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Characterization of saliva samples for biobanking purposes

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Running Title: Characterization of saliva samples for biobanking

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Abstract

Biobank saliva sample quality depends on specific criteria applied to the collection, processing and storage. In spite of the growing interest in saliva as a diagnostic fluid few biobanks currently store large collections of such samples. The development of a Standard Operating Protocol (SOP) for saliva collection and quality control is fundamental for the establishment of a new saliva biobank which stores samples available to the saliva research community. For the establishment of the SOP, different collection methods were tested using the total volume of protein obtained, protein content and protein profiles. Furthermore, the impact of the circadian variability, inter and intra individual differences on the study variables were also determined, as well as the saliva sample stability at room temperature.

Considering our results, a sublingual cotton-roll method for saliva collection proved to produce saliva with the best characteristics and should be applied in the morning, whenever possible. Our results also show that there is a larger variability in salivary proteins between individuals than in the same individual for a 5-month period. According to the electrophoretic protein profile protein stability is guaranteed for 24h at room temperature and the protein degradation profile and protein identification was characterized.

We conclude that it is possible to collect saliva with an easy and inexpensive protocol, resulting in saliva samples for protein analysis with sufficient quality for biobanking purposes.

Introduction

Biobanks comprise organized collections of biospecimens annotated with personal and clinical information and are a fundamental resource for high quality academic research and translational medicine [1–3]. The development and validation of analytical methods, diagnostic tests and biomarker discovery depends on good quality repositories [4]. Two main concerns of biobank managers are sample preservation and annotation quality and consistency, both of which are dependent on the standardization of collection, processing and storage protocols [1].

In the 2000s about 43 biobanks were created around the world [4]. In Portugal the major Biobank is established in the Lisbon Academic Medical Centre (IMM) [5] and has recently started to store saliva samples.

Several Omics saliva studies have led to the production of massive amounts of data collected and annotated in databases identifying and characterizing different salivary components (DNA, RNA, proteins, metabolites and microorganisms) [6]. Saliva reflects the health or disease state [7–18] of an individual and presents many advantages relative to other fluids such as to blood, serum or plasma, which include simple, non-invasive and safer sampling methods requiring minimal equipment and easy and inexpensive storage possibilities. Contrary to other sterile fluids, like CSF [4], blood, amniotic and pleural fluid, saliva is not sterile [19] and therefore subject to microbial degradation which influences sample quality [8]. Furthermore, some parameters such as diurnal, inter and intra individual variation on the different components haven´t been definitely established. In fact, even

though for some proteins and steroid hormones [9–11], for total volume [9,12] and total protein [18] there are data supporting diurnal variations, for other proteins the quantification seem to be independent of the circadian cycle [10,18].

The purpose of the present study is to develop a SOP for collection, processing and quality control to be used in the establishment of the Biobanco-IMM saliva collection. The establishment of the SOP is based on the selection of the collection method and site as well as the time of collection based on the impact of the circadian variability on the volume, protein concentration and protein profile of the collected saliva. The evaluation of the inter and intra individual variability and the sample stability at room temperature complements the evidence necessary to establish a SOP guaranteeing the preliminary characterization of the saliva to be stored.

Materials and Methods

Participants and ethics statements

Saliva samples were collected at Universidade Católica Portuguesa on 22 healthy volunteers (9 males and 13 females) aged 19 to 27 years old (mean=21 years; SD=2.34). This is a convenience sample representative of the university students. Donors consented to the collection and storage of the samples and associated data by signing an informed consent approved by the Ethics Commission of the Centro Hospitalar Lisboa Norte – Hospital de Santa Maria. The clinical database is authorized by the National Commission for Data Protection [5].

Saliva sample collection methods

Unstimulated Whole Saliva (WS) was collected from healthy subjects. Subjects were asked to refrain from eating, drinking or have oral hygiene procedures 1 hour prior to saliva collection. Before collection, subjects were asked to rinse the mouth with clean water for 30 seconds to remove desquamated epithelial cells, microorganisms and food and drink remnants. After the mouthrinse, subjects were asked to wait for a minute before collection. Three different methods were used: passive drooling, sublingual cotton-roll and vestibular cotton-roll.

i) Passive Drooling

A 50mL sterile tube was used to collect passive drooled saliva for 3 minutes. The tube was maintained on ice during collection to ensure the integrity of the sample.

ii) Cotton-roll Based Methods

Sublingual or vestibular saliva was collected with two cotton rolls placed under the tongue or the vestibular area respectively, for 2 minutes. The cotton rolls were placed inside a 15mL sterile plastic tube with a sterile 100 µl pipette tip in the bottom to facilitate saliva collection by centrifugation at 10000 x g's for 10 minutes at 4ºC. Total volume collected and protein concentration were measured and after re-suspension by vortex the total volume is aliquoted and stored at –80ºC.

Selection of collection method

A total of 36 UWS samples from 9 healthy subjects were characterized as to total volume of saliva and protein concentration.

Volume was measured using a micropipette and protein concentration was determined using the protein UV program of a NanoVue Spectrophotometer (Life Science, GE Healthcare, UK).

Statistical analysis for total volume was determined by non-parametric Freadman Test (Dunn's test for multiple comparisons) and statistical analysis for total protein was determined by parametric one-way ANOVA (Tukey test for multiple comparisons) after verifying data normality.

