Portugal), at the Portuguese Football Federation (Lisboa, Portugal), at the Municipality of Viseu and at Faculty of Dental Medicine of the Portuguese Catholic University (Viseu, Portugal).

Passive drooled saliva samples (2mL) were collected into 50mL sterile tube without stabilizers using a previous established protocol (24). Human nasopharyngeal/oropharyngeal swab specimens were collected from the same donors, according to the Portuguese Directorate-General of Health (DGS) guidelines and placed in viral transport medium. This study used 184 saliva samples for SARS-Cov-2 detection method development (160 SARS-CoV-2 negative samples and 24 positive samples); for the pool assays, 132 samples were analysed (130 negative and 2 positive). The community test was performed with 2017 saliva samples from 216 female and 1801 male volunteers. The average of ages was 17.1 (±10.7) years and 12.9 (±8.5) year respectively (S1 Table). Saliva collections in minors was executed under the supervision of the legal tutor.

This is a cross-sectional study focused on detecting SARS-CoV-2 viral load on saliva pools for population wide testing. To achieve this goal, we studied: 1) the potential of saliva for the detection of SARS-CoV-2 using a simplified procedure; 2) the sensibility/specificity of detection method in saliva; 3) the potential of pooling saliva specimens for the detection of SARS-CoV-2.

This study was carried out in accordance with the Helsinki Declaration and the Oviedo Convention. The ethical aspects of the present study were reviewed and approved by the Ethics Committee for Health at Centro Hospitalar Tondela Viseu. Written informed consent including purpose of the study, data confidentiality, rights of participation, and the right to withdraw from the study at any time was provided by every participant before study enrolment.

3.2 Sample storage and pre-treatment

Samples were sent to the lab on a refrigerated container to perform sample inactivation, RNA extraction and further SARS-Cov-2 detection by RT-PCR. All extracted RNA samples were stored at -80°C until analysis. All samples were destroyed upon completion of the study.

3.3 RNA extraction

To compare paired SAL and NPS samples, total RNA was extracted from 200 µl of the NPS viral transport medium and saliva samples, using RNAdvance Viral XP kit (Beckman Coulter, Indianapolis, United States) using Biomek i5 Automated Workstation liquid handling (Beckman Coulter, Indianapolis, United States) according to manufacturer's protocol.

To simplify procedures, reduce costs and time of analysis, we optimized a direct RT-PCR approach for SARS-Cov-2 detection combining proteinase K and heat-inactivation. For this purpose, a volume of 100 µL of sample (single or pooled) was mixed with 20 µL of Proteinase K (20 µg/mL, NZYTech, Lisboa, Portugal) followed by incubation at 57°C (15 min) and at 95°C for 15 min (enzyme and viral inactivation). This strategy was used to determine sensitivity and reproducibility for pooling assays.

3.4 SARS-CoV-2 detection

Viral load determination was performed using reverse transcription-polymerase chain reaction (RT-PCR) analysis using the Novel Coronavirus (2019-nCOV) RT-PCR detection Kit (Shanghai Fosun Long March Medical Science CO. Ltd, China) for O (ORFa1b), N (Nucleocapsid phosphoprotein coding gene) or E (Envelope) gene fragments of SARS-CoV-2, according to manufacturer's protocol. RT-PCR reactions were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, California, United States). Samples were considered positive, when amplification of two or three SARS-CoV-2 gene fragments were amplified below 40 cycles (CT value).

3.5 Assay design

For sensitivity determination we spiked infectious viral particles RNA UN kit SARS-CoV-2 Reference Material Kit) into healthy donor saliva at 1:10 ratio for contrived positive sample, after SAL and NPS COVID-19 detection. For reproducibility assay we performed two separate RT-PCR reactions for the same saliva samples. For evaluation of pooling saliva samples for SARS-CoV-2 screening prior to RNA extraction, we arbitrarily chose 2 positive samples and 130 negative samples, previously determined. A total of 32 pools of 10 samples (1 positive + 9 negative) and 32 pools of 20 samples (1 positive + 19 negative) were prepared. The negative samples were mixed into pools of different sizes containing equal volumes of 10 and 20 unique samples, 32 pools each. Negative pools were prepared with different samples to determine whether different negative-sample composition in the pool affected the detection of positive samples. Sensitivity and sensibility were determined as described (34–36).

3.6 Data treatment and statistical analysis

Average cycle threshold (CT) was calculated using Microsoft Office Excel 365 software (Microsoft, Redmond, WA). Kappa (K) statistics value, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) at two-sided 95% confidence interval (CI) using the Clopper and Pearson method, were analysed using Online GraphPad Prism version calculator (GraphPad Software, San Diego, CA, USA).

Pair analysis (Saliva and Nasopharyngeal Swab specimens) were conducted by Wilcoxon signed rank test. Pooling strategy statistical analysis was conducted by Mann-Whitney rank test. These analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA, USA).

4. Results

4.1 Saliva assay sensitivity

To determine the sensitivity of the detection of SARS-CoV-2 in saliva samples, 99 saliva samples were tested: 50 positive samples (to which a SARS-CoV-2 RNA template was added; these will be referred to as "spiked samples" from this point forward) and 49 negative samples. 48 of the 50 spiked samples were identified as positive for SARS-CoV-2, while 2 were recorded as (false) negatives (Table 5). We observed an almost perfect agreement (K = 0.96, CI 95%: 0.90–1.00) with 96% of sensibility (95% CI: 0.86–0.99) and 100% of specificity (95% CI: 0.93–1.00). The average CT of three genes were: O gene CT 32.7, E gene CT 33.3 and N gene CT 34.0.

Table 5. Sensitivity and specificity of SARS-CoV-2 detection on saliva samples by RT-PCR. Saliva samples were divided into 2 groups: a control group and a test group in which samples were spiked with a SARS-CoV-2 RNA template. Sensitivity and specificity were determined as described (34-36). CI – confidence interval.

Saliva samples	N	Saliva samples		Sensitivity (95% CI)	Specificity (95% CI)
		Detected	Not Detected		
Positive	50	48	2	96% (0.86 - 0.99)	100% (0.93 - 1.00)
Negative	49	0	49		

4.2 Assay reproducibility

To test the reproducibility of the method, we tested 60 saliva samples, divided into 2 groups (29 Sars-Cov-2 spiked and 31 Sars-Cov-2 negative). Each of these samples were tested twice (Table 6). Data shows an almost perfect agreement (K = 0.93, CI 95%: 0.84–1.00) as well as hight sensitivity (96.6%; CI 95% 0.82–0.99), specificity (96.8%; CI 95% 0.83–0.99), positive predicted value (96.6%; CI 95% 0.82–0.99), and negative predicted value (96.8%; CI 95% 0.83–0.99). A false negative and a false positive were detected on the second reaction.

Table 6. Determination of sensitivity and specificity of saliva testing. RP-PCR was used to detect SARS-COv-2 in saliva samples. Each sample was analyzed twice and treated as independent samples. Sensitivity, sensibility, positive predictive value (PPV) and the negative predictive value (NPV) (with a confidence interval (CI) of 95%) were determined according to [34-36]. CI – confidence interval.

Saliva samples	N	Saliva samples		Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
		Detected	Not Detected				
Positive	29	28	1	96.6% (0.82 - 0.99)	96.8% (0.83 - 0.99)	96.6% (0.82 - 0.99)	96.8% (0.83 - 0.99)
Negative	31	1	30				

No significant differences (p > 0.05, Fig 4) were detected when comparing both reactions on positive saliva specimens, by Wilcoxon matched pair signed rank test. The average CT of three genes were: O gene CT 29.6; E gene CT t 30.2 and N gene CT 29.4.

4.3 Comparison between saliva samples and nasopharyngeal swab samples

We evaluated the concordance between the detection of SARS-Cov-2 in saliva (SAL) and in nasopharyngeal (NPS) samples collected from the same patients (147 pairs of SAL and NPS samples, Table 7). Detection of SARS-CoV-2 on NPS samples showed a moderate agreement with SAL samples (κ = 0.58, CI 95%: 0.37–0.79). We also compared the CT values of the concordant positive SAL and NPS samples (10 positive pairs in NPS and SAL) by Wilcoxon matched pair signed rank test. A higher median viral load was seen for SAL (32.6) specimens compared with the median CT for NPS samples (28.9) with no significant differences ($p > 0.05$, Fig 5).

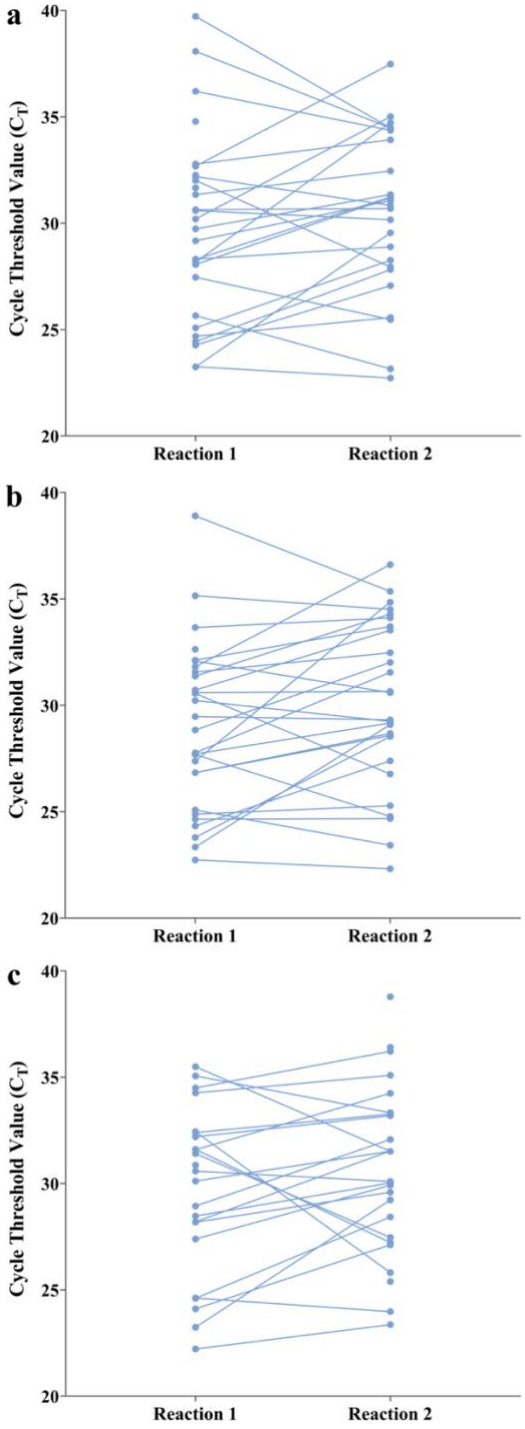

Figure 4. Cycle threshold (CT) values for SARS-CoV-2 E gene (a), N gene (b) and O gene (c) of paired saliva samples (N = 29), connected by a line, in two different RT-PCR reactions (Reaction 1 and Reaction 2), compared by Wilcoxon matched pairs signed rank test.

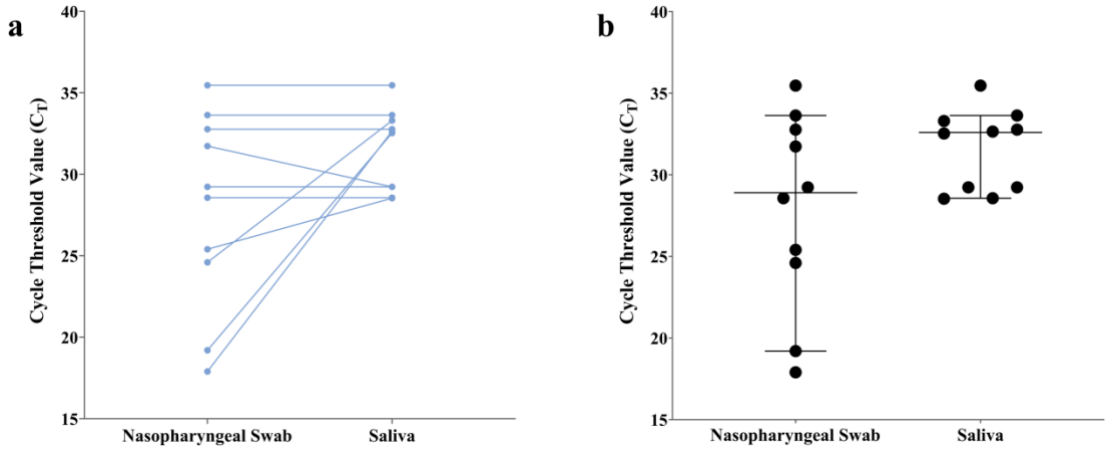

Figure 5. Comparison of Viral loads of SARS-CoV-2 between nasopharyngeal swab and saliva specimens. a) Ct values for paired NPS and SAL samples (10 pairs). Pairs are connected by a line. b) Scatter plot with the median with 95% CI on error bars. Statistical differences were determined by Wilcoxon matched pairs signed rank test.

Table 7. Determination of sensitivity and specificity in Saliva (SAL) using Nasopharyngeal (NPS) paired samples as gold standard. Sensitivity, sensibility, positive predictive value (PPV) and the negative predictive value (NPV) (with a confidence interval (CI) of 95%) were determined according to [34-36]. CI – confidence interval.

Saliva samples	**N**	**Nasopharyngeal Swab samples**		**Sensitivity (95% CI)**	**Specificity (95% CI)**	**PPV (95% CI)**	**NPV (95% CI)**
		Detected	Not Detected				
Positive	15	10	5	66.7% (0.33 - 0.82)	94.7% (0.91 - 0.99)	62.2% (0.38 - 0.88)	95.6% (0.89 - 0.98)
Negative	132	7	125				

4.4 Evaluation of the pooling saliva samples strategy for SARS-CoV-2 screening

Due to dilution pooling, it is expected a reduction on the sensitivity of SARS-CoV-2 detection by RT-PCR. To test this hypothesis, our pooling strategy was designed using 10 (Table 8) or 20 (Table 9) saliva samples per pool. Each "positive pool" contained a SARS-CoV-2 positive sample with an average of 25.6 CT. "Negative pools" had only SARS-CoV-2 negative samples (previously tested).

Table 8. Determination of sensitivity and specificity of SARS-CoV-2 detection on saliva pools (10 samples). Positive pools were constituted by 9 negative samples and 1 SARS-CoV-2 RNA spiked sample. The negative pools were constituted by 10 negative samples. A total of 260 saliva samples were randomly distributed in 32 pools of 10 samples. Sensitivity and specificity were determined as described [34-36]. CI – confidence interval.

Pool samples	N	Positive	Negative	Sensitivity (95% CI)	Specificity (95% CI)
Positive	32	31	1	96.9% (0.84 - 0.99)	99.9% (0.87 - 1.00)
Negative	32	0	32		

Table 9. Determination of sensitivity and specificity of SARS-CoV-2 detection on saliva pools (20 samples). Positive pools were constituted by 19 negative samples and one positive SARS-CoV-2 RNA spiked sample. The negative pools were constituted by 20 samples. A total of 260 saliva samples were randomly distributed in 32 pools of 20 samples. Sensitivity and specificity were determined as described [34-36]. CI – confidence interval.

Pool samples	N	Positive	Negative	Sensitivity (95% CI)	Specificity (95% CI)
Positive	32	28	4	87.5% (0.71 - 0.96)	99.9% (0.87 - 1.00)
Negative	32	0	32		

From the 32 "positive pools", each formed by 10 saliva samples (9 negative and 1 positive; Table 8), 31 pools were detected as "positive" (average CT of three genes: ORF1ab gene CT 26.7; Envelope gene CT 27.2 and Nucleocapsid gene CT 26.6) and 1 as "not detected". All 32 "negative pools" were detected as negative. Sensibility was determined to be 96.9% (95% CI: 0.84–0.99) and specificity was 99.9% (95% CI: 0.87–1.00). An almost perfect agreement was observed (κ = 0.97, CI 95%: 0.91–1.00).

We also tested 20-sample pools (n = 64; Table 9). From the 32 "positive pools" (pools containing 1 control positive sample), 28 were detected as positive (average CT of three genes: ORF1ab gene CT 27.5; Envelope gene CT 28.1 and Nucleocapsid gene CT 27.0) and 4 were reported as "not detected". All "negative pools" (n = 32) were detected as negative. Sensibility is 87.5% (95% CI: 0.71–0.96). Specificity is 99.9% (95% CI: 0.87–1.00). An almost perfect agreement was observed (κ = 0.88, CI 95%: 0.78–0.96).

The comparison of the Cycle Threshold value ORF1ab, Envelope and Nucleocapsid genes between 10-sample and 20-sample pools is shown on Fig 6. The 10-sample pool (20.93) showed slightly, non-significantly, lower CT genes mean comparing to pool of 20 (23.70).

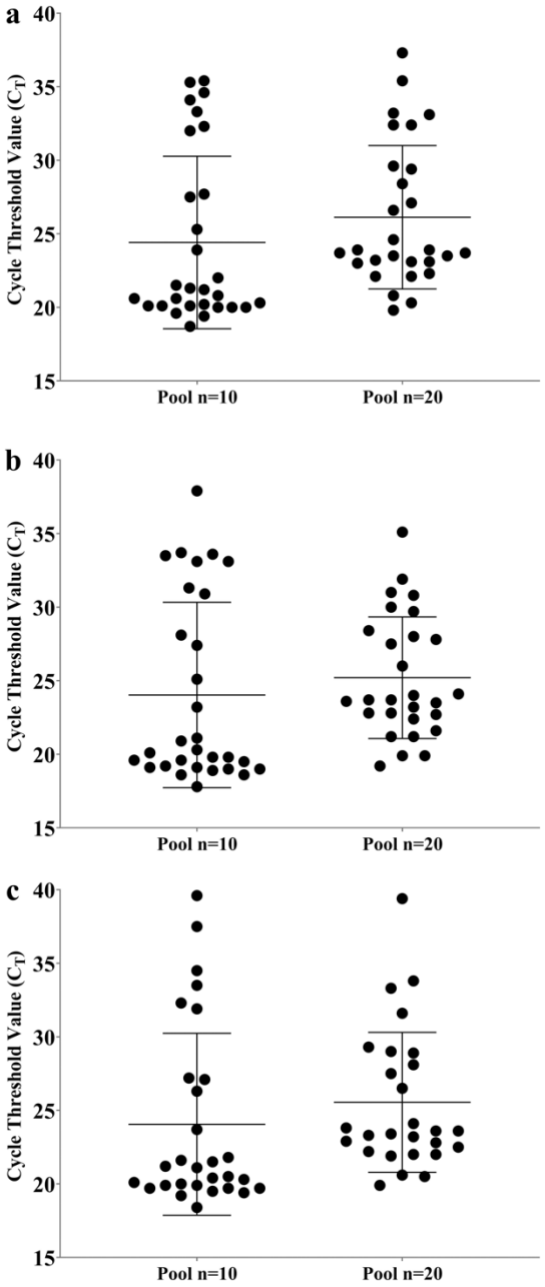

Figure 6. Comparison of Cycle Threshold (CT) values of 10- and 20-sample pools. CT values of Envelope (a), Nucleocapsid (b), and ORF1ab (c) genes were compared between the two types of samples by Mann-Whitney rank test.

