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Inter-individual variability of protein patterns in saliva of healthy adults

Mercedes Quintana^{a,b,c}, Olivier Palicki^{a,b,c}, Géraldine Lucchi^d, Patrick Ducoroy^d,
Christophe Chambon^e, Christian Salles^{a,b,c}, Martine Morzel^{a,b,c,*}

^aINRA, UMR 1129 FLAVIC, F-21000 Dijon, France

^bENESAD, UMR 1129 FLAVIC, F-21000 Dijon, France

^cUniversité de Bourgogne, UMR 1129 FLAVIC, F-21000 Dijon, France

^dCLIPP « Clinical Innovation Proteomic Platform », IFR 100 Santé-STIC, F-21000 Dijon, France

^eINRA, Plate-forme « Exploration du métabolisme », F-63122 St Genès Champanelle, France

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ABSTRACT

In order to document inter-individual variability in salivary protein patterns, unstimulated whole saliva was obtained from 12 subjects at 10 am and 3 pm of the same day. Saliva proteins were separated using two-dimensional gel electrophoresis, and semi-quantified using image analysis. One-way ANOVA was used to test the effects “time of sampling” and “subject”. Data were further explored by multivariate analyses (PCA, hierarchical clustering). Spots of interest were identified by mass spectrometry (MALDI-TOF MS/MS and nanoLC ESI-IT MS/MS). A dataset of 509 spots matched in all gels was obtained. There was no diurnal statistical effect on salivary patterns while inter-individual variability was high with 47 spots differentially expressed between subjects ($p < 1\%$). Clustering of these spots revealed that subjects could be discriminated first based on several proteins participating to the non-specific immune response (cystatins, lipocalin 1, parotid-secretory protein and prolactin-induced protein). Independently, subjects were also differentiated by their level of proteins originating from serum and involved in the immune system (complement C3, transferrin, IgG2), as well as the relative abundance of enzymes involved in carbohydrates metabolism (amylase and glycolytic enzymes). Inter-individual variability should be accounted for when searching for salivary biomarkers or when studying in-mouth biochemical mechanisms.

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1. Introduction

Studies on characterisation of human saliva proteome have recently flourished with the advent of efficient methods, combining electrophoretic or chromatographic separation of protein mixtures with mass spectrometry-based protein identification [1–5]. 2-D electrophoresis allows the comprehensive characterisation of relatively abundant proteins, while LC-MS may in some cases detect proteins present in extremely low quantities. Such highly resolutive and complementary techniques have revealed the complexity of

saliva. For example, a recent publication of the “Human Salivary Proteome Consortium” reports the identification of 1166 proteins from parotid and submandibular/sublingual salivas [6]. Even more proteins are expected in whole saliva, since it also contains proteins from other glands and from the gingival crevicular fluid, a transudate of blood plasma.

Besides being complex, saliva is also a dynamic fluid and its protein composition for a given subject may qualitatively vary. Thus, taste stimulation induces changes in proteome patterns [7]. Differences in the proportions of specific low molecular weight peptides have also been detected in parotid saliva at

* Corresponding author. INRA, UMR1129 FLAVIC, F-21000 Dijon, France. Fax: +33 380 69 34 03.

E-mail address: Martine.Morzel@dijon.inra.fr (M. Morzel).

different times of the day [8]. Other studies, although they did not strictly compare the same subjects, suggest that saliva protein composition may vary with age in infants. For example, the proportions of the various isoforms of Proline-Rich Proteins (PRPs) varied with post-conception age in newborns [9]. Finally, proteome pattern modifications were evidenced between groups of subjects either healthy or suffering from oral or systemic pathologies [10,11]. In addition to this plasticity in response to age, condition or environmental factors, one should also consider the variability from one subject to another due to genetic polymorphism. The highly polymorphic character of several salivary proteins, in particular PRPs, has long been recognised (for a review, see [12]).