After choosing the most suitable collection method, all subsequent procedures were completed with saliva collected by this method.

Circadian variability

Upon the establishment of the collection procedure (sublingual method), the circadian effect on saliva volume and protein concentration was assessed. The number of subjects was increased to 22 (the initial 9 subjects plus 13 other subjects) to gain statistical power. Statistical analysis was performed by Paired t-test. Saliva volume and total protein concentration were estimated as described above.

Inter-individual *versus* intra-individual variability

To evaluate if intra individual variability undermines the use of saliva as a marker of individual physiological and pathological status, samples from 8 healthy donors were collect at 11 different times for 5 months. Total saliva volume and total protein concentration were compared by a two-Way ANOVA considering days of collection as the row factor and different individuals as the column factor.

Statistical analyses

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, USA).

Protein profile analysis

In order to determine if there are characteristic variability patterns in the protein profiles obtained from healthy subjects, whole saliva from 22 individuals was analyzed by capillary electrophoresis using an Experion™ Automated Electrophoresis System (Biorad) with standard protein chips (Experion™ Pro260 Analysis Kit). Samples were analyzed according to Biorad technical specifications. Briefly, to all saliva samples, sample buffer with β-mercaptoethanol was added. The saliva samples and the ladder were subjected to the same denaturing conditions (95º for 10 min). The migration times and the concentration of each protein in the sample wells were normalized to the ladder using internal markers.

The protein profile and the quantification of the abundant protein bands were determined using the Experion™ Software, version 3.20.

Variability within each band was calculated by the variation coefficient of the Total Protein values relatively to all the proteins in each group of molecular weights (MW).

In order to analyze the protein profiles and the relationship between individuals according to protein concentration by MW, hierarchical clustering analysis (HCA) was computed by using PermutMatrix v1.9.3 (LIRMM, Montpellier, France, http://www.lirmm.fr/ caraux/PermutMatrix/) [20] using the Euclidean distance and Ward's method.

Saliva protein stability at room temperature

To assess the profile of salivary proteins degradation aliquots of saliva samples from 5 individuals were maintained for 0, 24, 48 and 72 hours at room temperature and the protein profile was evaluated as described above.

Identification of saliva proteins

For protein processing, samples were denatured using Laemmli buffer containing SDS with DTT, alkylated with acrylamide and resolved by gel-electrophoresis[14]. Entire gel lane was sliced, digested with trypsin and peptides were extracted. This complex mixture of peptides was then analyzed by LC-MS/MS micro-reversed-phase at low pH coupled to a high resolution mass spectrometer (Triple TOF™ 5600 ABSciex®). Peptide fragmentation spectra were generated for protein identification using ProteinPilot software (ABSciex®) against Uniprot.

Results

Selection of the most advantageous collection method

Comparing the vestibular, sublingual and drooling saliva collection methods, regarding total volume of saliva collected, there are extremely significant differences between the vestibular method and both sublingual and the drooling methods ($p<0.0001$). The sublingual method is the one which renders the smallest volume of saliva (593.4 µL (sublingual), 1460 µL (vestibular) and 1288 µL (drooling) **(Figure 1A)**. The volume collected by drooling is on average slightly higher than the volume collected by sublingual but the difference is not statically significant.

In terms of protein concentration, there is a slight difference between the sub lingual (2.05 mg) and vestibular methods (2.51 mg) ($p=0.0106$) and a larger difference between sublingual (2.05 mg) and drooling (2.98 mg) ($p=0.0001$) **(Figure 1B)**. The vestibular and drooling methods were not statistically different ($p=0.0503$).

Based on the data obtained, the sublingual method was selected as the most adequate for the establishment of the SOP with an average of 1.460 ± 0.522 ml of saliva collected in 2 minutes and 2.051 ± 1.004 mg/ml of total protein obtained.

Circadian variability

To ascertain if samples collected in the afternoon are different from samples from the same individual collected in the morning, the effect of circadian variability was determined regarding total volume of saliva and total protein (Figure 2). The total volume is different between morning and afternoon ($p=0.0119$) and although the volume of morning collections is lower, sample variability is smaller (Figure 2A).

Regarding total protein, there seems to be no circadian effect (Figure 2B).

Inter-individual *versus* intra-individual variability

The analysis of the variability of total volume of saliva collected and total protein between and within individuals was performed on a total of 88 samples collected from 8 healthy subjects at 11 different time points throughout 5 months (Figure 3). Statistically significant differences were obtained regarding total volume and total protein both between and within individuals ($p<0.0001$). However, the 2-way ANOVA results show that in both variables a higher percentage of the variation is attributed to the individual rather than collection time (Figure 3). This difference is higher if the total protein is considered (% of total variation due to different individuals is 39.43 vs 26.13 when the samples are from the same individual but collected at different times).

Protein profile variability

Knowing from the previous results that the protein concentration was variable between individuals (Figure 3), it was necessary to study the individual protein profiles to understand if differences were due to all or only some of the proteins.