5. Discussion

5.1 Saliva assay sensitivity and reproducibility

The performance characteristics of COVID-19 PCR on saliva and other less invasive sample types, including throat gargle (oral rinses or mouthwashes), has only recently been evaluated (14, 37). RNA extraction from saliva samples for SARS-CoV-2 detection and viral load analysis is usually subjected to expensive, time consuming, kit-dependent protocols. We applied the SalivaDirect (33) methodology for saliva treatment. This methodology does not require expensive saliva collection tubes containing preservatives nor does it require specialized reagents or equipment for nucleic acid extraction. This strategy granted the reliable analysis of a high number of samples at low cost (Fig 4).

To assess the quality of detection of SARS-CoV-2 in saliva samples we compared the sensitivity and reproducibility of this method to the gold standard (nasopharyngeal samples). Previous studies showed that sensitivity for detection of SARS-CoV-2 RNA in saliva samples compared with NPS ranges from around 70% to 100% (8, 12, 15, 16, 27, 37, 38) and our results are in agreement with these studies. Besides being self-collected, saliva is less prone to errors than nasopharyngeal swab. False negatives have been related to swab collection technique, due to the heterogenicity on the deep insertion, and to the anatomy of the patient, introducing a higher level of variability (1, 2, 27, 37).

Viral loads considering CT values are not only the threshold used for considering a test result positive but also frequently used as a comparison measure between test results. In the current study, the overall median CT in SAL was slightly higher than NPS, although the difference was not statistically significant ($p>0.05$). Silva et al (39) showed that nasopharyngeal and saliva viral load, are not equivalent measures of disease processes for COVID-19. The correlation between nasopharyngeal and saliva was low ($R = 0.61$) and while saliva viral load could significantly predict disease severity and mortality over age, nasopharyngeal viral load could not reliably distinguish severity or predict mortality (40). So far there is no consensus as to which of the sample types, SAL or NPS, has higher viral load values. Some studies have reported that higher viral loads were seen in patients with more severe disease. In our study, the CT values are not statistically different in SAL (reflecting a lower viral load) from NPS samples. Importantly, the range of viral load in the specimens in a lower number of samples can greatly affect the final calculated percent positive agreement because the specimens with higher viral loads are more likely to be detected by both NPS and SAL; therefore, studies with a higher median viral load across most specimens will show a higher percent positive agreement than a study with a lower median viral load (27).

5.2 Pooling saliva samples for SARS-CoV-2 screening

To date, only a few studies have evaluated the efficiency of SARS-CoV-2 detection in pooled saliva samples (13, 27–29). Some studies analysed the efficiency in different pooling sizes from 5 (27, 29), 10 (13, 27) to 20 (28) samples. If, on the one hand, pools with a smaller number of samples suggest greater sensitivity in detecting the virus, on the other hand, the cost reduction increases considerably with the increase in pool size. The ideal is to be able to increase the size of the pools without losing the ability to detect the virus. This is especially relevant in individuals with a low viral load. In

our study we tested pools of 10 and 20 saliva samples applying the methodology to real situations.

We showed that SARS-CoV-2 can be detected both in 10 and 20-sample saliva pools, although the sensitivity is 9.4% lower in the latter. The lower sensitivity on 20-sample pools is surely associated to the dilution factor. Previous studies also demonstrated an increase in Ct values after pooling (27–29). Not all studies used direct sample pooling, and some used extracted RNA to build the pools, with increase resource use (time and cost) and is prone to error. Pasomsub et al 2020 (29) used extracted RNA pooling strategy to build pools of 5 and 10 samples, achieving lower differences (0.1 and 1.4 respectively) in the CT values of individual samples and pool. These CT values are lower than saliva sample pooling. On other studies, an average increase 2–3 CT was obtained on 5- and 10-sample pools, and an increase of almost 4 CT, on 20-sample pools (13, 27, 29). Our saliva pooling strategy, resulted in only a slight increase on CT value of 1.2 CT (in 10 sample pools), and 1.9 CT in average (in 20 sample pools) compared to positive spike samples.

Recently some reports described saliva as an appropriate fluid for SARS-CoV-2 detection on patients with high viral load (27). Nonetheless, we were able to detect infected, but asymptomatic patients. One advantage of pool testing is its time and cost-effectiveness, allowing population-based asymptomatic screening or monitoring even when testing supplies are limited.

The pooling strategy described herein was applied to SARS-CoV-2 testing in a real-life scenario. Over 2000 individuals were tested with this strategy being possible to identify 3 asymptomatic SARS-CoV-2 carriers (0.15%), preventing outbreaks. The application of this strategy to a population of civil servants and junior athletes provided the economy of 1840 individual tests. Also, pools were built with saliva samples instead of extracted viral RNA. We demonstrated the efficiency of saliva pooling for the detection of SARS-CoV-2 heterogeneous population under investigation for COVID-19 in a low prevalence setting. Saliva pooling may facilitate the detection of the disease in suspected symptomatic patients during the disease outbreak, providing the advantages in the ease of specimen collection and resource conservation.

Nevertheless, some aspects of this study deserve attention. First, although almost 300 samples were used, the declining incidence of COVID-19 cases over the study period limited the number of positive samples enrolled in the study. Although saliva collection is easier and less prone to variation than nasopharyngeal collection, saliva can be a challenging sample when it comes to processing and analysis. Therefore, rigorous methods of homogenization and pipetting of samples must be followed. Furthermore, it is important that when using saliva samples, the CT used for considering a sample positive should be adjusted. Most detection kits are optimized for nasopharyngeal swabs, and as our and other studies suggest, CT tend to be higher in saliva samples. In this study the CT was increased from the recommended 32 cycles to 40 cycles guaranteeing the high sensibility and specificity of the SARS-CoV-2 detection.

6. Conclusions

In summary, we have demonstrated that saliva samples can be reliably used for SARS-CoV-2 detection, and that saliva-based large-scale population screening for COVID-19 with or without pooling is feasible, fast and leads to large economic and environmental impact reductions. We showed that saliva pools of either ten or twenty samples do not compromise the detection of SARS-CoV-2. The ease of saliva collection, and the pool strategy is an appealing method for mass-screening programs or sentinel surveillance, especially in resource-limited scenarios.

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Chapter IV - COVID-19 Salivary protein profile: unravelling molecular aspects of SARS-CoV-2 infection

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Chapter IV

1. Abstract

COVID-19 is the most impacting global pandemic of all time, with over 600 million infected and 6.5 million deaths worldwide, in addition to an unprecedented economic impact. Despite the many advances in scientific knowledge about the disease, much remains to be clarified about the molecular alterations induced by SARS-CoV-2 infection. In this work, we present a hybrid proteomics and in silico interactomics strategy to establish a COVID-19 salivary protein profile. Data are available via ProteomeXchange with identifier PXD036571. The differential proteome was narrowed down by the Partial Least-Squares Discriminant Analysis and enrichment analysis was performed with FunRich. In parallel, OralInt was used to determine interspecies Protein-Protein Interactions between humans and SARS-CoV-2. Five dysregulated biological processes were identified in the COVID-19 proteome profile: Apoptosis, Energy Pathways, Immune Response, Protein Metabolism and Transport. We identified 10 proteins (KLK 11, IMPA2, ANXA7, PLP2, IGLV2-11, IGHV3-43D, IGKV2-24, TMEM165, VSIG10 and PHB2) that had never been associated with SARS-CoV-2 infection, representing new evidence of the impact of COVID-19. Interactomics analysis showed viral influence on the host immune response, mainly through interaction with the degranulation of neutrophils. The virus alters the host's energy metabolism and interferes with apoptosis mechanisms.

Keywords: COVID-19; saliva; proteomics; interactomics; Oralint

2. Introduction

The emergence of the novel coronavirus disease 2019 (COVID-19), caused by the infection with SARS-CoV-2 coronavirus, created the urgency to develop new diagnostic and therapeutic targets, as well as identify preventive strategies (1). PCR-based diagnostics can be implemented and scaled quickly, but do not provide information on disease mechanisms (2,3).

Mass spectrometry (MS)-based proteomics does not depend on previous information and can be set up in an untargeted way. The sensitive and high-throughput mass spectrometers allow detection of even the faintest changes in host physiology. Currently, MS-based proteomic workflows are well established, and can be routinely used for definition of molecular pathways and pathogenesis, prognostic's biomarkers, drug discovery or repurposing and vaccine development (4,5). Concerning COVID-19, MS-based proteomics has the potential to unveil aspects of the disease, providing valuable information for understanding the molecular pathways disrupted by virus infection (4).

Serum (and plasma) are, by a long shot, the most studied and well-known fluids with regards to human diseases. This over-burden of information has the inborn drawback of decreasing research in other biological fluids. Alternative fluids of diagnosis, such as saliva, have clear advantages when compared to blood (6,7). Saliva has grown in popularity as a fluid of interest in recent years, due to its ease of collection via a non-invasive sampling procedure. Overall, saliva can be used to shed insight on illness development and pathophysiological mechanisms, in several (oral and systemic) diseases, including SARS-CoV-2 infection (8,9,10,11,12).

While proteomics has already proven to be a valuable tool for identifying biomarkers for many conditions, more strategies are still needed to functionally interpret proteomics data to clarify molecular changes of diseases such as COVID-19 and predict disease severity (11,13).

We hypothesize that SARS-CoV-2 induces characteristic proteome changes that can be detected in the saliva of infected patients. These molecular changes may shed light on the association of infection to host response.

3. Materials and Methods

3.1 Ethical Statement

This study was carried out in accordance with the Helsinki Declaration and the Oviedo Convention. The ethical aspects of this study were reviewed and approved by the Ethics Committee for Health at Centro Hospitalar Tondela Viseu. Written informed consent including the purpose of the study, data confidentiality, rights of participants, and the right to withdraw from the study at any time was provided by every participant or by their legal representatives or guardians before study enrolment.

3.2 Participant Enrolment

Five saliva samples (2 females and 3 males, aged 16 to 71) of a cohort of COVID-19 patients at Centro Hospitalar Tondela Viseu were collected. Furthermore, saliva samples from five healthy subjects (2 females and 3 males, aged 28 to 59) showing no evidence of oral and systemic pathologies or inflammatory processes, negative for SARS-CoV-2, were also collected. All samples were tested by PCR for SARS-CoV-2 presence using a previously established protocol for SARS-CoV-2 testing in saliva (9).

The COVID-19 patients exhibited a variety of symptoms: acute respiratory insufficiency syndrome (n = 1), cough (n = 2), dyspnea (n = 1), headache (n = 2) and myalgia (n = 3).

3.3 Saliva Collection

Passive drooled saliva samples (2 mL) were collected in 50 mL sterile tubes without stabilizers as described (9) and in agreement with the Portuguese healthcare guidelines (DGS Norma 004 2020). Samples were refrigerated and inactivated using 1% Triton X-100 detergent. Whole saliva was centrifuged at 10,000× g, 10 min, 4 °C. The supernatant was aliquoted and stored at −80 °C for analysis.

3.4 Sample Preparation

Protein concentration was determined using the Pierce BCA assay kit (ThermoFisher Scientific, Waltham, MA, USA), according to the manufacturers' instructions, and concentration was adjusted to 50 µg/mL. Saliva was analyzed by capillary electrophoresis (Experion™ automated capillary electrophoresis system, Bio-Rad, Hercules, CA, USA), and compared to the in-house profiles to confirm sample integrity.

For data-dependent acquisition (DDA) experiments, replicates from each condition were pooled into two different samples (COVID+ and COVID-) before sample processing. For data-independent acquisition (DIA), each sample was processed individually. Protein content (60 µg) from each sample was separated by SDS-PAGE (4–15%) for about 17 minutes at 110 V (Short-GeLC Approach (14) and stained with Coomassie Brilliant Blue G-250. For DDA experiments, each lane was divided into 5 gel pieces, and for DIA experiments into 3 gel pieces for further individual processing. After the distaining step, gel bands were incubated overnight with trypsin for protein digestion and peptides were extracted from the gel using 3 solutions containing different percentages of acetonitrile (30, 50, and 98%) with 1% formic acid. Acetonitrile was evaporated using a vacuum-concentrator, and peptides were re-suspended in 25 µL (DDA) or 30 µL (DIA) 2% acetonitrile/0.1% formic acid. Each sample was sonicated using a cup-horn (Ultrasonic processor, 750 W) for about 2 min, 40% amplitude, and pulses of 1 s ON/OFF. Ten microliters of each sample (DDA) or 5 µL (DIA) were analyzed by LC-MS/MS.

3.5 LC-MS Methodology

Samples were analyzed on a NanoLC™ 425 System (Eksigent®) coupled to a Triple TOF™ 6600 mass spectrometer (Sciex®, USA) equipped with an ESI DuoSpray™ Source (Sciex®, USA). The chromatographic separation was performed on a Triart C18 Capillary Column 1/32″ (12 nm, S-3µm, 150 × 0.3 mm, YMC) and using a Triart C18 Capillary Guard Column (0.5 × 5 mm, 3 µm, 12 nm, YMC) at 50 °C. The flow rate was set to 5 µL/min and mobile phases A and B were 5% DMSO plus 0.1% formic acid in water and 5% DMSO plus 0.1% formic acid in acetonitrile, respectively. The LC program was performed as followed: 5–30% of B (0–50 min), 30–98% of B (50–52 min), 98% of B (52–54 min), 98–5% of B (54–56 min), and 5% of B (56–65 min). The ionization source was operated in the positive mode set to an ion spray voltage of 5500 V, 25 psi for nebulizer gas 1 (GS1), 10 psi for nebulizer gas 2 (GS2), 25 psi for the curtain gas (CUR), and source temperature (TEM) at 100 °C. For DDA experiments, the mass spectrometer was set to scanning full spectra (m/z 350–2250) for 250 ms, followed by up to 100 MS/MS scans (m/z 100–1500). Candidate ions with a charge state between +1 and +5 and counts above a minimum threshold of 10 counts per second were isolated for fragmentation, and one MS/MS spectrum was collected before adding those ions to the exclusion list for 15 seconds (mass spectrometer operated by Analyst® TF 1.8.1, Sciex®). The rolling collision was used with a collision energy spread of 5. For SWATH experiments, the mass spectrometer was operated in a looped product ion mode and specifically tuned to a set of 42 overlapping windows, covering the precursor mass range of 350–1400 m/z. A 50 ms survey scan (350–2250 m/z) was acquired at the beginning of each cycle, and SWATH-MS/MS spectra were collected from 100–2250 m/z for 50 ms, resulting in a cycle time of 2.1 s.

3.7 Data Analysis

3.6.1 Ion-Library Construction (DDA Information)

A specific ion-library of the precursor masses and fragment ions was created by combining all files from the DDA experiments in one protein identification search using the ProteinPilot™ software (v5.0, Sciex®, USA). The paragon method parameters were the following: searched against the reviewed Human (SwissProt) database, cysteine alkylation by acrylamide, digestion by trypsin, and gel-based ID. An independent false discovery rate (FDR) analysis, using the target-decoy approach provided by Protein Pilot™, was used to assess the quality of identifications.

3.6.2 Relative Quantification of Proteins (SWATH-MS)

SWATH data processing was performed using SWATHTM processing plug-in for PeakViewTM (v2.0.01, Sciex®). Protein relative quantification was performed in all samples using the information from the protein identification search. Quantification results were obtained for peptides with less than 1% of FDR and by the sum of up to 5 fragments/peptide. Each protein was normalized for the total sum of areas for the respective sample. Protein quantities were obtained by the sum of up to 15 peptides/protein (Supplementary Tables S1–S4).

3.7 Protein Functional Analysis

Functional analysis of altered salivary proteins in COVID-19 identified by mass spectrometry was performed according to Figure 7.

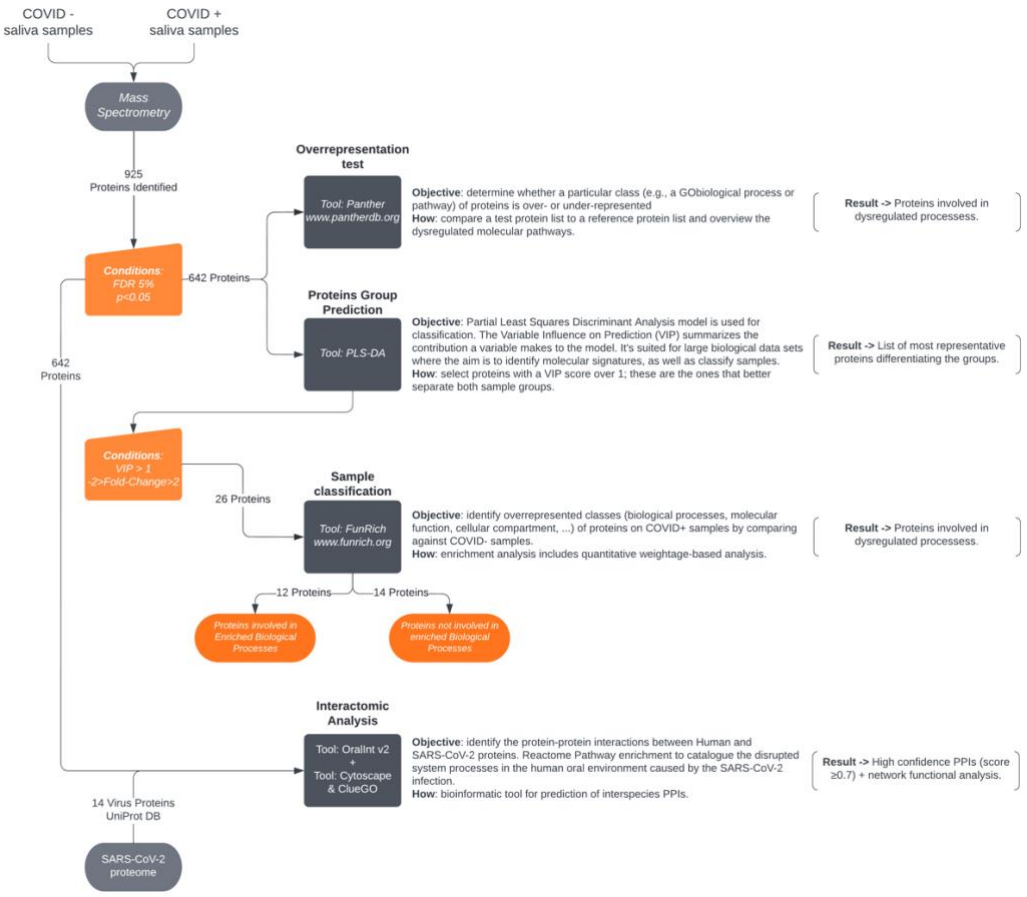

Figure 7. Scheme of the protein functional analysis and interactomics workflow. Proteins were identified by LC-ESI-TOF mass spectrometry. An initial dataset of 925 proteins was narrowed down via filtering by an FDR 5% and $p < 0.05$ conditions, resulting in 642 salivary proteins then analyzed (GO biological process) by Panther overrepresentation analysis. Proteins (26) with a VIP > 1 (PLS-DA model) and −2 > Fold-Change > 2 defined were used in sample classification analysis with the FunRich tool. The enrichment resulted in 12 proteins with dysregulated processes and with $p < 0.05$. Interactome analysis on the narrowed down dataset against the SARS-CoV-2 proteome.