In this context, any study based on proteome analysis of saliva, be it for diagnostics purposes [13] or comprehension of in-mouth mechanisms, needs to take into account variability in protein profiles within a group of subjects. However, very few studies provide data on this topic. Morales-Bozo et al. [14] used 1-D electrophoretic separation to characterise polypeptide salivary profiles in a healthy population, and found overall no age or sex effect but observed inter-individual variations. Variability was also examined using intact protein LC/ESI-TOF mass spectrometry [15], which technically restricts the observations to low molecular proteins (<15 kDa). The authors demonstrated that day-to-day intra-subject and even more so inter-subject salivary protein variation is significant. The objective of the present work was therefore to formally document inter-subjects variability in 2-D electrophoretic salivary patterns.

2. Materials and methods

2.1. Saliva collection and processing

Twelve volunteers (6 women, 6 men; 26–47 years old; non smokers; salivary flow in resting conditions $>0.5 \text{ ml min}^{-1}$) were recruited for the study. Written informed consent was obtained from all participants. Two sampling sessions took place at 10:00 am and 3:00 pm of the same day. Subjects were instructed to refrain from eating or drinking at least one hour before the sessions. They spat out saliva accumulating freely in their mouth as frequently as desired over a duration of 2 min. Samples were immediately centrifuged at 14,000 g for 20 min at 4 °C. The supernatant was submitted to ultrafiltration at 15,000 g for 30 min at 10 °C using spin columns with a 5 kDa cut-off (Vivaspin 500, Sartorius AG, Germany). Aliquots of the resulting protein extracts ($n=24$) were stored at -80 °C until analysis.

2.2. 2-DE analysis

The samples were analysed in series of 6 gels. Samples were randomly assigned to the series. Protein concentration was measured in the extracts following the Bradford's method. The first dimension was performed using 17 cm 3–10NL IPG strips (Bio-Rad) on a PROTEAN (Bio-Rad) IEF cell. Protein extracts were suspended in a buffer containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.1% w/v DTT, 1% v/v 3–10 carrier ampholytes and 0.3% v/v protease inhibitors (SIGMA). Strips were loaded with 150 μg of protein for analytical gels and

800 μg for preparative gels, and rehydrated at 20 °C for 13 h at 0 V and 8 h at 50 V. Isoelectric focusing was carried out at a final voltage of 8000 V for a total of 60 kWh. Thereafter, strips were equilibrated for 15 min and 20 min in two consecutive solutions of 6 M Urea, 50 mM Tris-HCl pH 8.8, 30% v/v glycerol, 2% w/v SDS, to which was added DTT at 1% w/v or iodoacetamide at 2.5% w/v. Strips were then placed on 12% SDS-polyacrylamide gels and electrophoresis was performed at 50 mA per gel on a PROTEAN II Multi Cell (Bio-Rad). Analytical gels were silver stained [16] and preparative gels were stained using the so-called Blue silver protocol [17].

2.3. Image analysis and statistical treatment

Gel digital images were acquired with the Image Scanner (Amersham Biosciences) and analysed using SameSpots software v.3.0. Quantities of matched spots were normalised by calculating the ratio of each spot's quantity to the total quantity of valid spots in a gel. Data were expressed in ppm. Finally, in order to make the variance independent of the mean, data were transformed into log before statistical treatment. The dataset was analysed using the Statistica software (StatSoft, Tulsa, OK, USA). It was first submitted to a Principal Component Analysis using all 509 spots as variables. Data were then submitted to one-way ANOVA testing three factors: time of sampling (morning/afternoon), gender, and subject. A spot was considered significant when it was associated to a p -value $<1\%$, and a fold-change above 2 for the two-level factors "time of sampling" and "gender". Specifically for the description of intra-individual variability, significant spots were selected and they were subjected to a Principal Component Analysis. Data for these 47 spots and 12 subjects were visualised by heatmap and ascendant hierarchical cluster analysis (method of Ward based on Euclidian distances) using the software PermutMatrix [18].