Figure 4 presents an example of the electrophoretic profile of the 8 healthy individuals used as saliva donors for Figure 3. It is obvious that in spite of similar protein profiles, there are proteins which are different between individuals even though all the individuals are considered healthy. To identify the different groups of proteins, the respective elecropherograms were analyzed. Nineteen protein groups were identified with the following apparent MW (9.2, 9.5, 9.9, 11.9, 13.3, 16.1, 22.3, 27.8, 34, 37.5, 46.5, 55.1, 61.7, 67.3, 70.1, 76, 79.1, 92.1, 157.6 kDa) and a variation coefficient was determined for each protein group (Figure 5). From the 19 protein groups identified 5 (13.3, 16.1, 27.8, 46.5 e 61.7 kDa) are present in all individuals studied and 2 other groups are present in 85% (9.5 e 70.1 kDa). From the proteins which are present in every individual the ones which show the least variation are of 16.1 and 61.7 kDa. The data from the MS analysis (Table 1) show that the possible proteins found within this range of MW are Calmodulin-like protein 3, Prolactin-inducible protein (PIP), Salivary acidic proline-rich phosphoprotein 1/2, Cystatin D, Cystatin-SA (Cystatin-2), Cystatin-SN (Cystain-SA-I), Cystatin-S (Cystatin-4), Cystatin-C (Cystatin-3), Profilin-1, Fatty acid-binding protein, Serum albumin, Ezrin (Cytovillin), Pyruvate kinase PKM and Alpha-amylase 1.

To verify if the different electrophoretic protein profiles are enough to distinguish individuals, a hierarchical clustering analysis according to Euclidean distance and Ward's method was performed (Figure 6). It is obvious that even within healthy individuals there are different groups in the electrophoretic patterns. The male

participants are all grouped within the same cluster (6, 10, 12, 13, 18, 19, 20, 21) while the older individuals formed a different group (11 and 17) with similar protein profiles. There were two individuals taking antidepressant medication which are also grouped (5 and 7). No relationship between the electrophoretic profile and smoking (3, 4, 5, 8, 10, and 11), alcohol consumption (2, 3, 4, 5, 6, 7, 8, 9, 10, and 11) or contraceptive medication (1, 2, 3, 4, 5, 8, 9, 11, and 17) was found.

Saliva protein degradation profile

Since saliva is a non-sterile biofluid, it becomes important to evaluate saliva stability at room temperature to identify how different protein bands are affected by degradation. Figure 7 shows the protein degradation profile of saliva samples from 5 individuals obtained by capillary electrophoresis. The protein MW groups defined above were used and the MW groups in which more degradation is apparent are 9.5; 22.3 and 46.5 kDa. According to the MS data (Table 1), the potential protein in these MW ranges are Acyl-CoA-binding protein (ACBP), Submaxillary gland androgen-regulated protein 3B (Proline-rich peptide P-B), Glutathione S-transferase P, Heat shock protein beta-1 (HspB1), Zymogen granule protein 16 homolog B, Interleukin-1 receptor antagonist protein (IL-1RN) and Alpha-1-antitrypsin. Additionally, the more conserved protein groups are 16.1 kDa (Calmodulin-like protein 3, Prolactin-inducible protein, Salivary acidic proline-rich phosphoprotein 1/2, Cystatin D, Cystatin-SA, Cystatin-SN, Cystatin-S, Cystatin-C, Profilin-1 and Fatty acid-binding protein) and 61.7 kDa (Serum albumin, Ezrin, Pyruvate kinase PKM and Alpha-amylase 1) (Figure 5) which probably means they are less susceptible to degradation.

Regardless the degradation profile of some protein MW bands, the degradation at room temperature is only significant after 24 hours (in this assay no enzyme activity inhibitors or antibacterial agents were added).

Identification of Saliva Proteins

A pool of 1012 peptides corresponding to 55 human proteins was identified (Table 3) and have previously been found in WS and are listed by OralCard [6].

Discussion

Considering our results, the sublingual cotton-roll method for saliva collection ensures a suitable sample volume and protein concentration in only 2 minutes.

These are important factors to take into account when saliva samples are collected for biobank storage purposes and the sublingual method chosen allows for the collection of the greater saliva volume even though the quantity of saliva is slightly lower than drooling. This difference may be due to the fact that the cotton-roll acts like a filter and helps to eliminate cell debris, membranes, protein aggregates and bacterial cells. This “filtration” is desirable and possibly contributes for a better quality of the sample and longer stability at room temperature. The sublingual collection method is also preferred to the vestibular one because the collection of saliva sublingually allows the collection from all glands and therefore is more representative of WS whereas the vestibular collection is mainly from the parotid glands (rich in amylase but poor in other proteins). Amylase has also been shown to be more prone to variations due to stress/anxiety or stimulation [21].

The collection protocol selected is simple, patient friendly and an easy method to perform in any laboratory requiring inexpensive minimal materials/equipment available in most labs. These are important considerations for large population studies.

One aspect to consider during collection is the circadian variation in protein concentration and content and the best time for sample collection should be defined based on these results. There have been many studies evaluating circadian variations in flow rate and saliva composition [18,22–27]. Our results showed significant differences between morning and afternoon sampling regarding the volume collected but not concerning protein concentration, which was also found by others [18,23].

In spite of the lack of variation in total protein there are studies showing circadian variations in several proteins [10,22,28]. Therefore, saliva collection should be performed in the morning, whenever possible, if a morning collection is not possible, it should still be done and the time of collection noted.