Class enrichment (biological process) of COVID+ saliva proteins was obtained via the PANTHER Overrepresentation test (PANTHER V 16.0) against salivary proteins identified in healthy subjects (deposit in SalivaTecDB (10,15,16)). Only the biological processes significantly altered with $p < 0.05$ were considered enriched. The binomial test with Bonferroni correction was used.

A bioinformatic approach, the PLS-DA model, was used to select the proteins capable of classifying the 2 groups of samples, based on variable influence on projections (VIP) values and FDR indexes. Proteins with a VIP value higher than one, an FDR of 5% and a p-value < 0.05 were selected for the functional analysis. These proteins functions were analyzed by a biological processes' enrichment analysis using the FunRich tool. Our dataset was analyzed against the FunRich curated database background. The gene ontology annotations including biological processes, the Gene Ontology database, HPRD, Entrez Gene and UniProt, were used to build the FunRich background database (15).

3.8 Human–SARS-CoV-2 In Silico Interactomics Analysis

The protein–protein interactions (PPIs) derived between humans and SARS-CoV-2 proteins were predicted using the OralInt tool developed by our group (8,10), which

allows the prediction of interspecies protein interactions. The input data was the human saliva proteome described in previous sections (642 proteins) as of July 2022 and the SARS-CoV-2 reference proteome deposited in UniProt (17) (14 proteins).

The predicted interactions were categorized and assessed based on the prediction score (0.9–1.0: very high confidence; 0.7–0.9: high confidence; 0.4–0.7: medium confidence; 0.1–0.4: low confidence). Interactions with scores of less than 0.7 were eliminated (Table S3).

A network of predicted high confidence PPIs (score ≥ 0.7) was created Using Cytoscape v.3.9.1 (18). Network analysis was performed using Cytoscape's Network Analyzer Tool to ease data interpretation, and quantitation data obtained by mass spectrometry were used to represent the degree of alteration of proteins after infection with SARS-CoV-2.

A network functional analysis was also performed using the Reactome Pathway enrichment (updated 8 February 2022) with ClueGo v2.5.8 + CluePedia v.1.5.9 (19) to catalogue the disrupted system processes in the human oral environment caused by the SARS-CoV-2 infection.

3.9 Statistical Analysis

A Kruskal–Wallis test was performed to select the proteins statistically different between the COVID+ and COVID- samples. Dunn's test of multiple comparisons, with Benjamini–Hochberg p-value adjustment, was performed to determine in which comparisons statistical differences were observed. The Mann–Whitney test was used for the binary comparisons. The multivariate analysis was performed in MetaboAnalyst as previously described (6).

4. Results and Discussion

We hypothesized that SARS-CoV-2 induces characteristic proteome changes that can be detected in the saliva of infected patients. We propose a methodological approach for the functional analysis of altered salivary proteins in COVID-19 identified by mass spectrometry to search for molecular changes associated with COVID-19. These molecular changes may shed light on viral particle entry mechanism, salivary markers for new diagnostic approaches (protein quantification), or the association of infection to host response.

The proteomic analysis of the 10 saliva samples (COVID+ and COVID-) resulted in the identification of 925 proteins with a 5% FDR (Supplementary Table S1), from which 642 have a $p < 0.05$ (Supplementary Table S2).

4.1 Protein Functional Analysis

Considering the initial dataset of 925 proteins, a pool of 642 were selected with 5% FDR to perform an enrichment analysis (PANTHER Overrepresentation test, Figure 7). The enrichment analysis resulted in 39 biological processes significantly altered with a 2.5-fold enrichment. Interestingly, three biological processes had over a four-fold enrichment, indicating that these are over-represented in our dataset: Immunoglobulin Production, Production of Molecular Mediator of Immune Response and Cell Recognition.

Twenty-six proteins contribute significantly to the PLS-DA model with a PC1 VIP score > 1.0 (Table 10): of these, twenty-two proteins are more abundant and four are less abundant in COVID+ samples than in healthy individual samples. Inter-alpha-trypsin inhibitor heavy chain H1 and Annexin A7 stand out by being the least and the most abundant in this dataset when comparing the two sample groups.

Table 10. List of the proteins with a VIP score > 1. These are the proteins that better differentiate COVID+ and COVID- samples. Shown are the UniprotKB AC code, protein names, gene names, fold change (COVID+/COVID-) and the biological process defined by the enrichment analysis. * Proteins involved in enriched biological processes.

UniprotKB AC	Protein Name	Gene Name	Fold Change	Biological Process
P19827	Inter-alpha-trypsin inhibitor heavy chain H1	*ITIH1*	−8.28	* Protein metabolism
P01023	Alpha-2-macroglobulin	*A2M*	−2.91	* Protein metabolism
Q96FX8	p53 apoptosis effector	*PERP*	−2.52	Apoptosis
P02788	Lactotransferrin	*LTF*	−1.53	Transport
Q99623	Prohibitin-2	*PHB2*	1.61	Mitochondrion organization
P29218	Inositol monophosphatase 1	*IMPA1*	1.64	* Energy pathways

Q9Y376	Calcium-binding protein 39	*CAB39*	1.77	Protein serine/threonine kinase activity
P13987	CD59 glycoprotein	*CD59*	1.84	* Immune response
P15153	Ras-related C3 botulinum toxin substrate 2	*RAC2*	1.88	Regulation of respiratory burst
Q86VR7	V-set and immunoglobulin domain-containing protein 10-like	*VSIG10L*	1.99	Cell adhesion molecule
P20061	Transcobalamin-1	*TCN1*	2.08	* Transport
P01706	Immunoglobulin lambda variable 2-11	*IGLV2-11*	2.17	Response to bacterium
P20340	Ras-related protein Rab-6A	*RAB6A*	2.18	Antigen receptor-mediated signaling pathway
P58499	Protein FAM3B	*FAM3B*	2.25	Antimicrobial response protein
Q9HC07	Transmembrane protein 165	*TMEM165*	2.42	Humoral immune response
Q9UBX7	Kallikrein-11	*KLK11*	2.61	* Protein metabolism
Q6UXB3	Ly6/PLAUR domain-containing protein 2	*LYPD2*	2.62	Mitotic cell cycle
Q04941	Proteolipid protein 2	*PLP2*	3.05	* Transport
P63000	Ras-related C3 botulinum toxin substrate 1	*RAC1*	3.06	Regulation of cell shape
A0A0C4DH68	Immunoglobulin kappa variable 2-24	*IGKV2-24*	3.09	Immune response
P39656	Oligosaccharyl transferase 48 kDa subunit	*DDOST*	3.15	* Energy pathways
P0DP04	Immunoglobulin heavy variable 3-43D	*IGHV3-43D*	3.37	Defense response to bacterium
Q08380	Galectin-3 binding protein	*LGALS3BP*	4.80	* Immune response

P0DOX2	Immunoglobulin alpha-2 heavy chain		4.86	Immune response
P19971	Thymidine phosphorylase	*TYMP*	5.89	Mitochondrial genome maintenance
P20073	Annexin A7	*ANXA7*	6.40	* Transport

Biological processes enrichment analysis (FunRich tool) of the PLS-DA relevant proteins showed that these proteins are involved in five main biological processes: Apoptosis, Energy Pathways, Immune Response, Protein Metabolism and Transport, in which 12 proteins are involved (Figure 8, Table 10). Transport is the process with the most proteins mapped (four), and apoptosis is the process with the least (one)

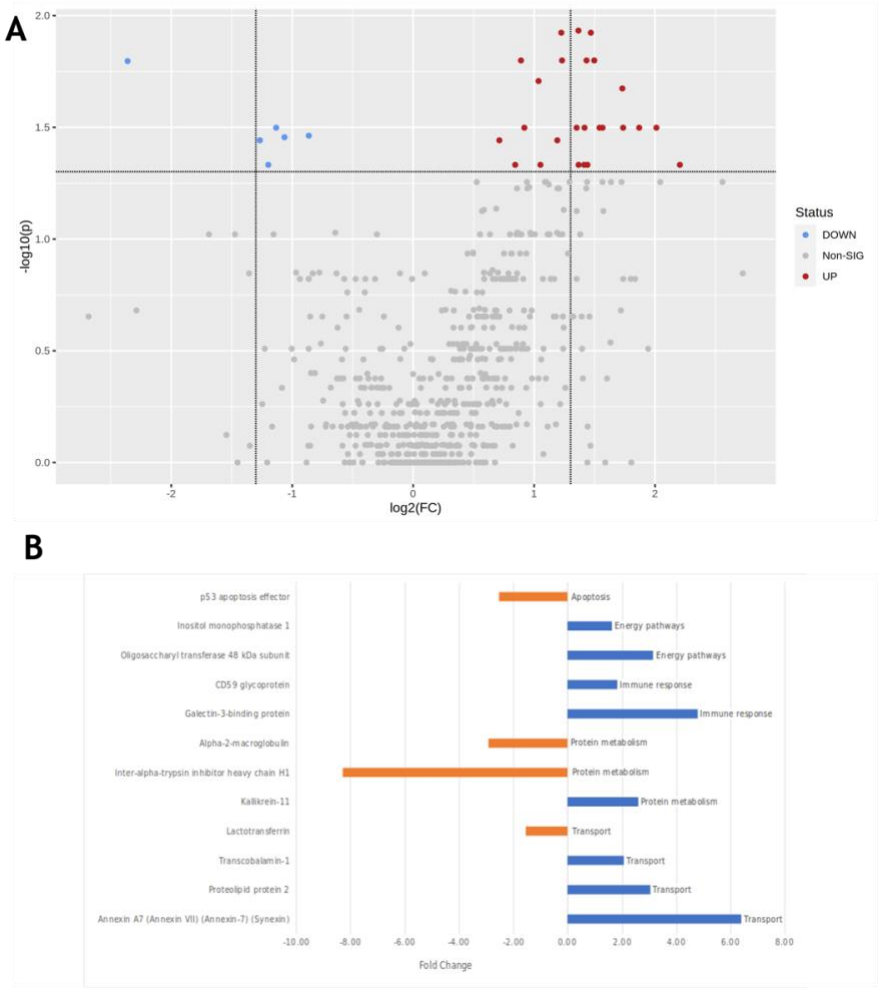

Figure 8. (A) Graphical representation of the protein distribution by protein expression (X axis) and VIP score (Y axis) variables in a Volcano plot. Blue and red dots represent up and down regulated proteins. Gray dots represent t=e proteins without regulation data. (B) Biological processes enrichment analysis graphical representation of the proteins with VIP score > 1 against the FunRich background database. The protein names are listed in the Y axis, with the respective fold change on the X axis. The biological process is indicated next to the graphic bar in A→Z order.

4.1.1 Apoptosis

A key component of a host's defense against viral infections is cell death (19). Apoptosis, pyroptosis and necroptosis are the three primary types of cell death that occur in virus-infected cells, and each is regulated by a different collection of host proteins (20).

Our results suggests that the p53 apoptosis effector, a component of intercellular desmosome junctions, was the main contributor for the observed dysregulation of apoptosis (Figure 8), playing a role as an effector in the TP53-dependent apoptotic pathway (17). Apoptosis is involved in COVID-19: the open reading frame (ORF) 3a protein of SARS-CoV (SARS 3a) can induce caspase activation (19). Simultaneously, it is known that p53 plays a role in SARS-CoV-2 ORF3a-induced apoptosis (21). So, in COVID-19, p53 interacts with the viral particles of SARS-CoV-2 at two different levels: a direct regulatory activity of p53 on SARS-CoV-2 replication, shared by the other coronaviruses, and more notably, the ability of SASR-CoV-2 to up-regulate the major p53 inhibitor MDM2. This down-regulation of the basal levels of p53, described as part of the survival strategy of SARS-CoV-2, is shared by other SARS-CoV viruses (22), and leads to perturbation of the tissue homeostasis, as mentioned above. The physiological significance of p53 basal activity is still emerging, as p53 suppression is linked to the disruption of tissue hemostasis. Low basal levels of p53 have been associated with respiratory disorders, suggesting a protective role for p53 in vascular hemostasis and inflammation of the lungs (23).

Our results show that in saliva, p53 apoptosis effector is less abundant (FC −2.52) in patients with COVID-19, corroborating the results obtained by Cui et al., 2021, and suggested by Milani et al., 2022 (22,23,24).

4.1.2 Energy Pathways

The risk of death by SARS-CoV-2 infection is higher in patients with energy metabolism-related chronic disorders, including diabetes (25). Our analysis showed that there is a dysregulation of energy processes after SARS-CoV-2 infection, which may help to explain the increased mortality in these patients (25,26). In fact, according to preliminary clinical data on COVID-19 patients, individuals with type 2 diabetes and other metabolic disorders that impair general metabolic health are more likely to experience a more severe infection course than those who were metabolically healthy before contracting the virus (26). This could be due to the impact of SARS-CoV-2 infection and disease outcome based on the energy metabolism equilibrium (25).

The oligosaccharyl transferase 48 kDa (OST) subunit and inositol monophosphatase 1 are the main contributors for the dysregulation of energy pathways processes observed in our analysis. Oligosaccharyl transferase 48 kDa subunit catalyzes the initial transfer of a glycan from the lipid carrier dolichol-pyrophosphate to an asparagine residue, the first step in protein N-glycosylation (17). Oligosaccharyl transferase gene DDOST is related with the innate immune system and with SARS-CoV-2 infection and was identified interacting with nonstructural proteins (NSP4) important for virus replication (27). In fact, reactome analysis (Figure 9) shows a maturation of the SARS-CoV-2 spike protein by N-glycosylation that seems to involve OST.

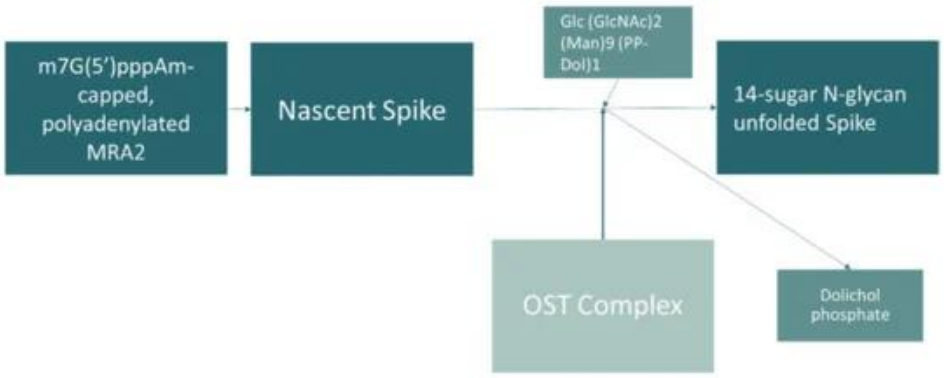

Figure 9. Pathway of spike protein glycosylation, according to Reactome.org (28). DDOST gene relation with SARS-CoV-2 infection: maturation of SARS-CoV-2 spike protein by N-glycosylation involving the oligosaccharyltransferase (OST) complex. Mammalian cells express OST complexes that contain a catalytic subunit and accessory proteins as the dolichyl-diphosphooligosaccharide–protein glycosyltransferase 48 kDa subunit (DDOST). DDOST catalyzes the initial transfer of a glycan from the lipid carrier dolichol-pyrophosphate to the nascent polypeptide chain. Image adapted from reactome.org (accessed on 22 February 2022).

It has been hypothesized that the blockage of the active site of oligosaccharyl transferase can significantly inhibit the infection of both SARS-CoV-2 and its variants (29,30,31). The increase on the quantity of oligosaccharyl transferase (FC 3.15) in saliva samples of COVID+ patients agree with data suggesting the involvement of OST in spike protein glycosylation. To our knowledge, our study is the first to identify and quantify this protein in the saliva of patients with COVID-19.

Inositol monophosphatase 1 (IMPA2) is responsible for the provision of inositol required for synthesis of phosphatidylinositol and polyphosphoinositides, being an important modulator of intracellular signal transduction via the production of the second messengers myoinositol 1,4,5-trisphosphate and diacylglycerol (17). Until now, there is no known direct relation with COVID-19, although it has been related with pulmonary complications with an important role in pulmonary arterial hypertension (32). Nonetheless, the increase (FC 1.64) of inositol monophosphatase 1 on the saliva of COVID-19 patients might be related to a defense response to the respiratory infection. It is known that myoinositol promotes the maturation of pulmonary surfactant phospholipids, regulating the synthesis of type II pneumocytes (33). Inositol promotes a mechanical stabilization of cell shape enabling alveolar cells to counteract collapsing forces.

4.1.3 Immune Response

An efficient elimination of invading pathogens requires a good synergy between innate and adaptive immune responses (34). A highly ordered cellular and molecular cascade is involved in the immune response controlling the balance of viral eradication vs. immunological harm (35). In fact, SARS-CoV-2 infection-associated immune responses are central to the pathogenesis of COVID-19 (36).