2.4. Mass spectrometry-based protein identification

Spots of interest were excised manually, washed in NH_4HCO_3 0.1 M for 10 min and dehydrated in ACN for 10 min. Dry spots were incubated successively in 10 mM TCEP/0.1 M NH_4HCO_3 for 30 min at 37 °C, in 55 mM iodoacetamide/0.1 M NH_4HCO_3 for 20 min, in 0.1 M NH_4HCO_3 for 5 min and in ACN for 5 min. Digestion was performed in two steps: spots were first pre-incubated for 30 min at 4 °C in 20 μL of a 40 mM of NH_4HCO_3 /10% ACN solution containing 10 ng/ μL of trypsin (V5280, Promega, USA). 15 μL of this solution was subsequently removed and 10 μL of 40 mM NH_4HCO_3 /10% ACN was added, prior to incubation at 37 °C for 2 h. Peptides were collected in the two successive supernatants obtained after addition of 0.5% TFA and ACN (1 μL and 10 μL , respectively) and sonication for 10 min.

For MALDI-TOF analysis, peptides were further concentrated on C18 beads (Invitrogen) following the manufacturer's instructions. 0.5 μL of the concentrate was loaded onto a Ground Steel target, mixed with 1 μL of matrix solution (3.5 mg/ml CHCA in ACN 50%, TFA 0.25%) and allowed to dry. The target was introduced in a mass spectrometer MALDI-TOF (Ultraflex, Bruker Daltonics, Bremen, Germany). Ionisation was performed in MS and MS/MS (PSD-LIFT technology) by irradiation of a nitrogen laser (337 nm) operating at 50 Hz. Data were acquired at

a maximum accelerating potential of 25 kV in the positive and reflectron modes. The MALDI mass spectra were calibrated using the Peptide Calibration Standards from Bruker Daltonics. The software packages Ultraflex version 3.0, Flex control, Flex Analysis and Biotoools version 3.1 were used to record and analyze the mass spectra. The database search was performed with Mascot in the MSDB database. Methionine oxidation was accepted as a variable modification and carbamidomethyl modification of cysteine as a global modification. Mass deviation tolerance was set at 80 ppm in MS mode and 0.5 Da in MS-MS mode.

When identification by MALDI-TOF proved unsuccessful, identification was also attempted using nano LC-ion trap MS/MS analysis. HPLC was performed with an ULTIMATE LC SYSTEM combined with Famos autosampler and Switchos II microcolumn switching for preconcentration (LC Packings, Amsterdam, The Netherlands). Six μl of the supernatant containing peptides were loaded on the column PEPMAP C18, 5 μm , 75 μm ID, 15 cm (LC Packings) using a pre-concentration step in a micro pre-column cartridge (300 μm ID, 1 mm). Supernatants were loaded on the pre-column at 30 $\mu\text{L}/\text{min}$. After 3 min, the pre-column was connected with the separating column and the gradient was started at 200 nL/min. The buffers were 5% ACN, 0.5% HCOOH in water (A) and 5% H₂O, 0.5% HCOOH in ACN (B). A linear gradient from 10 to 90% B for 45 min was applied. For ion trap MS, a LCQ DECA with a nano electrospray interface (Teruo Fisher Scientific, Les Ulis, France) was used. Ionisation (2.2 kV ionisation potential) was performed with a liquid junction and a non-coated capillary probe (New Objective, Cambridge, USA). Peptide ions were analyzed by the data-dependent “triple play” method: (i) full MS scan (m/z 400–1400), (ii) zoomscan (scan of the major ion with bigger resolution), (iii) MS/MS of this ion. Identification of peptides was performed with Mascot 2.2, restricting the taxonomy to *Homo sapiens* (216,961 sequences) in the protein NCBI nr 20080912 database. Mass deviation tolerance was set at 0.4 Da. Protein identification was validated when at least two peptides originating from one protein showed significant identification scores.