One of the concerns of saliva as a diagnostic fluid is the fact that within the same individual its composition varies throughout time. Our results demonstrate that the variation between individuals is larger than the variation within the same individual for a period of 5 months (total volume and total protein). Similar results have been obtained by other authors regarding specific salivary components [29]. These results reinforce the potential use of saliva to monitor physiological/pathological status changes in individuals.

Regarding the protein profile there are proteins which are present in all individuals. The proteins with the least variation in healthy subjects might have an

added potential to identify phenotypical variations. Our results show that the proteins which are less variable are involved in maintaining oral health and immune response (cystatins) [30] as well as chronic inflammation and diabetes (Profilin-1) [31]. Another proteins identified as less variable is Alpha-amylase 1 which has been studied as a marker for inflammatory status [32], tumor proliferation [33] and stress [34] Our results show the potential of saliva use in these applications.

The clustering analysis of the electrophoretic protein profiles showed that even within healthy individuals there are different protein profiles. These profiles may be associated to distinct variables such as gender, age, medication (antidepressants) and sample stability that may influence protein composition of saliva. Therefore electrophoretic protein profiles of human saliva may be used to monitor sample quality and pathological alterations of the “normal” protein profile, as long as certain variability factors are controlled. However, these applications require confirmation with more specific and detailed data.

Sample degradation at room temperature was shown to occur mainly in the first 24 hours and some proteins bands were more susceptible to degradation. If the research is to be directed to proteins within these MW values sample stability is a major concern and the addition of protease inhibitors is recommended.

Furthermore, the protein bands more conserved between individuals seem to be less prone to degradation which makes them especially suitable for physiological and sample quality markers.

As far as we know this is the first study where a saliva collection method was tested and evaluated considering not only the variability in total protein, volume collected and electrophoretic profile but also relating it to circadian rhythm, sample quality management and protein degradation.

Our results show that it is possible to obtain saliva samples suitable for biobanking storage by an easy and inexpensive method, as long as the appropriate controls, inclusion and exclusion criteria are taken into account to diminish the heterogeneity between different studies. Additionally, the association of a protein profile to each sample stored highly increases the amount of information available for future sample selection, incrementing the sample usability.

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Figure Legends

Figure 1– Comparison between the three collection methods considering the Total Volume of saliva collected **(A)** and Total Protein Concentration **(B)**. Statistical analysis for Total Volume was determined by non-parametric Freadman Test (Dunn's test for multiple comparisons) and statistical analysis for Total Protein was determined by parametric one-way ANOVA (Tukey test for multiple comparisons). Statistical analysis performed on data from 36 saliva collections for each method.

Figure 2– Circadian variability regarding Total Volume **(A)** and Total Protein Concentration **(B)** for morning and afternoon collections. Statistical analysis was performed by Paired t test.

Figure 3– Box-plot of Inter-individual and intra-individual variability on Total Volume **(A)** and Total Protein Concentration **(B)**. The results correspond to saliva samples from 8 healthy donors from which samples were collected at 11 different times (throughout 5 months). Statistical analysis was performed by a two-Way ANOVA considering the days of collection as the row factor and the different individuals as the column factor.

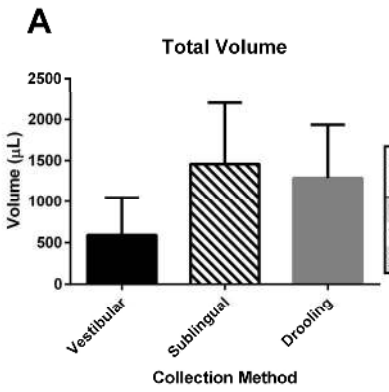
Figure 4- Inter-individual variability of protein electrophoretic profile. The protein profile from 8 different individuals (the same of Figure 3) analysed by capillary electrophoresis using the Experion BioRad System. **(L)** Ladder with a range from 10 to 150 kDa.

Figure 5- Protein band variability to identify the protein groups which are the most different between individuals (n=22) the bar indicate the %CV for each group of proteins. **+**protein bands present in all individuals studied.

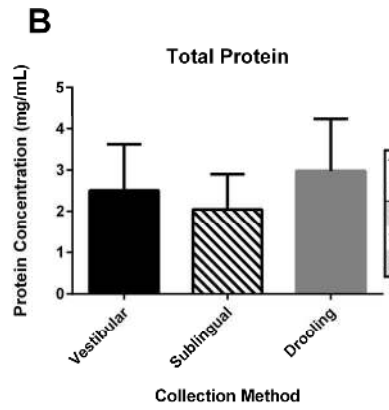
Figure 6- Comparison of individual's saliva regarding the electrophoretic profile. Each column represents the data for one subject. Rows represent protein groups by MW.

Protein concentration in each protein band from capillary electrophoresis profile is presented and the color code is graduated from black (minimum concentration) to light grey (maximum concentration). Cluster analysis performed with PermutMatrix using Ward's minimum variance method according to Euclidean distance. (n=22)

Figure 7– Protein degradation profile of five saliva samples exposed to room temperature during 0, 24, 48 and 72 hours analysed by capillary electrophoresis using the Experion BioRad System. The values of each box correspond to means of 5 individuals with min and max values indicated as error bars. **+**Protein bands with higher degradation.