Our functional analysis shows that galectin-3 binding protein and CD59 glycoprotein may be key modulators of the immune response biological process in COVID-19. Galectin-3 binding protein promotes integrin-mediated cell adhesion and may stimulate host defense against viruses and tumor cells (17). Galectin-3 proteins were proposed to act as alarmins due to amplifying inflammatory responses during sepsis and several types of infection (37,38,39). Interestingly, a high abundance (higher than 30.99 ng/mL) determined in serum can predict severity disease state (40).

Furthermore, galectin-3 orchestrates the inflammation response by activating innate immune cells and releasing different cytokines, including IL-6 and TNF alpha which are present in high concentrations in severe COVID-19 cases (36,37,38). Galectin-3 has also been proposed as a biomarker of the inflammatory status in COVID-19 patients. Cervantes-Alvarez et al., 2022 (39) have suggested that plasma levels of this protein have shown a strong correlation with lung fibrosis progression, a consequence of COVID-19. Additionally, galectin-3 has been found elevated in the serum of COVID-19 patients (41). In our study, galectin-3 was more abundant in COVID-19 group samples, with a fold change of 4.80.

CD59 glycoprotein is a potent inhibitor of the complement membrane attack complex (MAC) action. CD59 acts by binding to the C8 and/or C9 complements of the assembling MAC, thereby preventing incorporation of the multiple copies of C9 required for complete formation of the osmolytic pore. The CD59 glycoprotein is involved in signal transduction for T-cell activation complexed to a protein tyrosine kinase (17). The activity of CD59 in COVID-19 stills unveiled. However, many enveloped viruses are assembled in lipid rafts, which contain CD59 proteins (42). The lipid rafts are a cholesterol-rich domain found on the surface of cells, normally aggregating the TCR-antigen ligation site. This structure ensures the optimal immunological synapse on T-cell activation and antigen-specific signaling (43). It is also known that CD59 inhibits the formation of complement membrane attack complex pores in the membranes of expressing cells: it is a ‘suicide inhibitor’ (44). Our results shows that CD59 was more abundant in the COVID-19 sample group (FC 1.84). This result is not in agreement with Ramlal et al., 2020, who claimed that there is an under regulation of the CD59 gene during the COVID-19 infection (45). Ramlal’s investigation cannot be directly compared with our proteomic profiling of saliva since Ramlal characterized the transcriptome of nasopharyngeal swabs. In this study, SNPs from CD55 were found to negatively affect expression levels which are associated with a high risk of negative outcome (46).

4.1.4 Protein Metabolism

During infection, alterations in host metabolism occur at all levels—cellular, tissue, organ and physiological. Recent data from different infectious illnesses have shown that metabolic processes are significant mediators of host defense systems that guard against the physiological damage that happens during infections and subsequently permit survival. Impaired protein metabolism is a metabolic abnormality linked with severe COVID-19, characterized as increased protein and muscle breakdown, reduced muscle synthesis and increased synthesis of acute phase proteins (47).

The enrichment analysis showed that kallikrein 11 protein, inter-alpha-trypsin and alpha-2-macroglobulin are the main contributors for the dysregulation of this biological process.

Kallikrein 11 is a multifunctional protease that cleaves the kallikrein substrate and trypsin (17). Bradykinin production by the kallikrein/kinin system is involved in acute respiratory distress syndrome (ARDS) of bacterial sepsis origin and may be a driver of the COVID-19 ARDS-like lung injury. These peptides have chemotactic properties, induce increased vascular permeability, and activate immune and endothelial cells leading to inflammation and cyto-/chemokine expression (48).

Kallikrein 11 (KLK11) has not been associated with COVID-19 until now, but there are a few studies showing the relation of the kallikrein–renin pathway with the

infection by SAR-CoV-2. Correlations of this system, specifically the consumption of pekilocerin, with patient death due to respiratory failure have already been reported (49).

Lipcsey et al., 2021, suggested that the strong activation of the kallikrein/kinin system can be the main driver of the ARDS-like condition in COVID-19 (49). This pathway was also linked to COVID-19 by Carvalho et al. (2021), relating the activation of a kallikrein-like effect (TMPRSS2 transmembrane serine protease 2) to an increase in the production of bradykinin, leading to inhibition of the ACE2 pathway, the main entry of SARS-CoV-2 in the cells (48,50). This interaction was described also by Sidarta-Oliveira et al., 2020, defining its expression mainly to alveolar area (49). The kallikrein-11 increase (FC 2.61) in saliva of COVID-19 patients may be related to an active virus replication and cell entry.

Inter-α-trypsin inhibitor heavy chain H1(ITIH1) acts as a carrier of hyaluronan in serum or as a binding protein between hyaluronan and other matrix proteins, including those on cell surfaces in tissues to regulate the localization, synthesis and degradation of hyaluronan, which are essential to cells undergoing biological functions. ITIH1 contains a peptide that potentially stimulates a broad spectrum of phagocytotic cells (17). The function of inter-α-trypsin inhibitor heavy chain 1 has not yet been described in COVID-19. Nonetheless, in severe COVID-19 patients, ITIH1 is downregulated (50) and is more abundant in COVID-19 survivors group compared with the and non-survivors (51). Interestingly, according to our work, inter-α-trypsin inhibitor heavy chain H1 was found to be less abundant in the saliva of COVID-19 patients than in healthy patients (FC −8.28), which could be related to the severity of the disease.

Alpha-2-macroglobulin (α2-M) inhibits all four classes of proteinases by a unique ‘trapping’ mechanism. This protein has a peptide stretch, called the ‘bait region’, which has specific cleavage sites for different proteinases. When a proteinase cleaves the bait region, a conformational change is induced in the protein trapping the proteinase. The entrapped enzyme remains active against low molecular weight substrates (activity against high molecular weight substrates is greatly reduced). Following cleavage in the bait region, a thioester bond is hydrolyzed and mediates the covalent binding of the protein to the proteinase (17). An interaction with α2-M was tested for human immunodeficiency virus type 1, and a cleavage was shown in the bait region (52,53). Alpha-2-macroglobulin is known by the capacity of maintaining the hemostatic balance and moderating innate immunity (54,55), preventing structural damage during inflammation by inhibiting the expression of proteases by leukocytes that have been activated and also suppressing proteases released by invasive microbes (56). This protein also participates in the regulation of inflammatory mediators. In COVID-19, α2-M is attached on the luminal surface of endothelial cells (57). Seitz et al., 2020, suggested that a higher abundance of α2-M during SARS-CoV-2 infection in childhood favors a positive disease outcome (55). Oguntuyo et al. (58) determined that a higher abundance of alpha-1-antitrypsin and α2-M in serum could inhibit the SARS-CoV-2 particle invasion. The observed downregulation of alpha-2-macroglobulin in COVID-19 saliva (FC –2.91) agrees with an active infection.

4.1.5 Transport

To complete their life cycles, beta-CoVs as SARS-CoV-2 and HCoV-OC43, use an overlapping collection of host factors. These are genes involved in transport and biosynthetic processes (59). These findings imply that SARS-CoV-2 is dependent on

unique intracellular host factors and complexes that regulate intracellular transport (59).

Rab GTPases and Rab GTPase regulatory proteins, which govern intracellular transport, anchoring and exocytosis of secretory vesicles, are another group of important SARS-CoV-2 host factors (59).

Our enrichment analysis showed that annexin A7, proteolipid protein 2, transcobalamin 1 and lactotransferrin are the main contributors for the dysregulation of transport (Table 10).

Annexin A7 (ANXA7) is a calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis (17). Annexin A7 is part of a calcium-binding protein family implicated in vesicle transport and apoptosis. Exocytosis, glutamate release and N-Methyl-D-aspartic acid (NMDA) trafficking are all functions of ANXA7 (60). This supports the theory that there can be molecular mimicry of SARS-CoV-2 targeting ANXA7-expressing cells in the brain, resulting in neurological injury (60). The interaction of human annexin A7 and SARS-CoV-2 ORF1ab was described by Venkatakrishnan et al., 2020 (61), which is in accordance with the higher levels of annexin A7 (FC 6.40) in COVID-19 patients. Annexins A2 and A5 were associated with COVID-19 (62). Annexin A2 is critical for fibrinolysis in the lung by acting as a co-receptor that activates endogenous tissue plasminogen activator (t-PA) to lyse clots and promote fibrin clearance. Its inhibition might also explain the diffuse alveolar damage, ARDS and pulmonary fibrosis seen in severe cases of COVID-19 (62).

The proteolipid protein 2 (PLP2) (17,63) was defined as important for viral progression and linked to regulation of mitochondrial function in SARS-CoV-2 (64). Although the link between PLP2 and COVID-19 is not well established yet, we found that this protein is more concentrated in COVID-19 saliva samples (FC 3.05). This can represent a suppression of antiviral mechanisms and active infection progression.

Transcobalamin 1, also known as haptocorrin, is encoded by the TCN1 gene. This gene encodes a member of the vitamin B12-binding protein family (17). The association of TCN1 with COVID-19 was defined by its involvement in neutrophil-mediated immune responses (65,66). Distinct types of enzymes related to limiting host infection are expressed by neutrophil granulocytes. The cytotoxic chemicals influence the inflammatory response when they are released from the granules. As commonly related, COVID-19 patients manifest perivascular infiltrates surrounding the capillaries in the lungs. In these patients, TCN1 is up-regulated (66), as it is in COVID19+ saliva (FC 2.01). The correlation of TCN1 with type 2 Diabetes Mellitus on COVID-19 outcome is being studied (26). Higher levels of vitamin B12 were related with expanded seriousness of COVID-19 (67). On the other hand, in a plasma proteome profiling of COVID-19 contaminated patients, transcobalamin 2 was found to be elevated compared to the control group (68).

Lactotransferrin is known by its broad-spectrum antiviral activity as well as immunomodulatory and anti-inflammatory actions (69). It can simultaneously counteract the inflammatory and iron homeostasis disorders caused by bacterial and viral attacks (70). Lactotransferrin has been associated to COVID-19 pathology as a possible treatment (69). Recent in vitro studies demonstrated its antiviral activity against SARS-CoV-2 (69,70,71). The lower quantity of lactotransferrin in the saliva of COVID-19+ patients (FC –1.53) is related to the presence of an active viral infection.

4.2 Other Proteins

PLS-DA analysis is a well-known tool for analyzing multidimensional data. It incorporates a discriminatory algorithm that identifies the variables that allow the distinction between groups. A VIP score measures a variable's importance in the PLS-DA model, summarizing the contribution of each variable to the model. Proteins with a VIP > 1, but that do not account for the enrichment of the biological processes, are listed in Table 10, and include several immunoglobulins in patients with COVID-19. Until now, none of the identified immunoglobulins identified were related to COVID-19. Interestingly a similar immunoglobulin, the lambda variable 3–25, was identified as a COVID-19 characteristic protein that may indicate the progression of the two stages of the COVID-19 disease (72).

Ras-related C3 botulinum toxin substrate 1 (RAC1) is a plasma membrane-associated small GTPase that, when active, binds to a variety of effector proteins regulating cellular responses from secretory processes to phagocytosis of apoptotic cells, and neurons adhesion, migration and differentiation (17). Ras-related C3 botulinum toxin substrate 2 (RAC2) is involved in inflammation-mediated lung damage (73). The link to COVID-19 was assigned by Wang et al., 2020 (74), with neutrophil activation after the SARS-CoV-2 invasion by the RAC2 gene.

Thymidine phosphorylase (TYMP) encodes an angiogenic factor which promotes angiogenesis in vivo and stimulates the in vitro growth of a variety of endothelial cells (17). TYMP has a role in the systemic immune response to SARS-CoV-2 infection. Because red blood cells do not produce TYMP and TYMP is not secreted, it is hypothesized that that the elevated plasma TYMP concentration in COVID-19 patients was caused by either platelet-rich thrombolysis or organ injury. TYMP expression is high in both platelets and the lung (75), as well as in saliva (5.89 times more abundant than in the saliva of healthy patients, Table 10).

Ly6/PLAUR domain-containing protein 2 (LYPD2) is predicted to be extracellular or in the plasma membrane (17). LYPD2 is highly expressed in transcriptional profiles in non-classical monocytes from patients with COVID-19 (76). The gene encoding for this protein is differentially expressed in two neutrophil subpopulations (77). Non-classical monocytes may ingest virally infected apoptotic endothelial cells (78). The 2.62-fold increase of LYPD2 in COVID-19 saliva samples may represent an active immune system fighting against infection in the COVID+ sample group, but further validation is needed.

Transmembrane protein 165 (TMEM165) is a transmembrane protein produced in fibroblasts that has been linked to bacterial infections in the lungs (17). SARS-CoV-2 interactome reveled the interaction between ORF3a and TMEM165, which is consistent with the knowledge that ORF3a hijacks the HOPS (homotypic fusion and vacuole protein sorting) complex and RAB7, required for membrane contact between autophagosomes and lysosomes for autolysosomes to develop (79). The increase on TMEM165 abundance in the saliva of COVID-19 patients (FC 2.42) is consistent with an active infection.

Metabolism regulating signaling molecule B (FAM3B) induces apoptosis of alpha and beta cells in a dose- and time-dependent manner (17). FAM3B expression in the endocrine pancreas is stimulated by hyperglycemia and proinflammatory cytokines. FAM3B promotes insulin secretion under physiological settings, but it is also a secreted cytokine-like protein that can cause cellular death. Increased FAM3B production is linked to pancreatic cell dysfunction, hyperglycemia and insulin resistance, implying a

role in the control of glucose and lipid metabolism. Fraser et al. (80) has suggested FAM3B as a protein biomarker able to predict survival/death of patients with COVID-19. In this study, COVID-19 patients had a 2.25-fold increase of FAM3B.

Ras-related protein Rab-6A is a protein transport and regulator of membrane traffic from the Golgi apparatus towards the endoplasmic reticulum (17), found interacting with SARS-CoV-2 RNA in infected human cells (81). This interaction was also mentioned in a study from Pereira et al., 2021 (82): RAB6A was found interacting with SARS-CoV-2 cells associated with vesicle trafficking (81). Viral infection may alter the host cell's exosomal-loading processes, resulting in alterations in the protein and nucleic acid content of extracellular vesicles (EVs). It indicates that infected cells produce modified EVs rather than relying on viral content. As a result, as compared to EVs that do not include infected cells, these changed EVs may influence the host immunological response (83). Our results show RAB6A had a 2.18-fold change, indicating a vesicle trafficking compromise.

V-Set and immunoglobulin domain containing 10-like (VSIG10) is expressed in the esophagus (and many other oral and digestive tissues) and is involved in anti-inflammatory responses. It is the first time that this protein is related to COVID-19 (two-fold increase in COVID-19 patients).

Calcium-binding protein 39 (CAB39) enables kinase binding activity and protein serine/threonine kinase activator activity (17). This protein was associated with SARS-CoV by a functional enrichment pathway (84) and is less abundant in SARS-CoV-2 blood samples (85). We detected a 1.77-fold increase of CAB39, potentially indicating that different body fluids may show different trends for the same protein.

Prohibitin 2 (PHB2) is involved in defense response to viruses (17). It may contribute to viral replication by arresting normal host cell functions (86). The interaction of nnon-structural protein 2 (NSP2) of SARS-CoV with prohibitin 2 was shown by Cornillez-Ty CT. et al. (2009) (87). It is hypothesized that coronavirus proteins may impact key mitochondrial functions such as respiration, but also lipid homeostasis and innate immunity. Indeed, mitochondria are involved in both lipogenesis and lipolysis, and prohibitin expression has been shown to impact lipid accumulation and degradation (88).

4.3 Human–SARS-CoV-2 In Silico Interactomics Analysis

The human–SARS-CoV-2 interactome was predicted using OralInt v 2.0 (12). Protein quantitation data obtained by mass spectrometry were used to represent the degree of alteration of proteins after infection with SARS-CoV-2.

OralInt (12) (https://bioinformatics.ua.pt/software/oralint/, accessed on 22 February 2022) is an algorithm designed for interactome prediction: it allows the identification of key proteins involved in pathologies which may be used in diagnostics or as therapeutic targets, as well as the proposal of microbial infection mechanisms.

The in silico interactomics analysis predicted a total of 10,914 protein–protein interactions (PPIs), with 263 of these PPIs showing a high level of confidence (SCORE ≥ 0.7). The 14 virus proteins interact with 100 human proteins found altered in the saliva of COVID-19 patients (Figure 10). Only 5 of these 100 human proteins were identified in SARS-CoV-2 infection pathway and related pathways by the PathCards (genecards.org accessed on 22 February 2022): Ubiquitin-60S ribosomal protein L40, beta-2-microglobulin, ubiquitin-conjugating enzyme E2 variant 1, 14-3-3 protein

beta/alpha and dolichyl-diphosphooligosaccharide protein glycosyltransferase subunit 1. The remaining 95 proteins might be related to not yet known molecular changes of SARS-CoV-2 infection.

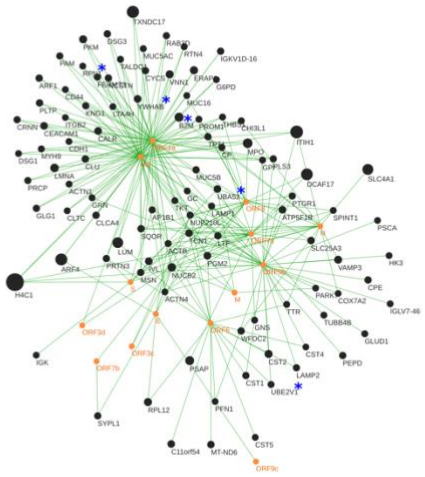

Figure 10. High-confidence network of interactions (263 interactions) resulted from the input of 642 human proteins and the SARS-CoV-2 reference proteome (14 proteins) predicted by OralInt. Network visualization was done using Cytoscape. SARS-CoV-2 proteins in orange, human proteins in black. Hub proteins in bold. Node size is proportional to the absolute value of the fold change. * Represent the five proteins already identified in the SARS-CoV-2 pathway by PathCards.

4.3.1 Viral Hub Proteins

Viral hub proteins interact mainly with proteins from human immune system, suggesting a direct influence on inflammation and host defense modulation. It is worth noting that this is the first time that ADP-ribosylation factor 4 (cellular trafficking) has been associated with COVID-19.