3. Results

3.1. Protein concentration in the protein extracts

Protein concentration in the extracts ranged from 2.09 to 8.58 $\mu\text{g } \mu\text{l}^{-1}$, at the exception of one subject who showed a concentration of 26.1 $\mu\text{g } \mu\text{l}^{-1}$ (average of the morning and afternoon samples). Excluding data from this outlier, protein concentration in the extracts was on average $4.68 \pm 1.83 \mu\text{g } \mu\text{l}^{-1}$ ($n=11$) and $5.38 \pm 1.84 \mu\text{g } \mu\text{l}^{-1}$ ($n=11$) for morning and afternoon samples, respectively, and $4.95 \pm 1.71 \mu\text{g } \mu\text{l}^{-1}$ ($n=12$) and $5.13 \pm 2.04 \mu\text{g } \mu\text{l}^{-1}$ ($n=10$) for females and males, respectively. Differences between genders or between morning and afternoon samples were not significant.

3.2. Variability in protein patterns

3.2.1. Global proteome patterns

509 spots matched across all gels were retained for the analysis. Projection of observations (gels) on the PCA plot of the proteome

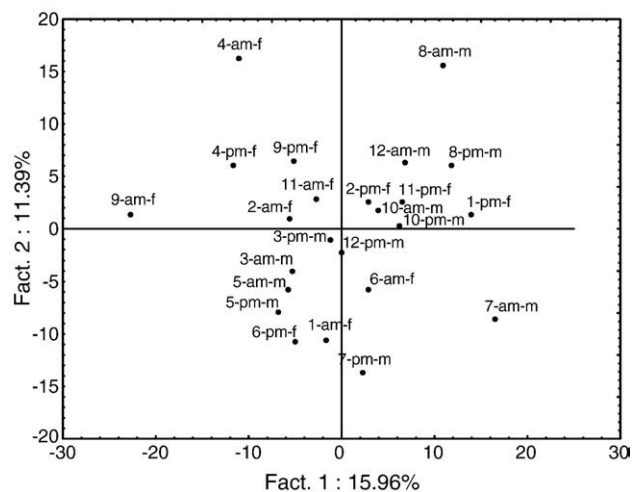


Fig. 1 – Principal component analysis scores plot derived from the entire set of proteome data for 12 subjects. Observations are labelled as follows: subject number (1 to 12) – time of sampling (am/pm) – gender (m/f).

data are shown in Fig. 1. This plot displays the first 2 principal components, which account for 27% of variation in the samples. The plot provides a first overall picture of variability within samples. Considering in particular the two potential factors of variation “time of sampling” and “gender”, it is clear that they are not preponderant factors in samples’ variation since they do not separate samples in two distinct groups on the represented 2D space. This is confirmed by results of ANOVA: no spots were considered significantly different between the two times across the whole group of subjects, and only 3 spots (one unidentified, one cystatin SN, one parotid secretory protein) were significantly different between males and females, all over-expressed in females.

Concerning intra-individual variability, the two samples corresponding to the same subject are in general located at a moderate distance. Some subjects are characterised by very reproducible proteome patterns (e.g. subject number 10) while others are more variable in their patterns between morning and afternoon (e.g. subject number 1).

3.2.2. Inter-subject variability

Subjects’ specificities were the greatest source of variability in the data since 47 spots were differentially expressed between subjects (Table 1). Observing the experimentally estimated vs theoretical molecular weight of identified proteins, it appeared that four spots (195, 280, 297, 468) were putative fragments, i.e., their estimated molecular weight were largely below the molecular weight of the native protein. However, for spot 195 identified as α -amylase, results of MS identification showed that peptides both at the N- and C-terminal extremities were matched to the sequence of the entire protein. The discrepancy between the estimated and theoretical MW was therefore probably due this isoform of amylase being submitted to internal deletions prior to secretion in the oral cavity, as speculated by Hirtz et al. [19]. Therefore, apart from three fragments, all identified spots corresponded to intact proteins. When projecting observations on the PCA space generated by those 47 spots (Fig. 2), i.e., focusing on data

Table 1 – List of spots differentially represented ($p < 1\%$) between subjects: identifications and descriptors of proteins (accession number, estimated vs theoretical MW in kDa), minimum (min), maximum (max) and median values in ppm and fold change between the minimum and maximum values in ppm (FC max).