Dunn's multiple comparisons test	Mean	Significance	Summary	Ajusted P value
Vestibular vs. Sublingual	593.4 vs 1460	Yes	****	< 0.0001
Vestibular vs. Drooling	593.4 vs 1288	Yes	****	< 0.0001
Sublingual vs Drooling	1460 vs 1288	No	ns	> 0.9999



Tukey Multiple Comparison Test	Mean	Significance	Summary	Ajusted P value
Vestibular vs. Sublingual	2.51 vs 2.05	Yes	*	0.0106
Vestibular vs. Drooling	2.51 vs 2.98	No	ns	0.0503
Sublingual vs Drooling	2.05 vs 2.98	Yes	***	0.0001

Figure 1– Comparison between the three collection methods considering the Total Volume of saliva collected (A) and Total Protein Concentration (B). Statistical analysis for Total Volume was determined by non-parametric Freadman Test (Dunn's test for multiple comparisons) and statistical analysis for Total Protein was determined by parametric one-way ANOVA (Tukey test for multiple comparisons). Statistical analysis performed on data from 36 saliva collections for each method.
179x153mm (300 x 300 DPI)

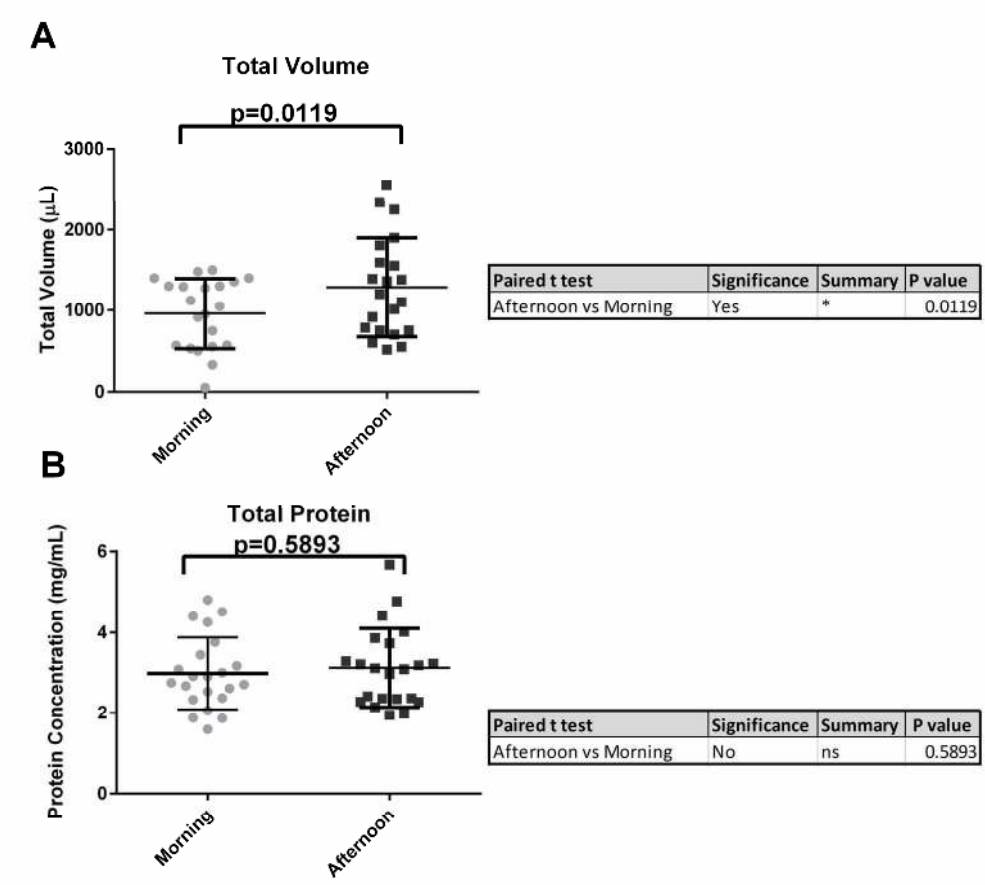
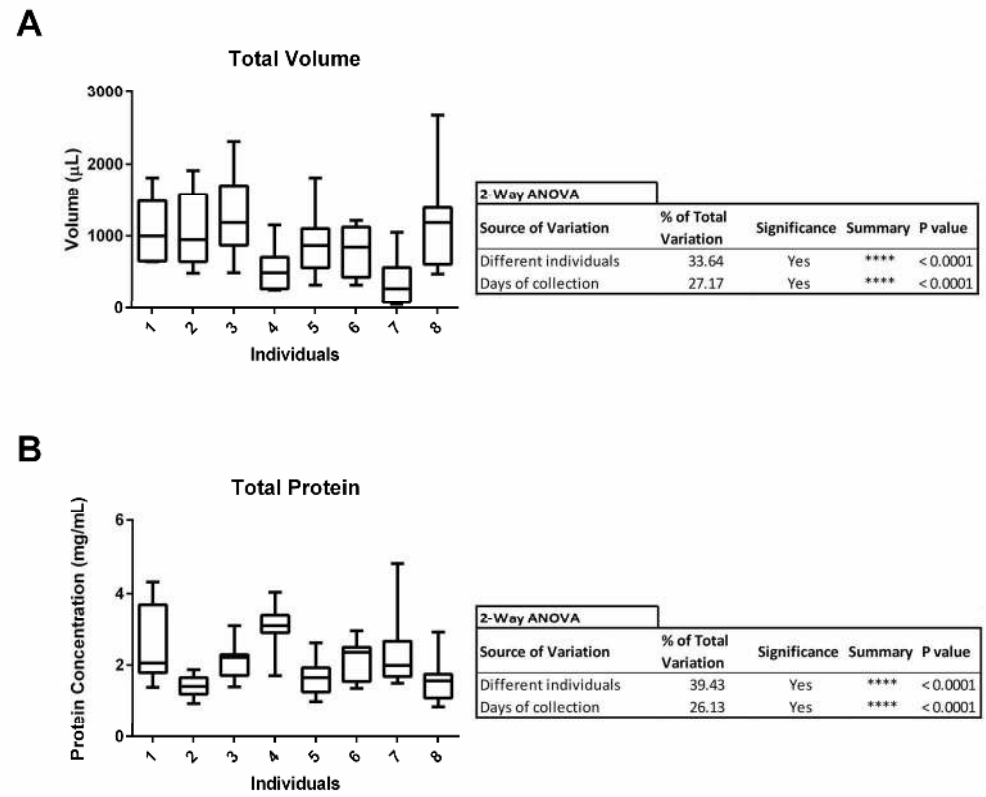