The hub proteins ORF1a and rep are involved in a high number of interactions with human proteins (histone H4 (H4); inter-alpha-trypsin inhibitor heavy chain H1; lumican (LUM); myeloperoxidase (MPO); beta-2-microglobulin (B2M); ADP-ribosylation factor 4 (ARF4) and thioredoxin domain-containing protein 17 (TXNDC17); Figure 10). ORF1a and rep are multifunctional virus proteins involved in the transcription and replication of viral RNAs. Both proteins inhibit antiviral response triggered by innate immunity or interferons (88,89,90) and may play a role in the modulation of the host cell survival signaling pathway.

ORF1a and rep interact with histone H4 (H4). Histone H4 has the most potent antiviral activity among histones and has been shown to promote neutrophil activation and consequently the early phase of the innate immune response to influenza A virus (91,92,93,94). This study shows a reduction in histone H4 in patients with COVID-19 (FC −14.47), which may prevent an effective elimination of the virus at an early stage of infection.

Inter-alpha-trypsin inhibitor heavy chain H1 (ITIH1) (FC −8.28) contains a phagocytosis uptake signal motif which could stimulate a broad spectrum of phagocytotic cells. We hypothesize that a reduction in ITIH1 may compromise the phagocytosis of viral particles. This is supported by the reduction in apoptosis observed

in the functional analysis (Figure 8) which is known to precede the phagocytosis of viruses already observed regarding influenza A virus (92).

Lumican (LUM) (FC −5.90) has been found to be critical for the host immune innate response (95); therefore, lower lumican levels may act as an additional biomarker of inflammation in the COVID-19 patients.

Myeloperoxidase (MPO) (FC −4.44) plays a role in the host defense system of polymorphonuclear leukocytes. It is responsible for microbicidal activity against a wide range of organisms (Figure 10—Innate immune system). In the stimulated PMN, MPO catalyzes the production of hypochlorous acid, in physiologic situations, and other toxic intermediates that greatly enhance PMN microbicidal activity (96). The production of MPO can be activated by histone H4 and, since it is largely reduced in patients with COVID-19 (FC −14.47), this can lead to a reduction of MPO and, consequently, diminish the antiviral responsiveness to SARS-CoV-2.

Beta-2-microglobulin (B2M) is a component of the class I major histocompatibility complex (MHC) involved in the presentation of peptide antigens to the immune system (Figure 10). B2M is also essential for the correct subcellular distribution of both hereditary hemochromatosis protein (HFE) and hepcidin, two proteins critical for iron homeostasis (97). An increase in B2M and consequently in hepcidin can lead to the marked hypoferremia that seems to characterize severe COVID-19, and may contribute to worsening prognosis by impairing not only response to hypoxia, but also immune function (98). Finally, it should be considered that low iron status can theoretically impair the efficacy of COVID-19 vaccination (99). In this study, B2M was found to be increased in the saliva of patients with COVID-19 (FC 3.78), which agrees with the work of Conca and colleagues (100), who related the serum levels of this protein with the severity of the disease. Our work demonstrates that it is possible to measure the levels of this protein in a less invasive way in saliva samples, maintaining the trend observed in serum (101).

Thioredoxin domain-containing protein 17 (TXNDC17) (FC 7.59) has not yet been linked to COVID-19, although it is known to modulate TNF-alpha signaling and NF-kappa-B activation (102), which have a role in cytokine storm syndrome, associated with greater severity in COVID-19-related symptoms. Therefore, therapeutics that reduce the levels of TXNDC17 may be considered in the treatment of COVID-19.

ADP-ribosylation factor 4 (ARF4) is a GTP-binding protein involved in protein trafficking. To our knowledge, the direct correlation between ARF4 and SARS-CoV-2 infections has not yet been established. However, there is evidence that hepatitis C virus (HCV) infection is associated with an upregulation of ARF4, which promotes HCV replication (99). The pronounced increase in ARF4 (FC 7.37) observed in our study suggests that SARS-CoV-2 may employ a replication-promoting mechanism similar to HCV, as both are Group IV viruses ((+)ssRNA). This may provide new therapeutic targets for SARS-CoV-2 antiviral therapy.

4.3.2 Human Hub Proteins

The functional analysis of the high-confidence interaction network between human saliva proteins and SARS-CoV-2 proteins shows that many of these interactions are associated with proteins from the human immune response (transcobalamin 1 (TCN1) and nucleobindin-2 (NUCB2)), mainly through the degranulation of neutrophils.

Furthermore, the virus alters the host's energy metabolism and interferes with apoptosis mechanisms (Figure 11 and Section 3.1).

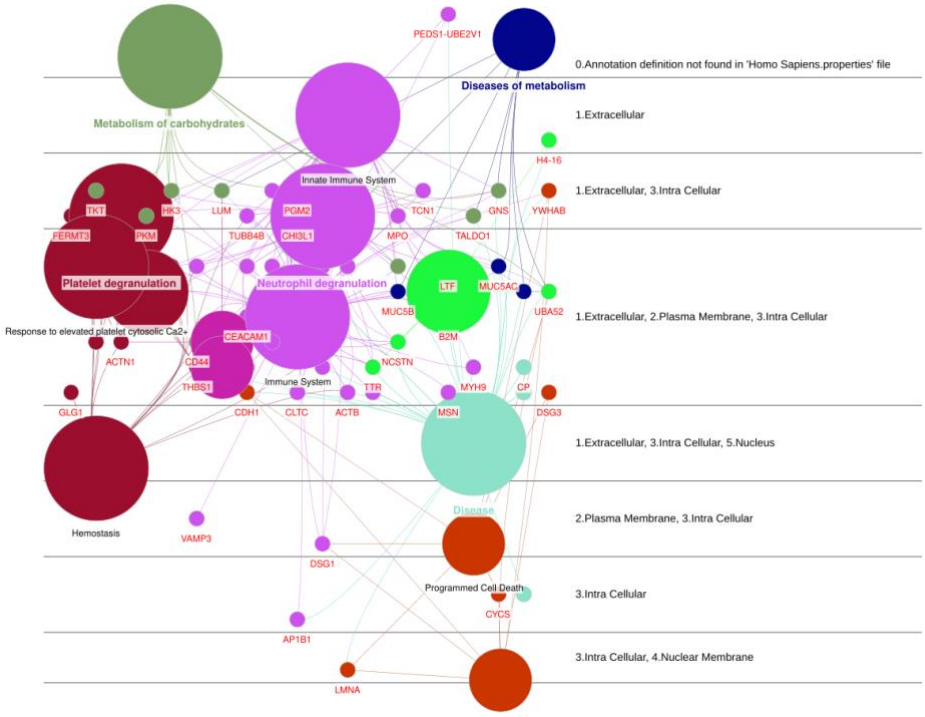

Figure 11. Functional analysis of the high-confidence interaction network of human saliva proteins with SARS-CoV-2 proteins. Reactome pathway analysis, using ClueGo + CluePedia; visualization with Cerebral View.

The association of the NUCB2 protein (FC −3.04) with COVID-19 is not yet established. It is known that this protein may be involved in the regulation of inflammation, immune functions, host defense and apoptosis through mediating TNF-alpha receptor (TNFR1) release (103). The decrease in NUCB2 in patients infected with SARS-CoV-2 (FC −3.04) may contribute to the cytokine storm characteristic of the most severe forms of COVID-19, as a decrease in the amount of TNFR1 can cause an increase in the production of TNF-alpha to compensate for the decrease in receptor availability.

TCN1 (FC 2.08) is a major constituent of secondary granules in neutrophils, is involved in neutrophil-mediated immune responses (Figure 11) and has been previously found upregulated in nasopharyngeal swabs from SARS-CoV-2 patients (67). This study shows that these alterations can also be observed in saliva samples and corroborate other studies that hypothesized that the increase in TCN1 may be related to a generalized hyperinflammatory state characteristic of COVID-19 (66).

5. Conclusions

Although we consider that, in the future, further validation—with larger sample groups, that allow correlating molecular data with patient symptoms—should be considered, this study is a proof of concept of the molecular aspects of SARS-CoV-2 infection applying a proteomic approach on saliva.

We have identified 26 proteins altered in patients with COVID-19, which may be indicative of alterations resulting from the disease. Fourteen had already been described in other types of samples but not in saliva. On the other hand, 10 had never been associated with SARS-CoV-2 infection before, representing new evidence of the impact of COVID-19 on the host. As expected, many of the proteins altered upon infection with SARS-CoV-2 are related to the immune system, and the interactome analysis shows that the virus directly interacts with human immune system proteins. We showed that the virus alters the host's energy metabolism and interferes with apoptosis mechanisms. We also showed that many of the proteins that are altered upon infection with SARS-CoV-2 are involved in apoptosis, transport and signal transduction, revealing the broad spectrum of alterations that COVID-19 represents.

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Chapter V - Saliva for Diabetes non-invasive diagnostics and monitoring

The work presented in this chapter is being submitted.

1. Abstract

Type 2 Diabetes Mellitus is a chronic metabolic disease and is a major risk to health due to its characteristic long-term complications. It's estimated that around 537 million people live with diabetes worldwide and this number will keep increasing. Because of the dysregulation of glucose homeostasis characteristic of Diabetes, complications such as retinopathy, nephropathy and neuropathy or more severe like strokes and myocardial infarction are common.

The diagnosis and glucose concentration monitoring in diabetes are well established. However, monitoring the many complications of diabetes remains a challenge, compromising patients' prognosis and quality of life.

We present a hybrid proteomic strategy for the identification of salivary markers of T2DM and its complications. Mass spectrometry resulted in the identification of 917 proteins from which 622 have a 5% Normalized FDR. PANTHER analysis defined 16 disrupted biological processes associated to diabetes. Metabolic processes, response to stimulus, immune system processes and signalling can be highlighted by the known relation with T2DM. FunRich enrichment identified 11 deregulated biological processes highlighting 20 proteins directly related to complications in diabetes. Diabetic retinopathy, metabolic syndrome, insulin resistance, molecular impact of glucose and insulin homeostasis dysregulation, atherosclerosis, diabetic foot ulcer, protein catabolism and salivary gland function can be monitored using saliva. Albumin and Lactotransferrin concentrations are altered on diabetic patients' saliva. The alterations of these proteins are common to different complications and can be considered generic saliva indicators of diabetes complications.

We conclude that saliva has the potential to identify several molecules altered in diabetic patients compared to non-diabetic patients and that may be biomarkers not only of diabetes but also of the different complications of this disease. These results corroborate that saliva is a good diagnostic and monitoring fluid for diabetes and its complications.

2. Introduction

Type 2 Diabetes Mellitus (T2DM) is a chronic metabolic disease, defined by hyperglycaemia caused by abnormalities in insulin secretion, insulin action, or both. Diabetes is a major risk to health due to its characteristic long-term complications (1–3). The International Diabetes Association estimated a prevalence of 537 million people living with diabetes worldwide in 2021 and projected an increase of 46% by 2045 (4). The extension of this situation has been described as epidemy, due to the overwhelm of the healthcare systems (5).

Frequently, Diabetes Mellitus has complications associated. Those can be grouped in “microvascular diseases” (due to damages to small blood vessels) and “macrovascular diseases” (due to damages to the arteries) (6,7). Microvascular complications include retinopathy, nephropathy, and neuropathy (6,8). The major macrovascular complications include accelerated cardiovascular disease resulting in myocardial infarction and cerebrovascular disease manifesting as strokes (6,9). In opposite, diabetic ketoacidosis from exceptionally high blood glucose concentrations (hyperglycemia) and coma as the result of low blood glucose (hypoglycemia) are fatal (6). T2DM is also characterized by comorbidities such as obesity and cardiovascular disease. Micro-vascular and neuronal changes are resultant from the metabolic dysregulation of Diabetes Mellitus, compromising the ability of multiple normal organ functioning (2). Uncontrolled hyperglycaemia and induced metabolic disorders ultimately lead to micro- and macrovascular complications (e.g., angiopathy or retinopathy) (10).

Type 2 Diabetes Mellitus has an established screening and diagnostic protocol. Diabetes can be diagnosed either by the haemoglobin A1C criteria or plasma glucose concentration (after 12h fasting or 2-hour plasma glucose) (11). After a diagnosis of Diabetes Mellitus, the patients require to self-monitoring plasmatic glucose concentrations, regularly. The estimation of plasmatic glycated haemoglobin, and lipid levels is also necessary (11) for the evaluation of disease progression. Although the diagnosis of diabetes and blood glucose monitoring are well established, monitoring the many complications of diabetes remains a challenge (10).

Saliva is being addressed as a relevant biofluid for diagnostics due to its characteristics: it is safe and easy to collect, collection is non-invasive, and it contains many of blood and systemics molecules frequently used as biomarkers of diagnosis (2,3,5). But, although salivary diagnostics is increasingly being considered, monitoring pre- and established diabetes and its complications in saliva is yet to be validated (5,12). The identification of markers of prognosis could support the development of “point-of-care” tests that a physician could apply to follow up the patient and alert for behavioural changes or adjustment of diabetes control medication.

The aim of this study was the identification of biomarkers in saliva that allow monitoring of diabetes and its complications. We tested the hypothesize that saliva provides the biological information – proteins – to identify characteristic proteome changes induced from type 2 diabetes. The outcome may contribute to knowledge on developing new approaches to further study this disease.

3. Materials and Methods

3.1 Ethical Statement

This study was carried out in accordance with the Helsinki Declaration and the Oviedo Convention. Saliva samples were collected following the guidelines for the protection of personal data and ethical issues in use at the Biobank of the Institute of Molecular Medicine (IMM) of the Faculty of Medicine, University of Lisbon (http://biobanco-imm.biobanco.pt/documents). Written informed consent including the purpose of the study, data confidentiality, rights of participants, and the right to withdraw from the study at any time was provided by every participant or by their legal representatives or guardians before study enrolment.

3.2 Participant Enrolment

Five saliva samples (3 females and 2 males aged 69 to 82) of a cohort of T2DM patients from Centro de Saúde Dão Lafões, were collected. Saliva samples from five healthy subjects (3 females and 2 males aged 8 to 39) showing no evidence of oral and systemic pathologies after clinic evaluation, were also collected.

3.3 Saliva Collection

Cotton roll-based saliva samples were collected with two sterile cotton rolls placed under the tongue, for 2 minutes. The cotton rolls were placed inside a 15 mL sterile plastic tube with a sterile 100 mL pipette tip in the bottom to facilitate saliva collection by centrifugation at 10,000 g for 10 minutes at 4 ºC (13). Samples were then refrigerated and inactivated using 1% Triton X-100. Whole saliva was centrifuged at 10,000 ×g, 10 min at 4 °C. The supernatant was aliquoted and stored at -80 °C for analysis.

3.4 Sample preparation and LC-MS methodology

Protein concentration was determined using the Pierce BCA assay kit (ThermoFisher Scientific), according to the manufacturers' instructions and concentration was adjusted to 50 mg/mL. Saliva was analyzed by capillary electrophoresis (Experion™ automated capillary electrophoresis system, Bio-Rad Laboratories), and compared to the in-house profiles to confirm sample integrity.

Sample preparation for data-dependent acquisition (DDA) experiments and data-independent acquisition (DIA), as well as LC-MS methodology was performed as described previously (14).

3.5 Data analysis

3.5.1 Ion-Library construction (DDA information)

A specific ion-library of the precursor masses and fragment ions was created by combining all files from the DDA experiments in one protein identification search using the ProteinPilot™ software (v5.0, Sciex). The paragon method parameters were the following: searched against the reviewed Human (SwissProt) database, cysteine alkylation by acrylamide, digestion by trypsin, and gel-based ID. An independent False Discovery Rate (FDR) analysis, using the target-decoy approach provided by Protein Pilot™, was used to assess the quality of identifications.

3.1.2 Relative quantification of proteins (SWATH-MS)

SWATH data processing was performed using SWATHTM processing plug-in for PeakViewTM (v2.0.01, Sciex®). Protein relative quantification was performed in all samples using the information from the protein identification search. Quantification results were obtained for peptides with less than 1% of FDR and by the sum of up to 5 fragments/peptide. Each protein was normalized for the total sum of areas for the respective sample. Protein quantities were obtained by the sum of up to 15 peptides/protein.

3.6 Protein functional analysis

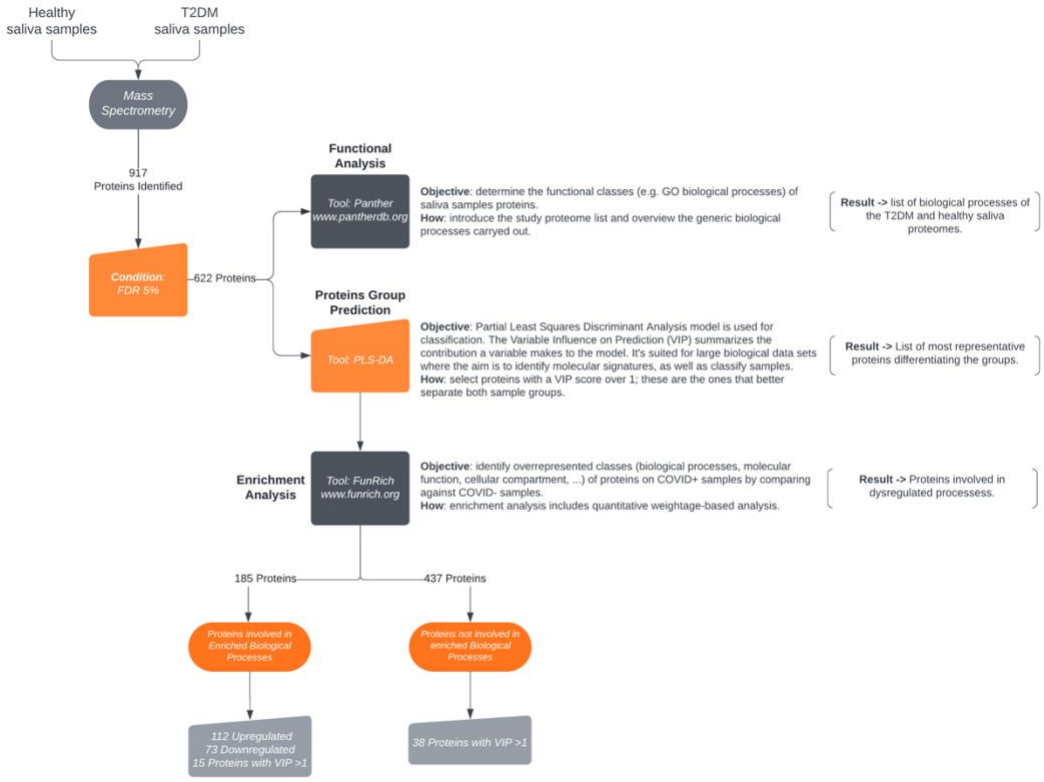


Figure 12. Scheme of the protein functional analysis workflow. Proteins were identified by LC-ESI-TOF mass spectrometry. An initial dataset of 917 proteins was narrowed down filtered by an FDR 5%, resulting in 622 salivary proteins then descripted according to GO biological process by PANTHER tool followed by an enrichment analysis by FunRich tool. Group analysis by PLS-DA model defined 53 proteins with a VIP score>1, 15 of which are also included in the enriched biological processes. The enrichment analysis resulted in 185 proteins, with dysregulated processes with a gene expression >10%. Adapted from "COVID-19 Salivary Protein Profile: Unravelling Molecular Aspects of SARS-CoV-2 Infection".