Spot	Identification, accession number and abbreviation	Estimated vs theoretical MW	min (ppm)	max (ppm)	median (ppm)	FC max
16	Glyceraldehyde 3-P dehydrogenase (gi120649)-GAPDH	38.7/35.9	616	8845	3320	14.34
25			449	4468	1304	9.93
51			196	2013	296	10.25
60			154	1253	669	8.12
62			97	419	188	4.32
68			127	931	487	7.31
72	Lipocalin 1 (gi 4504963)	15.8/17.5 ^a	321	2232	581	6.95
93			1154	3387	1708	2.93
149			1492	11669	5738	7.82
174	Transferrin (gi 136191)	72.3/77.0	1253	5460	2026	4.36
176	Cystatin D (gi 118186)	11.7/13.9 ^a	2055	6542	2862	3.18
183			459	7050	2820	15.35
195			925	2461	1487	2.66
204	α -amylase (gi 157833830)	33.3/55.7	108	757	172	7.01
210			739	3309	1829	4.48
219			107	806	344	7.48
223	Cystatin SN (gi 118188)	13.3/14.3 ^a	154	976	249	6.32
244	Prolactin-inducible protein (gi 134170) — PIP	16.6/16.6	1085	14561	2124	13.41
256	α -amylase (gi 157833830)	48.3/55.7	322	913	441	2.83
257	Prolactin-inducible protein (gi 134170) — PIP	18/16.6	1382	9629	5036	6.96
280	Parotid secretory protein -fragment (gi 16755850) — PSP	12.5/27.1	303	1722	824	5.67
297	Complement C3-fragment (gi 179665)	70.5/189.3	804	3375	2215	4.19
300	Parotid secretory protein (gi 16755850) — PSP	32.0/27.1	106	709	127	6.65
313			424	1311	757	3.09
344			3016	13912	6786	4.61
362			286	1412	463	4.92
399			521	4109	1607	7.87
410			373	1786	1194	4.79
421	Cystatin D (gi 118186)	11.7/13.9 ^a	421	1571	804	5.51
424			834	2446	1734	2.93
425			21	101	44	4.70
429			169	677	362	4.01
430			365	1603	736	4.39
434			274	1547	680	5.63
442	Immunoglobulin γ 2 heavy chain (gi 184758) — IgG2	56.5/35.8	3314	10017	4642	3.02
460	Prolactin-inducible protein -fragment (gi 134170) — PIP	11.2/16.6	597	2178	1408	3.65
468			1509	11583	7107	7.67
470			8597	22410	13736	2.61
471	Prolactin-inducible protein (gi 134170) — PIP	17.1/16.6	1066	7035	2345	6.60
472			329	1362	957	4.14
476			1945	5680	4122	2.92
477	α -amylase (gi 157833830)	54.2/55.7	196	843	425	4.29
483	Prolactin-inducible protein (gi 134170) — PIP	15.5/16.6	1105	13004	1960	11.77
485			559	2230	1292	3.99
487	Phosphoglycerate mutase (gi 49456447)	29/28.8	1041	3188	2038	3.06
498	Enolase 1 (gi 119339)	50.7/47.2	580	2603	1633	4.49
503	Lipocalin 1 (gi 4504963)	14.8/17.5 ^a	819	6211	3272	7.58

^aThe reported theoretical molecular weight is that of the mature chain of the protein.

allowing the discrimination between subjects, the morning and afternoon samples for one given subject were closely grouped. When comparing these results with those presented in Fig. 1, this translates the fact that the subject-specific signature constituted by those spots is more conserved within the day than the global proteome patterns.