Figure 2– Circadian variability regarding Total Volume (A) and Total Protein Concentration (B) for morning and afternoon collections. Statistical analysis was performed by Paired t test.
180x165mm (300 x 300 DPI)



2-Way ANOVA

Source of Variation	% of Total Variation	Significance	Summary	P value
Different individuals	33.64	Yes	****	< 0.0001
Days of collection	27.17	Yes	****	< 0.0001

2-Way ANOVA

Source of Variation	% of Total Variation	Significance	Summary	P value
Different individuals	39.43	Yes	****	< 0.0001
Days of collection	26.13	Yes	****	< 0.0001

Figure 3– Box-plot of Inter-individual and intra-individual variability on Total Volume (A) and Total Protein Concentration (B). The results correspond to saliva samples from 8 healthy donors from which samples were collected at 11 different times (throughout 5 months). Statistical analysis was performed by a two-Way ANOVA considering the days of collection as the row factor and the different individuals as the column factor.
167x140mm (300 x 300 DPI)

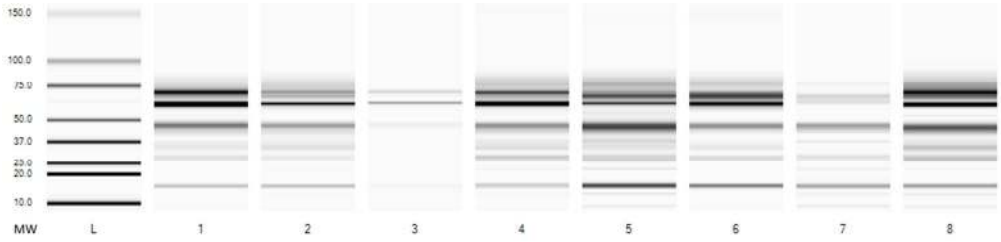


Figure 4- Inter-individual variability of protein electrophoretic profile. The protein profile from 8 different individuals (the same of Figure 3) analysed by capillary electrophoresis using the Experion BioRad System. (L) Ladder with a range from 10 to 150 kDa.
261x62mm (96 x 96 DPI)

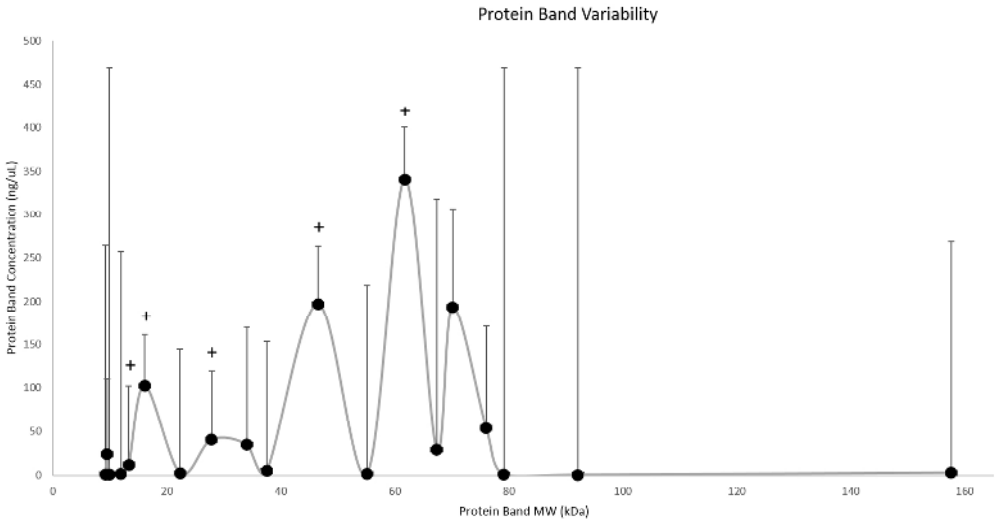


Figure 5- Protein band variability to identify the protein groups which are the most different between individuals (n=22) the bar indicate the %CV for each group of proteins. +protein bands present in all individuals studied.
419x218mm (96 x 96 DPI)