Functional analysis of salivary proteins in T2DM identified by mass spectrometry was performed according to figure 12.

Narrowed down proteome biological processes classification was obtained via the PANTHER Gene List Analysis (PANTHER V 17.0) (15). Only the PANTHER GO-SLIM biological processes were considered. The dataset followed an enrichment analysis of biological processes' using FunRich tool against FunRich curated database as background (16). PLS-DA model, was used to select the proteins capable to classify the 2 groups of samples, based on variable influence on projections (VIP) values. Proteins with the biological process enriched were discussed according to their relationship with T2DM.

3.7 Statistical analysis

Kruskal-Wallis test was performed to select the proteins statistically different between the T2DM and Healthy samples. Dunn's test of Multiple Comparisons, with Benjamini-Hochberg p-value adjustment, was performed to know in which comparisons statistical differences were observed. Mann-Whitney test was used for the binary comparisons. The multivariate analysis was performed in MetaboAnalyst as previously described (17).

4. Results and Discussion

We hypothesized that Diabetes Mellitus type 2 induces characteristic proteome changes that can be detected in saliva. We used a methodological approach for the functional analysis of altered salivary proteins identified by mass spectrometry to point out or clarify molecular changes associated with T2DM. These molecular changes may shed light on the metabolic dysregulations induced by Diabetes Mellitus or allow the identification of salivary markers for disease monitoring and its complications.

The analysis of the 10 saliva samples (T2DM and Healthy) resulted in the identification of 917 proteins from which 622 have a 5% Normalized FDR.

PANTHER gene list analysis has successfully mapped 611 from the 622 proteins which participate in 16 biological processes (Figure 13). Metabolic processes, response to stimulus, immune system process and signalling have a known relation with T2DM (18).

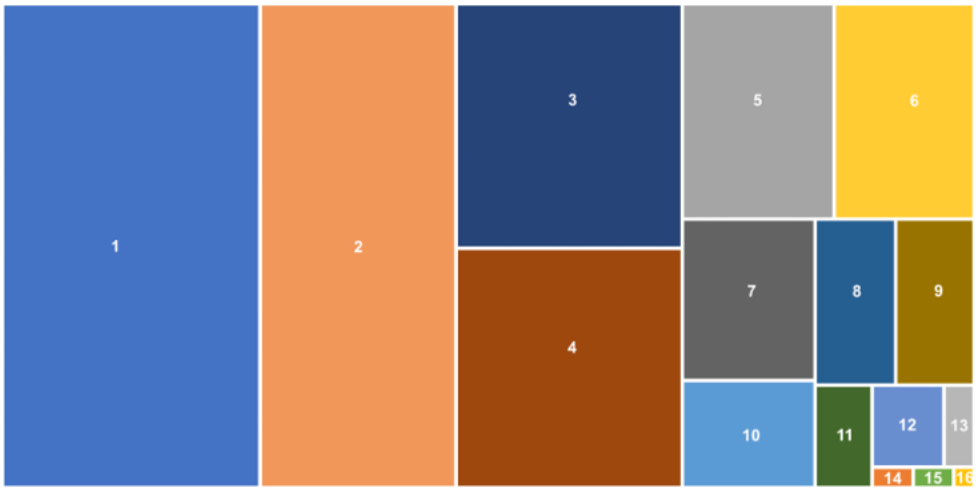


Figure 13. Tree map of PANTHER Gene List biological processes result. The numeration is in ascending order according to the number of genes involved in the respective biological process. The box size follows the same pattern. 1 – Cellular process (297 genes); 2 – Metabolic process (226 genes); 3 - Biological regulation (132 genes); 4 – Response to stimulus (129 genes); 5 – Localization (78 genes); 6 – Immune system process (72 genes); 7 – Signaling (51 genes); 8 – Biological process involved in interspecies interaction between organisms (34 genes); 9 – Multicellular organismal process (32 genes); 10 – Developmental process (31 genes); 11 – Locomotion (14 genes); 12 – Biological adhesion (14 genes); 13 – Growth (6 genes); 14 – Reproductive process (2 genes); 15 – Reproduction (2 genes) and 16 – Biomineralization (1 gene).

Enrichment analysis (FunRich) resulted in 185 proteins across 11 disrupted biological processes. In figure 14 the proteins involved in the enriched biological processes are represented according to their fold change: 112 are increased and 73 are decreased in the saliva of T2DM patients.

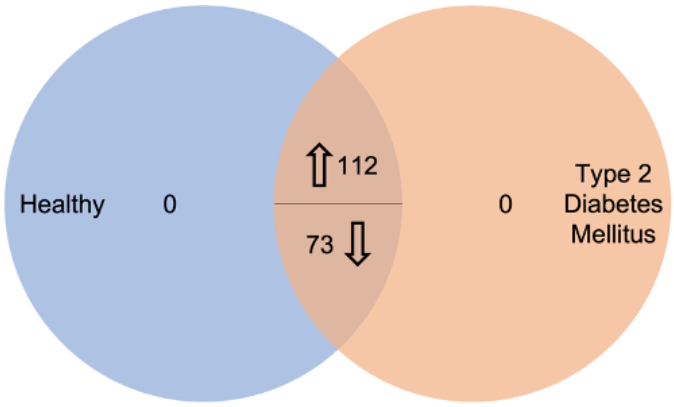


Figure 14. Venn diagram representing the proteins resulting from the enrichment analysis in FunRich tool.

The biological processes identified as disrupted in T2DM and the number of proteins involved in each are shown in figure 15. In a summary assessment of the enrichment analysis, the mechanism most affected is inflammatory response. Other mechanism that was found altered in DMT2 patients were Detection of chemical stimulus involved in sensory perception of bitter taste, Cellular response to starvation, Maintenance of mitochondrion location and Oligosaccharide metabolic process.

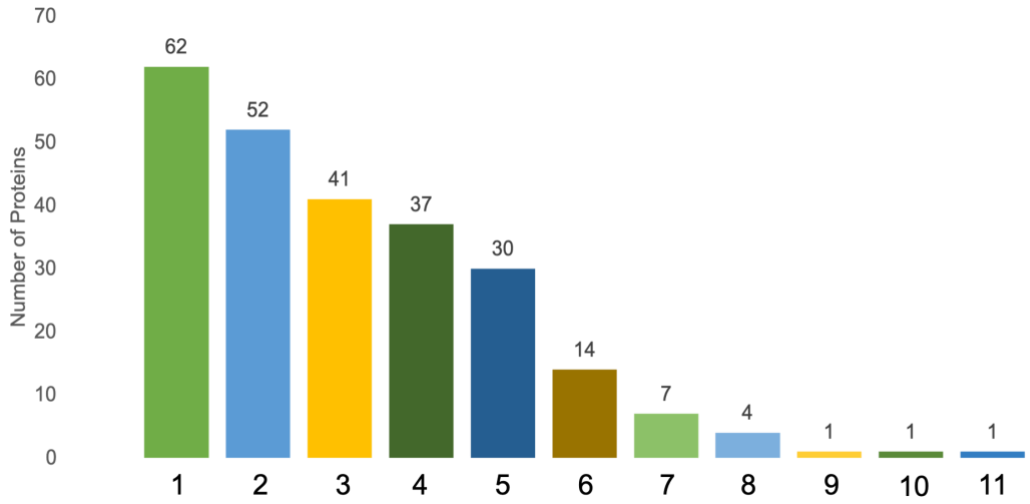


Figure 15. Biological processes enrichment analysis graphical representation of the proteins with gene expression >10 against the FunRich background database. The biological process is indicated in the bottom of graph in descending number of proteins involved. 1 - Innate immune response; 2 - Proteolysis; 3 - Adaptative immune response; 4 - Defense response to bacterium; 5 - Negative regulation of apoptotic process; 6 - Retina haemostasis; 7 - Detection of chemical stimulus involved in sensory perception of bitter taste; 8 - Cellular response to starvation; 9 - Maintenance of mitochondrion location; 10 - Negative regulation of programmed cell dead and 11 - Oligosaccharide metabolic process.

4.1 Protein enrichment analysis

Eleven biological processes are significantly altered (enrichment analysis, Biological Process Enrichment, Figure 16), with a minimum of 10% of differential expression.

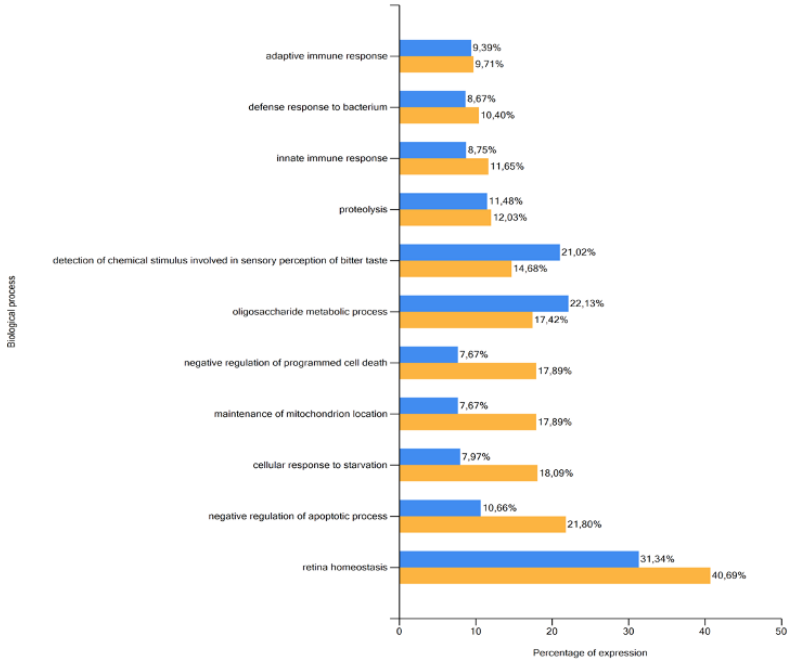


Figure 16. Biological Processes enrichment comparison between Healthy (blue) and Type 2 Diabetes Mellitus (orange) relative to the percentage of expression analysis against the UniProt background database. The biological processes are listed in the Y axis, with the respective fold change on the X axis. The biological process is indicated next to the graphic bar in A→Z order.

4.1.1 Retina homeostasis

The retina homeostasis is dysregulated in T2DM which is related to retinopathy, one of the most well-known complications of diabetes. We identified 8 proteins, highlighting Lactotransferrin, Heat shock protein beta-1 and Lipocalin-1 considered to provide reliable information's to monitor this condition and prevents diabetic retinopathy (table 11).

Table 11. List of proteins related with retina homeostasis process dysregulation in T2DM (p value <0.05). Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (T2DM/Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis. Only proteins with a VIP > 1 are shown.

UniProtKB AC	Protein name	Gene name	Fold change	VIP
P04792	Heat shock protein beta-1	HSPB1	-2.49	1.08
P25311	Zinc-alpha-2-glycoprotein	AZGP1	-1.56	1,60
P02787	Serotransferrin	TF	1.66	1.49
P02768	Albumin	ALB	2.23	1.59
P01860	Immunoglobulin heavy constant gamma 3	IGHG3	2.87	1.05
P02788	Lactotransferrin	LTF	5.25	2.02
Q96DA0	Zymogen granule protein 16 homolog B	ZG16B	5.70	1.27
P31025	Lipocalin-1	LCN1	6.02	1.44

Retina requires a healthy choroid to nourish the different outer retinal layers (19). Lactotransferrin (LTF), present in the human retina (19), has been proofed successful in the treatment of lesions in choroidal neovascularization in mice (20) and in parallel, when coated, demonstrated to be efficient on the treatment of the dry eye (21). In our study this protein was identified upregulated in T2DM saliva samples (FC = 5.25) being one of the protein contributors to the separation of study groups (VIP = 2.02). This protein can be an important marker to monitor the choroidal health, knowing that a disruption in homeostasis can lead to diabetic retinopathy, a microvascular complication of diabetes (19,22).

Heat shock protein beta-1 (HspB1) is upregulated upon optic nerve injury. This suggests that there might have a protective value for retinal ganglion cells after optic nerve injury (23). In a similar perspective, HspB1 has been shown to play a vital role in maintaining the integrity of retinal capillary endothelial cells. Proinflammatory cytokines are generally upregulated in the diabetic retina, reducing the levels of HspB1 in retinal capillary endothelial cells, which could lead to their apoptosis in diabetic retinopathy (24). This reduction has been observed in our results with a downregulation on T2DM saliva samples (FC=-2.49). The fact that pro-inflammatory cytokines are elevated can be the reason to have the LTF elevated on T2DM samples, with the main role of mediator of the innate immune response.

We identified an increase of Lipocalin-1 (LCN1) concentration on T2DM saliva samples (FC=6.02). To our knowledge, this protein has not yet been linked to T2DM. It belongs to a family of transport proteins and scavenges lipid from the corneal surface (25). Lipocalin-2 (LCN2) levels were significantly increased in the whole retinae of the rats after induction of retinal degeneration, compared to the controls. It has been suggested to be an upstream regulator of apoptosis of retinal degeneration (26). LCN2 is instrumental for the progression of diabetic complications like encephalopathy and peripheral neuropathy (27). The LCN2 upregulation observed after the retinal degeneration can be correlated with the LCN1 increased on our T2DM samples.

We identified albumin (ALB) upregulated (FC=2.23) in saliva and was previously suggested as diabetic retinopathy biomarker in serum (28).

Lactotransferrin, Heat shock protein beta-1 and Heat shock protein beta-1 are markers of changes in retina homeostasis process and consequently could be used for monitoring diabetic retinopathy complication of diabetes.

4.1.2 Negative regulation of apoptotic process

In T2DM the apoptotic process is one of the processes involved in the dynamic regulation of pancreatic ß-cell mass. Defects in ß-cells cause insulin resistance and inability to increase the insulin production (29). The number of ß-cells can increase (hyperplasia) by active replication and/or neogenesis. This increase is balanced by reduction in cell number via cell death that may results from necrosis and/or apoptosis. It is apoptosis that plays a critical role in the reduction of ß-cell mass occurring in patients with type 2 diabetes and subsequent progressive worsening of glycaemic control (29).

The negative regulation of apoptotic process was shown dysregulated in T2DM. We identified 19 proteins, highlighting the Endoplasmic reticulum chaperone BiP, Albumin and Lactotransferrin (table 12).

Table 12. List of proteins related with negative regulation of apoptotic process dysregulation in T2DM (p value <0.05). Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (T2DM/Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis. Only proteins with a VIP > 1 are shown.

UniProtKB AC	Protein name	Gene name	Fold change	VIP
Q04760	Lactoylglutathione lyase	GLO1	-2.73	1.48
P09211	Glutathione S-transferase P	GSTP1	-2.71	1.09
P04792	Heat shock protein beta-1	HSPB1	-2.49	1.08
P11021	Endoplasmic reticulum chaperone BiP	**HSPA5**	**-2.48**	**1.31**
O95865	Dimethylarginine dimethylaminohydrolase 2	DDAH2	-2.44	1.42
P63104	14-3-3 protein zeta/delta	YWHAZ	-2.34	1.15
P05120	Plasminogen activator inhibitor 2	SERPINB2	-1.62	1.08
P16070	CD44 antigen	CD44	1.16	0.99
P07996	Thrombospondin-1	THBS1	1.86	1.32
Q8NBS9	Thioredoxin domain-containing protein 5	TXNDC5	1.90	1.21
P02768	Albumin	ALB	2.23	1.59
P04040	Catalase	CAT	2.45	1.38
P14780	Matrix metalloproteinase-9	MMP9	2.75	1.32
P21333	Filamin-A	FLNA	2.87	1.13
P05164	Myeloperoxidase	MPO	4.72	1.11
P02788	Lactotransferrin	LTF	5.25	1.48

Lactotransferrin promotes apoptosis and the elevated levels have been related with neutrophil dysfunction in T2DM (30,31). In fact, LTF has been suggested as a biomarker to monitor the development or progression of diabetes and its complications (30). In opposite side, Endoplasmic reticulum chaperone BiP (HSPA5), is suggested to be a protector against ER stress, required for the ß-cells dysregulated apoptosis and consequent T2DM development (32).

It can be hypothesized that a failure of negative regulation of apoptotic process can lead to necrosis of ß-cells. The apoptosis has already been found highly activated in T2DM individuals (29). In saliva we identified LF upregulated (FC = 5.25; VIP = 2.02) sustaining the assumptions of previous authors. HSPA5 identified as protector of ER is underregulated on T2DM saliva samples (FC = -2.48; VIP > 2.02), suggesting an active ß-cell apoptosis on patients.

It's important to point out that negative regulation of programmed cell death process was found dysregulated as well. These two processes have a relation since both may contribute to a unfunctional equilibrium in the ß-cell mass and consequent glycaemic control. Albumin (ALB) plays a role as a negative inflammation biomarker, was the only protein (p value < 0.05) identified in this process. Low albumin concentration was associated with an adverse metabolic profile (33) on saliva samples, ALB was found elevated (FC = 2,23) suggesting an active fighting inflammation ongoing (34).

Proteins Lactotransferrin, Endoplasmic reticulum chaperone BiP and Albumin are markers of changes in negative regulation of apoptotic process and consequently may

be good candidates for monitoring the metabolic syndrome (glycaemic metabolic deregulation process) in diabetes.

4.1.3 Cellular response to starvation

Starvation is one of the earliest forms of insulin resistance and is associated with increased lipid metabolism molecules (lipolysis, plasma fatty acid, ketone bodies and intramyocellular lipids). This increased lipid load is related with reduced insulin sensitivity and consequently with conditions such as T2DM. The dysfunctions in this lipid metabolism led to insulin resistance, but the mechanism remains unclear (35).