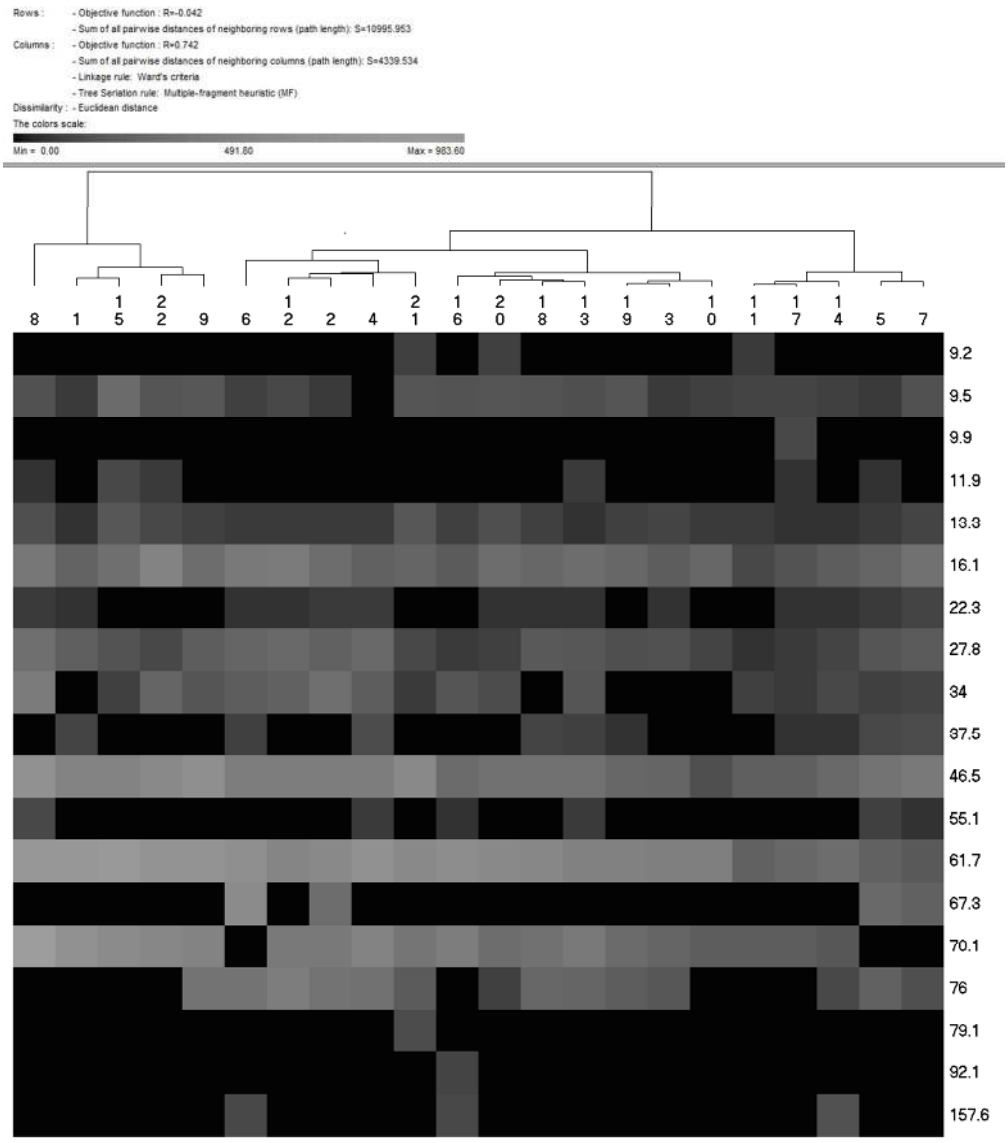


Figure 6- Comparison of individual's saliva regarding the electrophoretic profile. Each column represents the data for one subject. Rows represent protein groups by MW.
Protein concentration in each protein band from capillary electrophoresis profile is presented and the color code is graduated from black (minimum concentration) to light grey (maximum concentration). Cluster analysis performed with PermutMatrix using Ward's minimum variance method according to Euclidean distance. (n=22)
300x347mm (96 x 96 DPI)