The negative regulation of cellular response to starvation was shown altered in T2DM. We highlight Glutamine synthetase as the protein dysregulated in this process (table 13).

Table 13. List of proteins related with cellular response to starvation process dysregulation in T2DM (p value <0.05). Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (T2DM/Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis. Only proteins with a VIP > 1 are shown.

UniProtKB AC	Protein name	Gene name	Fold change	VIP >1
P15104	Glutamine synthetase	GLUL	-1.62	1.20
P02768	Albumin	ALB	2.23	1.59

Glutamine synthetase (GLUL) is involved in the catalysation of glutamate to glutamine (36). The glutamate is directly dependent of leptin to uptake in astrocytes in a time-dependent manner (37). The rapid glutamate capture by astrocyte's indicates that leptin could reduce the stimulatory effects of glutamate at nearby synapses, thereby reducing appetite (37). The rapid rise of insulin with feeding promotes uptake and storage of ingested energy in fat and muscle. The rapid fall of insulin with starvation promotes catabolism (38). Both leptin and insulin play key roles in signalling the physiologic transition from energy sufficiency to deficient energy/starvation (38). In our work, GLUL was found decreased in saliva (FC = -1.62) of T2DM patients which can be a consequence of starvation stimulus dysregulation with a constant GLUL depletion.

Glutamine synthetase is a marker of changes in cellular response to starvation process. Being less abundant in saliva, it may be good for monitoring insulin resistance mechanisms process in diabetes.

4.1.4 Maintenance of mitochondrion location

The T2DM is characterised by mitochondrial dysfunction and consequent homeostasis disruption, with elevated production of reactive oxygen species (ROS) and low levels of ATP (39). Downregulation of genes involved in mitochondrial biogenesis and oxidative phosphorylation has been observed in T2DM (39).

The maintenance of mitochondrion location is characterized by the conservation of specific location within a cell and prevented from moving elsewhere. We identified albumin in both processes contributing to this dysregulation (table 14).

Table 14. List of proteins related with maintenance of mitochondrion location process dysregulation in T2DM (p value <0.05). Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (T2DM/Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis. Only proteins with a VIP > 1 are shown.

UniProtKB AC	Protein names	Gene Names	Fold Change	VIP >1
P02768	Albumin	ALB	2,23	1,59

Diabetic retinopathy is inversely linked with the level of Serum Albumin in patients with T2DM (28). Mitochondrial oxidative stress increase leads to a change in mitochondrial function (40). In saliva serum albumin is decreased (FC = 2.23), suggesting an agreement with previous discoveries (39). Being a marker of the oxidative stress characteristic of diabetes (41) a preventive monitorization of Albumin could avoid this microvascular disease appearance.

4.1.5 Detection of chemical stimulus involved in sensory perception of bitter taste

Taste receptors in the gustatory system are expressed in the gastrointestinal tract and influence nutrients' intake. Different taste stimulates specific taste receptors that release metabolic hormones, modulating insulin expression with impact on glucose homeostasis. In T2DM some taste receptors are altered, and this is linked with glucose and insulin dysregulation (43). The detection of the chemical stimulus involved in sensory perception of bitter taste seems to be dysregulated, as suggested by the enrichment analysis (Figure 12). We identified Cystatin-SN, Zinc-alpha-2-glycoprotein, and Carbonic anhydrase 6 in this dysregulation process (table 15).

Table 15. List of proteins related with detection of chemical stimulus involved in sensory perception of bitter taste process dysregulation in T2DM (p value <0.05). Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (T2DM/Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis.

UniProtKB AC	Protein name	Gene name	Fold change	VIP
P01037	Cystatin-SN	CST1	-2.18	1.09
P25311	Zinc-alpha-2-glycoprotein	AZGP1	-1.56	1.60
P23280	Carbonic anhydrase 6	CA6	10.52	0.43

Cystatin-SN (CST1) is more abundant in saliva of individuals experiencing bitter tastes (44). In T2DM, Cystatin-SN is up regulated in patients with burn mouth sensation, attributed to diabetic peripheral neuropathy (45,46). This could reflect a defensive reaction against an on-going inflammation (46). Peripheral neuropathy causes pain, dysesthesia, and loss of sensation (45). In our study, CST1 is less abundant (FC = -2.18) in the saliva of DMT2 patients, which is contrary to that found in previous study (44). This observation can be related with the results of Baron, et al. 1999 (47) who reported the depletion of cystatin SN in periodontally diseased patients.

Zinc-alpha-2-glycoprotein (AZGP1) has a direct relation with sensory perception of bitter taste (48). It has been suggested as a potential obesity gene in plasma and may predispose to the development of obesity (49). AZGP1 plays a role in the regulation of adipose tissue mass, stimulating lipolysis, inhibiting lipid accumulation in adipose

tissue, regulating serum lipid values and influencing the secretion of other adipokines (50). This protein was found decreased (FC = -1.56) in saliva samples.

Carbonic anhydrase 6 (CA6) is a key factor responsible for the reduction of hepatic glucose production (51) and has been suggested as a marker for several complications of diabetes, namely retinopathy, kidney disease, neuropathy and cardiovascular disease (52). In saliva, CA6 was more abundant (FC = 10.52) in DMT2 patients, in agreement with previous studies (52) .

Proteins Cystatin SN, Zinc-alpha-2-glycoprotein and Carbonic anhydrase 6 are markers of changes in the detection of chemical stimulus involved in sensory perception of bitter taste and consequently will be good for monitoring the molecular impact of glucose and insulin homeostasis dysregulation in diabetes.

4.1.6 Adaptative and Innate immune response

Both innate and adaptive immunity have an essential role in the progression of T2DM, based on the effects induced by the abnormal differentiation of components of the immune system in the development of insulin resistance (53). Alterations on the proliferation of T cells and macrophages, and impairment in function of NK cells and B cells, which represents abnormal innate and adaptive immunity, are frequent phenomena in individuals suffering from T2DM (53).

The proteins involved in the adaptative, and innate immune response were shown dysregulated in enrichment analysis. Among the altered proteins in this biological process, we highlight Fibrinogen alpha chain, Fibrinogen beta chain and Bactericidal permeability-increasing protein (table 16).

Table 16. List of proteins related with adaptative and innate immune response process dysregulation in T2DM (p value <0.05). Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (T2DM/Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis. Only proteins with a VIP > 1 are shown.

UniProtKB AC	Protein name	Gene name	Fold change	VIP
O00584	Ribonuclease T2	RNASET2	-2.68	1.69
P01871	Immunoglobulin heavy constant mu	IGHM	-2.34	1.02
P01782	Immunoglobulin heavy variable 3-9	IGHV3-9	-2.00	1.38
A0A0B4J1X5	Immunoglobulin heavy variable 3-74	IGHV3-74	-1.94	1.15
P01721	Immunoglobulin lambda variable 6-57	IGLV6-57	-1.47	1.33
P06331	Immunoglobulin heavy variable 4-34	IGHV4-34	-1.31	1.03
P17931	Galectin-3	LGALS3	-1.13	1.15
Q14508	WAP four-disulphide core domain protein 2	WFDC2	1.29	1.00
A0A0C4DH25	Immunoglobulin kappa variable 3D-20	IGKV3D-20	1.35	1.27
P05109	Protein S100-A8	S100A8	1.38	1.03
P01700	Immunoglobulin lambda variable 1-47	IGLV1-47	1.41	1.44
P01601	Immunoglobulin kappa variable 1D-16	IGKV1D-16	1.50	1.12

P05156	Complement factor I	CFI	1.62	1.08
P31151	Protein S100-A7	S100A7	1.68	1.25
P02743	Serum amyloid P-component	APCS	1.73	1.66
P0C0L4	Complement C4-A	C4A	1.74	0.99
P06702	Protein S100-A9	S100A9	1.77	1.11
P01859	Immunoglobulin heavy constant gamma 2	IGHG2	1.90	1.26
P0DOX5	Immunoglobulin gamma-1 heavy chain		1.99	1.31
A0A0C4DH36	Probable non-functional immunoglobulin heavy variable 3-38	IGHV3-38	2.03	1.25
P02671	Fibrinogen alpha chain	FGA	2.11	1.06
Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1	2.43	1.34
P10909	Clusterin	CLU	2.50	1.27
P01860	Immunoglobulin heavy constant gamma 3	IGHG3	2.87	1.05
P80188	Neutrophil gelatinase-associated lipocalin	LCN2	2.89	1.94
O75594	Peptidoglycan recognition protein 1	PGLYRP1	2.91	1.32
P0DOY2	Immunoglobulin lambda constant 2	IGLC2	3.08	1.86
P12724	Eosinophil cationic protein	RNASE3	3.25	2.02
P49913	Cathelicidin antimicrobial peptide	CAMP	3.35	1.22
P15814	Immunoglobulin lambda-like polypeptide 1	IGLL1	3.38	1.38
P05089	Arginase-1	ARG1	3.71	1.10
P04003	C4b-binding protein alpha chain	C4BPA	3.78	1.65
P02675	Fibrinogen beta chain	FGB	4.06	1.38
P26583	High mobility group protein B2	HMGB2	4.32	1.62
P02788	Lactotransferrin	LTF	5.25	1.48
Q9NP55	BPI fold-containing family A member 1	BPIFA1	5.84	0.99
P05155	Plasma protease C1 inhibitor	SERPING1	6.58	1.55
P0C0L5	Complement C4-B	C4B	7.02	1.55
P17213	Bactericidal permeability-increasing protein	BPI	14.99	1.49

Fibrinogen alpha chain (FGA) and Fibrinogen beta chain (FGB) are involved in the disruption of the adaptative and innate immune responses. Fibrinogen is a thromboplastic and inflammatory marker and plays a pivotal role in the progression of atherosclerosis (31,54). Fibrinogen function as a crucial modulator of haemostatic balance and inflammatory processes in diabetes (54). Fibrinogen was found increased in blood in different moments of T2DM: before the onset of T2DM, and during the

transition from pre-T2DM to T2DM (31,54,55). In our study FGA and FGB were found elevated in saliva of the patients with T2DM (FC = 2.11 and FC = 4.06).

The bactericidal permeability-increasing (BPI) protein is an endogenous protein with antimicrobial properties that in T2DM can reduce inflammation (56). In saliva BPI was found overexpressed (FC = 14.99), probably a reflection of the body's attempt to fight the inflammatory state characteristic of diabetes.

Fibrinogen alpha chain and Fibrinogen beta chain are markers of changes of the adaptative and innate immune response process and consequently may be good markers for monitoring atherosclerosis complications (haemostatic balance and inflammatory processes) in diabetes (54).

4.1.7 Defense response to bacterium

In Diabetes, insulin deficiency and hyperglycaemia cause the disruption of immune system mechanisms such as cytokine production, immune cells function and phagocytosis (57).

The defense response to bacteria was shown dysregulated in enrichment analysis. Between the altered proteins in this biological process, we highlight Neutrophil elastase, Protein S100-A8 and Protein S100-A9 as the most representative proteins contributing to this dysregulation (table 17).

Table 17. List of proteins related with Defense response to bacterium process dysregulation in T2DM (p value <0.05). Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (T2DM/Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis. Only proteins with a VIP > 1 are shown.

UniProtKB AC	Protein name	Gene name	Fold change	VIP
P15515	Histatin-1	HTN1	-4.52	1.46
P01871	Immunoglobulin heavy constant mu	IGHM	-2.34	1.02
P01782	Immunoglobulin heavy variable 3-9	IGHV3-9	-2.00	1.38
A0A0B4J1X5	Immunoglobulin heavy variable 3-74	IGHV3-74	-1.94	1.15
P06331	Immunoglobulin heavy variable 4-34	IGHV4-34	-1.31	1.03
P05109	Protein S100-A8	S100A8	1.38	1.03
P06702	Protein S100-A9	S100A9	1.77	1.11
P01859	Immunoglobulin heavy constant gamma 2	IGHG2	1.90	1.26
A0A0C4DH36	Probable non-functional immunoglobulin heavy variable 3-38	IGHV3-38	2.03	1.25
P01860	Immunoglobulin heavy constant gamma 3	IGHG3	2.87	1.05
P80188	Neutrophil gelatinase-associated lipocalin	LCN2	2.89	1.94
P0DOY2	Immunoglobulin lambda constant 2	IGLC2	3.08	1.86
P49913	Cathelicidin antimicrobial peptide	CAMP	3.35	1.22
P15814	Immunoglobulin lambda-like polypeptide 1	IGLL1	3.38	1.38
P00738	Haptoglobin	HP	4.43	1.54
P05164	Myeloperoxidase	MPO	4.72	1.11
P08246	Neutrophil elastase	ELANE	8.02	1.14

Neutrophil-mediated immunity is the first host defense response against any infection (58). Neutrophils combat against pathogens via three independent mechanisms – a) phagocytosis, b) degranulation and c) by producing extracellular traps (59,60). Metabolic changes in T2DM increases the formation of advanced glycation end products, leading to a) increased production of superoxide, b) activation of inflammatory pathways, c) defects in innate immune cell function as in neutrophils and d) abnormal host responses (59,60). In hyperglycaemia, T2DM patients react poorly to infections (59). We found that, in saliva, Neutrophil elastase is elevated (FC = 8.02) supporting the hypothesis that T2DM reprograms metabolism in innate immunity keeping the active inflammation state leading to recurrent infections (59).

Diabetic foot ulcer is characterized by the unbalance immune system and is a common complication in T2DM individuals. The host open wounds are an entry for different pathogens that take advantage of a compromised immune system. Although the underlying pathophysiological mechanisms of diabetic foot still incomplete, the relation of proteins S100-A8 and S100-A9 levels in serum and the healing process was already reported (61,62). The data is not consensual as the levels of both these proteins in serum were lower in T2DM individuals using a proteomics approach (62) and higher using an immune-specific quantitative assays (61). In saliva of DMT2 patients, both were more abundant: S100-A8 (FC = 1.38) and A9 (FC = 1.79). This upregulation can stimulate the inflammatory response, causing the release of proinflammatory cytokines. This unbalance prevents the normal wound cicatrisation (62).

Given the above, we suggest that neutrophil elastase and proteins S100-A8 and S100-A9 may be considered as markers of changes in defense response to bacterium process and consequently adequate for monitoring inflammation state and wound healing evolution in diabetes.

4.1.8 Proteolysis

Insulin resistance of T2DM patients, lead to an increase in proteolysis and a decrease in protein synthesis (63). Enrichment analysis shows that proteolysis is dysregulated in T2DM (table 18).

Table 18. List of proteins related with proteolysis process dysregulation in T2DM (p value <0.05). Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (T2DM/Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis. Only proteins with a VIP > 1 are shown.

UniProtKB AC	Protein name	Gene name	Fold change	VIP
P55786	Puromycin-sensitive aminopeptidase	NPEPPS	-2.34	1.22
Q9BQR3	Serine protease 27	PRSS27	-2.22	1.24
Q9H4A4	Aminopeptidase B	RNPEP	-2.09	1.03
P28838	Cytosol aminopeptidase	LAP3	-1.87	1.28
P21128	Uridylate-specific endoribonuclease	ENDOU	-1.58	1.19
Q9UBR2	Cathepsin Z	CTSZ	-1.39	1.14
Q9UBX7	Kallikrein-11	KLK11	-1.34	1.16
P48147	Prolyl endopeptidase	PREP	-1.27	1.23
Q9NY33	Dipeptidyl peptidase 3	DPP3	-1.25	1.06
Q14508	WAP four-disulfide core domain protein 2	WFDC2	1.29	1.00

P05156	Complement factor I	CFI	1.62	1.08
P31944	Caspase-14	CASP14	1.77	1.19
Q9UKR3	Kallikrein-13	KLK13	1.88	1.02
P07339	Cathepsin D	CTSD	1.94	1.56
O60235	Transmembrane protease serine 11D	TMPRSS11D	1.97	1.54
P08311	Cathepsin G	CTSG	2.01	1.20
P22894	Neutrophil collagenase	MMP8	2.35	1.63
P14780	Matrix metalloproteinase-9	MMP9	2.75	1.32
Q9UL52	Transmembrane protease serine 11E	TMPRSS11E	2.79	1.20
Q9GZN4	Brain-specific serine protease 4	PRSS22	2.91	1.15
P08603	Complement factor H	CFH	3.30	1.19
P08519	Apolipoprotein(a)	LPA	3.48	1.21
P01024	Complement C3	C3	3.62	1.68
P02788	Lactotransferrin	LTF	5.25	1.48
P31025	Lipocalin-1	LCN1	6.02	1.44
P08246	Neutrophil elastase	ELANE	8.02	1.14
P24158	Myeloblastin	PRTN3	9.49	1.41

T2DM is the result of two defects: a reduction in the ability of insulin to stimulate glucose utilization (insulin resistance) and inadequate pancreatic ß-cell insulin secretion in response to hyperglycemia (64). Neutrophil elastase and Matrix mettaloproteinase-9 shown an important role in digestion with insulin consumption, something that is not occurring in T2DM individuals (64). Matrix mettaloproteinase-9 and Neutrophil elastase are upregulated in plasma of T2DM individuals exhibiting an increased proteolytic activity and a decrease in the density of the insulin receptor (64). In saliva of T2DM patients these two were more abundant as well.

4.2 Protein enrichment analysis

In addition to the proteins that participate in the enriched biological processes in our analysis, we also studied the role of proteins with VIP>1 that are not associated with the enriched processes because they alone are good at discriminating molecular changes in diabetic individuals compared to non-diabetic individuals. Thirty-eight proteins with a VIP > 1 (Figure 12), but that do not account for the enrichment of the biological processes, are listed in table 19.

Table 19. List of proteins with a VIP score > 1 not listed in the enrichment analysis. These are the proteins that better differentiate T2DM and Healthy samples. Shown are the UniProtKB accession number (AC), protein names, gene names, fold change (T2DM/Healthy) and the variable importance projection (VIP) score defined by the PLS-DA analysis.