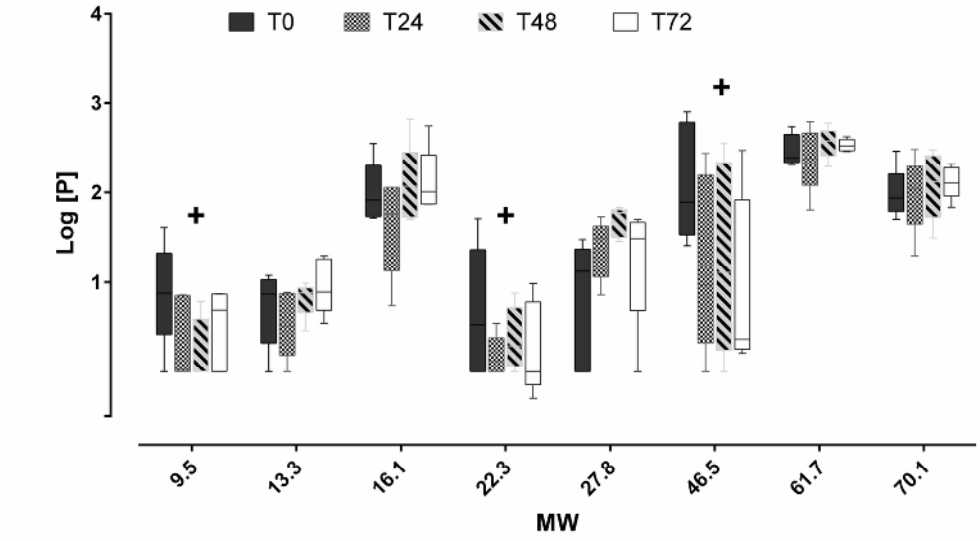


Figure 7– Protein degradation profile of five saliva samples exposed to room temperature during 0, 24, 48 and 72 hours analysed by capillary electrophoresis using the Experion BioRad System. The values of each box correspond to means of 5 individuals with min and max values indicated as error bars. +Protein bands with higher degradation.
131x79mm (300 x 300 DPI)

Table 1– Human salivary proteins identified by Mass Spectrometry analysis.

Mol. Wt. (kDa)	*UniProt Code*	*Protein Name*	OralCard [8]	*Identified in whole saliva*
161	A8K2U0	Alpha-2-macroglobulin-like protein 1	x	x
118	P22314	Ubiquitin-like modifier-activating enzyme 1	x	x
103	P55786	Puromycin-sensitive aminopeptidase (PSA)	x	x
86	P06396	Gelsolin (AGEL)	x	x
83	P01833	Polymeric immunoglobulin receptor (PIgR)	x	x
80	P22079	Lactoperoxidase (LPO)	x	x
78	P02788	Lactotransferrin (Lactoferrin)	x	x
77	Q08188	Protein-glutamine gamma-glutamyltransferase E	x	x
69	P02768	Serum albumin	x	x
69	P15311	Ezrin (Cytovillin)	x	x
58	P14618	Pyruvate kinase PKM	x	x
58	P04745	Alpha-amylase 1	x	x
57	P07237	Protein disulfide-isomerase (PDI)	x	x
54	Q9UBG3	Cornulin	x	x
53	P52209	6-phosphogluconate dehydrogenase	x	x
50	P80303	Nucleobindin-2	x	x
49	P01871	Ig mu chain C region	x	x
49	Q8N4F0	BPI fold-containing family B member 2	x	x
47	P01009	Alpha-1-antitrypsin	x	x
44	Q9UIV8	Serpin B13	x	x
43	P30740	Leukocyte elastase inhibitor (LEI)	x	x
39	P04083	Annexin A1	x	x
38	P01876	Ig alpha-1 chain C region	x	x
38	Q6P5S2	Protein LEG1 homolog	x	x
37	P00338	L-lactate dehydrogenase A chain (LDH-A)	x	x
37	P01877	Ig alpha-2 chain C region	x	x
36	P01857	Ig gamma-1 chain C region	x	x
36	P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	x	x
35	P23280	Carbonic anhydrase 6	x	x
34	P25311	Zinc-alpha-2-glycoprotein (Zn-alpha-2-GP)	x	x
29	P06870	Kallikrein-1	x	x
28	P31947	14-3-3 protein sigma	x	x
27	Q96DR5	BPI fold-containing family A member 2	x	x
23	P09211	Glutathione S-transferase P	x	x
23	P04792	Heat shock protein beta-1 (HspB1)	x	x
23	Q96DA0	Zymogen granule protein 16 homolog B	x	x
20	P18510	Interleukin-1 receptor antagonist protein (IL-1RN)	x	x
18	P01591	Immunoglobulin J chain	x	x
18	Q9UHA7	Interleukin-36 alpha	x	x
18	Q9UBC9	Small proline-rich protein 3	x	x
17	P27482	Calmodulin-like protein 3	x	x
17	P12273	Prolactin-inducible protein (PIP)	x	x
17	P02810	Salivary acidic proline-rich phosphoprotein 1/2	x	x
16	P09228	Cystatin-SA (Cystatin-2)	x	x
16	P01037	Cystatin-SN (Cystain-SA-I)	x	x
16	P01036	Cystatin-S (Cystatin-4)	x	x
16	P01034	Cystatin-C (Cystatin-3)	x	x
15	P07737	Profilin-1	x	x
15	Q01469	Fatty acid-binding protein	x	x
13	P06702	Protein S100-A9 (Calgranulin-B)	x	x
11	P01040	Cystatin-A (Cystatin-AS)	x	x

11	P05109	Protein S100-A8 (Calgranulin-A)	x	x
10	P07108	Acyl-CoA-binding protein (ACBP)	x	x
8	P02814	Submaxillary gland androgen-regulated protein 3B (Proline-rich peptide P-B)	x	x
7	P15515	Histatin-1 (Histidine-rich protein 1)	x	x

Protein identification was performed as previously described[14] using shortGeLC-MS/MS, and Protein Pilot software (v5, ABSciex) with an independent false discovery rate (FDR) based on a target-decoy approach used to assess the quality of the identifications (see material and methods section for details)