UniProtKB AC	Protein name	Gene Name	Fold change	VIP
P04280	Basic salivary proline-rich protein 1	PRB1	-20	2.0
P18510	Interleukin-1 receptor antagonist protein	IL1RN	-5.88	2.0
P43251	Biotinidase	BTD	-3.57	2.0
P14550	Aldo-keto reductase family 1 member A1	AKR1A1	-2.78	2.0

A8K2U0	Alpha-2-macroglobulin-like protein 1	A2ML1	-2.78	2.0
Q6ZVX7	F-box only protein 50	NCCRP1	-2.78	2.0
P36952	Serpin B5	SERPINB5	-2.70	2.0
P13716	Delta-aminolevulinic acid dehydratase	ALAD	-2.27	2.0
Q9Y5Z4	Heme-binding protein 2	HEBP2	-2.04	2.0
P06870	Kallikrein-1	KLK1	-1.59	2.0
Q16658	Fascin	FSCN1	-1.56	2.0
O95274	Ly6/PLAUR domain-containing protein 3	LYPD3	-1.45	2.0
Q13162	Peroxiredoxin-4	PRDX4	1.97	1.9
P43652	Afamin	AFM	2.18	2.0
P02679	Fibrinogen gamma chain	FGG	2.34	2.0
P02763	Alpha-1-acid glycoprotein 1	ORM1	2.42	2.0
P01011	Alpha-1-antichymotrypsin	SERPINA3	2.60	2.0
P01009	Alpha-1-antitrypsin	SERPINA1	2.75	2.0
P84077	ADP-ribosylation factor 1	ARF1	2.82	1.8
P61224	Ras-related protein Rap-1b	RAP1B	2.95	2.0
Q9HC84	Mucin-5B	MUC5B	3.11	1.8
P02790	Hemopexin	HPX	3.38	1.8
P04217	Alpha-1B-glycoprotein	A1BG	3.39	1.8
P02751	Fibronectin	FN1	3.46	2.0
Q6UX06	Olfactomedin-4	OLFM4	3.52	2.0
P55058	Phospholipid transfer protein	PLTP	3.72	2.0
P04196	Histidine-rich glycoprotein	HRG	3.79	1.8
P36222	Chitinase-3-like protein 1	CHI3L1	3.85	1.9
P48595	Serpin B10	SERPINB10	4.06	2.0
P41218	Myeloid cell nuclear differentiation antigen	MNDA	4.16	2.0
P02749	Beta-2-glycoprotein 1	APOH	4.36	1.8
Q99880	Histone H2B type 1-L	H2BC13	4.88	1.0
P01031	Complement C5	C5	5.54	2.0
Q05315	Galectin-10	CLC	6.18	2,0
P02647	Apolipoprotein A-I	APOA1	7.74	1.7
P59665	Neutrophil defensin 1	DEFA1; DEFA1B	8.00	2.0
P69905	Hemoglobin subunit alpha	HBA1; HBA2	9.20	2.0
P68871	Hemoglobin subunit beta	HBB	22.7	2.0

Basic salivary proline-rich protein 1 (PRB1) is expressed in large amounts in the salivary glands (parotid glands). It has been proven that there is a relation of diabetes with hypofunction of the salivary glands and with diabetes medication (65). In saliva this protein was found highly downregulated (FC = -20). Therefore, this protein could be of interest as a biomarker of the impact of diabetes on salivary gland function or to monitor anti-diabetes medication is being detrimental to gland function, which could have a major impact on a person's oral and systemic health. This information is

important to allow the medical doctor to act on the adjustment or reduce this medication.

Alpha-2-macroglobulin-like protein 1 (A2ML1) was suggested as a potential biomarker for diabetic retinopathy and other diabetic complications (66). A2ML1 is upregulated in plasma of diabetic patients with retinopathy (66). In our sample group none of the volunteers suffer from retinopathy and, in fact, A2ML1 was less abundant in the saliva samples of T2DM patients (FC = -2.78).

Alpha-1-antitrypsin (SERPINA1) is a defense response protein involved with pro-inflammatory immune response, previously reported to be associated with T2DM, is increased in proliferative diabetic retinopathy (67). In saliva this protein was also found more abundant in the saliva of T2DM patients (FC = 2.75)

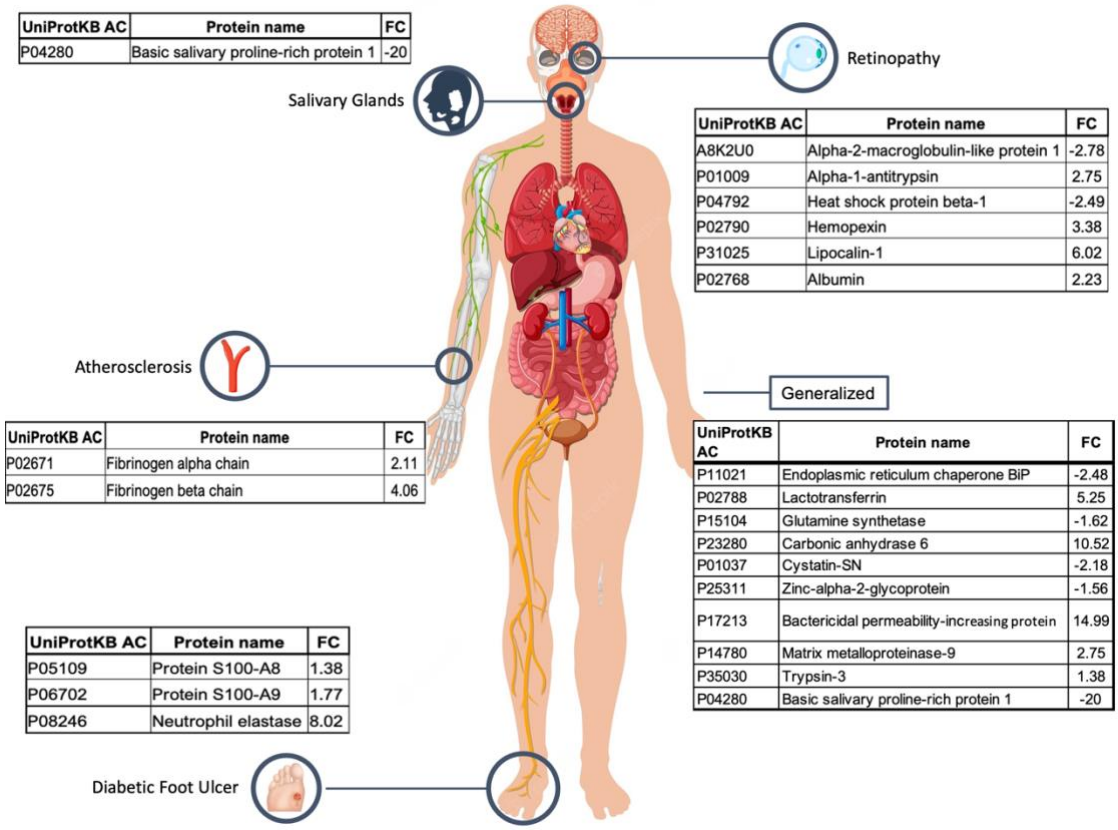
Hemopexin (HPX) is a scavenger of heme, highly toxic due to its ability to cause protein aggregation and contribution to oxidative stress (68). High levels of HPX were found in the saliva of patients with proliferative diabetic retinopathy (68). In our study this protein was found upregulated as well (FC = 3.38). Higher levels of HPX were found also in urine and linked with uncontrolled diabetes (69).

It’s important to point out that both Hemoglobin subunit (alpha and beta) were identified with a high upregulation, that surely can be related with the common oral manifestation of diabetes such as periodontitis [characterised by bleeding gums (70)].

5. Conclusions

This study proves the enormous potential of saliva as a diagnostic fluid. In it we can find several proteins that were found altered in serum, plasma, and urine of diabetic patients compared to non-diabetic patients and that may be biomarkers not only of diabetes but also of the different complications of this disease (table 10). Although this study identified many biomarkers that could serve as a basis for the development of new tests supporting a more complete diagnosis of diabetes and its complications, this is a pilot study that needs validation in a larger number of samples allowing a stronger correlation of the molecular data with patient complications.

In saliva, we have identified 20 proteins altered in patients with T2DM that reflect conditions dispersed for different body locations: arteries (atherosclerosis), eye (retinopathy), diabetic foot ulcer (foot), metabolic syndrome (generalized), insulin resistance (generalized), molecular impact of glucose and insulin homeostasis dysregulation (generalized), haemostatic balance and inflammatory processes (generalized), protein catabolism (generalized) and salivary gland function (salivary glands) (Figure 17). These proteins may be indicative of complications resulting from the disease and therefore should be considered as potential biomarkers.



UniProtKB AC	Protein name	FC
P04280	Basic salivary proline-rich protein 1	-20

UniProtKB AC	Protein name	FC
A8K2U0	Alpha-2-macroglobulin-like protein 1	-2.78
P01009	Alpha-1-antitrypsin	2.75
P04792	Heat shock protein beta-1	-2.49
P02790	Hemopexin	3.38
P31025	Lipocalin-1	6.02
P02768	Albumin	2.23

UniProtKB AC	Protein name	FC
P02671	Fibrinogen alpha chain	2.11
P02675	Fibrinogen beta chain	4.06

UniProtKB AC	Protein name	FC
P11021	Endoplasmic reticulum chaperone BiP	-2.48
P02788	Lactotransferrin	5.25
P15104	Glutamine synthetase	-1.62
P23280	Carbonic anhydrase 6	10.52
P01037	Cystatin-SN	-2.18
P25311	Zinc-alpha-2-glycoprotein	-1.56
P17213	Bactericidal permeability-increasing protein	14.99
P14780	Matrix metalloproteinase-9	2.75
P35030	Trypsin-3	1.38
P04280	Basic salivary proline-rich protein 1	-20

UniProtKB AC	Protein name	FC
P05109	Protein S100-A8	1.38
P06702	Protein S100-A9	1.77
P08246	Neutrophil elastase	8.02

Figure 17. Suggested saliva biomarkers according to the localization of the altered process.

The overall proposed panel of biomarkers of the different complications of diabetes, includes that both Albumin and Lactotransferrin that are common to some of these processes. Therefore, these proteins can be considered more generic indicators of diabetes complications but not specific markers of one of these complications.

The identification of a panel of biomarkers for T2DM complications in saliva is a pilot study that needs validation in a larger number of samples allowing a stronger

correlation of the molecular data with patient complications. These results can serve as a basis for the development of new tests to support a more complete diagnosis of diabetes and its complications, and a point-of-care for close monitoring and providing frequent data to the physician.

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Chapter VI - General conclusions and future perspectives

In recent years, saliva has been considered as a diagnostic material in different diseases (15). However, much remains to be done to make saliva a reality in clinical practice not only in terms of diagnosis but also as an informative fluid for the clarification of molecular mechanisms or the identification of therapeutic targets. Our work aimed to contribute to this goal by studying and providing evidence of the various aspects of the potential of saliva as a diagnostic fluid for COVID-19, but also as a source of molecular information to clarify molecular mechanisms associated with SARS-CoV-2 infection and complications of Type 2 Diabetes mellitus.

The COVID-19 pandemic brought the necessity to search for new solutions in the identification of the SARS-CoV-2 (58,60). The gold standard was established by the authorities with nasopharyngeal and throat swabs collection and PCR based assays (60). The swab sample collection demonstrated to be painful, difficult to perform specially in children and elder people and required trained personnel, that were in risk themselves during the procedure (48). The socio-economic and health system overburden, had other impacts such as environmental with the tests residual waste (58,75). Accordingly, we purposed to develop a strategy to detect SARS-CoV-2 in saliva and expand it to a strategy for wide population testing. In order to achieve this objective a digital platform to annotate the patient information with the clinical data association as well as laboratory results was built. The comparison between saliva and nasopharyngeal samples demonstrated that saliva can be reliably used for SARS-CoV-2 detection, and that saliva-based large-scale population screening for COVID-19 with or without pooling is feasible, fast and leads to large economic and environmental impact reductions. The viral loads were lower in saliva compared to the nasopharyngeal samples. This finding was documented and related with the symptoms evolution, with saliva showing higher viral load on the first week of symptoms (76). We showed as well that saliva pools of either ten or twenty samples do not compromise the detection of SARS-CoV-2. The ease of saliva collection, and the pool strategy is an appealing method for mass-screening programs or sentinel surveillance, especially in resource-limited scenarios. It is important to refer that most of extraction and PCR amplification kits available at the time were optimized for nasopharyngeal samples, far most viral concentrated samples comparing with the saliva component complexity. Since the sensibility in saliva was not optimized, we considered that the RT-PCR cycle count threshold (measure to define the virus presence or absence in the sample) should be increased for the saliva samples, otherwise many would be considered negative by mistake, especially the ones with lower viral load.

Despite the virus identification is well established, most of the molecular alterations induced by SARS-CoV-2 infection remains to be clarified (77,78). A comprehensive identification and understanding of how SARS-CoV-2 interact and modulates the host biological mechanisms is essential for developing effective strategies for disease therapies and drug development (79). Very little is known about how saliva can be used to detect these characteristic proteome changes of infected patients (80). Mass spectrometry-based proteomics are sensible techniques allowing the detection of small changes on the host physiology (81,82). Therefore, we proposed to develop a proof-of-concept strategy in identification of the molecular aspects of SARS-CoV-2 infection applying a proteomic approach on saliva. In our study, 26 high interest proteins able to differentiate both sample groups were identified. Fourteen had already been described in other types of samples but not in saliva. On the other hand, 10 had never been associated with SARS-CoV-2 infection before, representing new evidence of the impact of COVID-19 on the host. As expected, many of the proteins altered upon

infection with SARS-CoV-2 are related to the immune system, and the interactome analysis shows that the virus directly interacts with human immune system proteins. We showed that the virus alters the host's energy metabolism and interferes with apoptosis mechanisms. Interestingly, other studies documented a higher risk of death by SARS-CoV-2 infection in patients with energy metabolism-related chronic disorders, including diabetes (83). Our analysis showed that there is a dysregulation of energy processes after SARS-CoV-2 infection, which may help to explain the increased mortality in these patients (83,84). In fact, according to preliminary clinical data on COVID-19 patients, individuals with type 2 diabetes and other metabolic disorders that impair general metabolic health are more likely to experience a more severe infection course than those who were metabolically healthy before contracting the virus (84). This could be due to the impact of SARS-CoV-2 infection and disease outcome based on the energy metabolism equilibrium (83). We also showed that many of the proteins that are altered upon infection with SARS-CoV-2 are involved in apoptosis, transport and signal transduction, revealing the broad spectrum of alterations that COVID-19 represents. The interactome analysis, assisted by the Oralint algorithm, demonstrated that the 14 SARS-CoV-2 proteins (interacts with 100 human proteins, and only 5 of these where already been associated with SARS-CoV-2 infection pathway and related pathways by the PathCards.

Although the diagnosis of diabetes is now well established, much remains to be done in terms of monitoring the evolution of the disease and its various complications. Saliva can play an important role in this monitoring in an easy and non-invasive way performed by the family doctor or by the patient themselves in the comfort of their own home. Uncontrolled hyperglycaemia together with induced metabolic disorders will ultimately lead to micro- and macrovascular complications (87). Therefore, we developed a proof-of-concept strategy in identification of biomarkers which may be indicative various diabetes complications. Functional and enrichment analysis along with bibliography support we proposed a panel of 21 proteins directly related to different complications in diabetes. Diabetes is a long term known disease, unfortunately a very few studies were performed in saliva so far. We fill a gap and provide new highlights on saliva as a fluid for different diabetes complications. Further studies can lead to the development of a point-of-care device for regular monitoring of salivary markers and informative for the family doctor to understand and regulate the medication and diet.

In conclusion, with this work we confirmed the potential of saliva as fluid of interest both in diagnosis and in the discovery of new disease insights. Saliva was crucial in demonstrating the flexibility and capacity of its use demonstrated by the development of a reliable SARS-CoV-2 detection, the identification and discussion of molecular aspects of the viral infection in host and with host, as well as the discovery of new highlights in diagnosis and monitoring of Type 2 Diabetes Mellitus disease and its complications.

Future Perspectives

Saliva has been suggested as an excellent non-invasive diagnostic material and can be employed in the early detection and monitoring of numerous diseases, such as infectious or metabolic disorders. Although saliva collection is easier and less prone to variation, it can be a challenging sample when it comes to processing and analysis. In this regard, further work on decreasing the viscosity and complexity of saliva prior to the analysis must be done.

In SARS-CoV-2 identification within saliva the cycle threshold used for considering a sample positive should be adjusted. Most detection kits are optimized for nasopharyngeal swabs, and as our and other studies suggest, cycle threshold tend to be higher in saliva samples.

Despite the identification of SARS-CoV-2 in saliva has been proved successful, new strategies should be studied, preparing a new COVID-19 wave or another viral pandemic. The sensitivity of SARS-CoV-2 in a complex fluid such as saliva, should be taken in consideration when developing extraction and assay kits. Large-Scale comparative studies could be performed to optimize this sensitivity and implement low-cost monitoring tests in underdeveloped countries.

This work resulted also on the identification of high interest proteins able to differentiate COVID-19 and healthy individuals. The identification with quantification of COVID-19 and Health salivary proteome allowed to have a glimpse of what is the role on disrupted mechanisms. The interactome analysis increased the knowledge on the number and processes of human proteins involved on the SARS-CoV-2. Being this study a proof-of-concept, a relatively small number of samples was used in the proteomic trial and may have an impact on the results. Although molecular pathways differentiation between COVID-19 and control patients were suggested by other studies, we suggest targeted studies with more sample to assure the hypothesis suggested on our study.

In the future, several directions should be taken to completely unveil the mechanisms of SARS-CoV-2 infection. An interesting perspective to follow is a large-scale study with more complete data on symptomatology and comorbidities to better understand the relationship between different variables and changes in the molecular mechanisms of SARS-CoV-2 infection.

The identification of a panel of biomarkers for T2DM complications in saliva is a pilot study that needs validation in a larger number of samples allowing a stronger correlation of the molecular data with patient complications. These results can serve as a basis for the development of new tests to support a more complete diagnosis of diabetes and its complications, and a point-of-care for close monitoring and providing frequent data to the physician. The Type 2 Diabetes Mellitus study is being prepared and will be submitted soon.

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Appendix

Chapter III - Population wide testing pooling strategy for SARS-CoV-2 detection using saliva

The following table S1 is deposited in an excel file on the journal website:

Table S1| Population data from community testing with pooled sample analysis strategy. link

Chapter IV - COVID-19 Salivary protein profile: unravelling molecular aspects of SARS-CoV-2 infection

The following tables (S1, S2, S3 and S4) are deposited in a ZIP file named "Supplementary File 1" on the journal website:

Table S1| LC-MS identification table with Protein and Peptide summary. link

Table S2| Proteins identified with an FDR 5% and $p<0.05$. link

Table S3| Predicted high confidence PPIs. link

Table S4| MS Report COVID positive vs COVID negative sample group. link