Among significant spots, some spots were particularly variable in the population considered, as for example the spot 183 (cystatin D) which proportion varied more than 15-fold between the subjects with the highest and lowest

quantities respectively. Fig. 3 represents a zoom of the gel region containing this particular spot for the 12 subjects, illustrating the large variability in abundance of this particular isoform of cystatin D. It was represented as an extremely faint spot corresponding to minute quantities in some subjects, and as a pronounced spot in others. Finally, Fig. 4 shows the heatmap representation of the 47 significant spots and 12 subjects. Two main clusters of spots emerge, which clearly correspond to different functional group. Thus, the first cluster groups various enzymes (amylase, enolase 1, glyceraldehyde

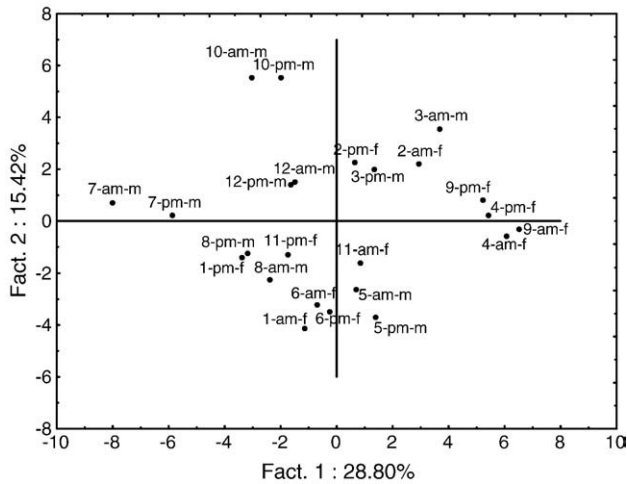


Fig. 2 – Principal component analysis scores plot derived from a dataset made of 47 spots differentially represented between subjects ($p < 1\%$), for 12 subjects. Observations are labelled as in Fig. 1.

3-phosphate dehydrogenase and phosphoglycerate mutase) and proteins originating from serum (transferrin, complement C3, immunoglobulin gamma2), while the second cluster is composed of many isoforms of four proteins: cystatins (D and SN), prolactin-inducible protein, parotid secretory protein and lipocalin1. As to the clustering of subjects, it does not reveal straightforward groups, which again confirms the high inter-individual variability. Looking in more details, it is however possible to find subjects with comparable profiles. For example, in the sub-cluster including spots 297 to 434 (framed in Fig. 4), subjects 10, 12, 7 and 1 tend to show a low abundance of

these proteins in contrast to subjects 4 and 9 who are characterised by a high proportion of these proteins.

4. Discussion

This study was designed to characterise variability in whole saliva proteome 2-D electrophoretic patterns of healthy adults. Overall and across the group of subjects, we found that there was no diurnal effect and a very moderate gender effect on salivary profiles, while inter-individual variability was significant.

Circadian and diurnal variations in saliva composition have been largely documented, in particular in relation to the neuroendocrine system function. For example, variations have been reported in the levels of hormones such as cortisol [20], melatonin [21] and leptin [22]. When it comes to salivary proteins and peptides present at higher concentrations, a general trend repeatedly reported is an increase of protein content in whole saliva during the day (e.g. [23]). Here, although there was a slight increase of protein content in the extracts, the difference remained statistically non-significant. This is not entirely surprising given the fact that only 5 h separate the two sampling points. The literature also reports quantitative variations in specific protein compounds, such as α -amylase [23,24], immunoglobulin A [25] or histatins [26,27] but not of other constituents, namely kallikrein [23] or statherin [26]. This suggests indirectly that salivary profiles vary within a day. However, direct information on diurnal profiles, i.e., adjusting for differences in protein concentration, is not plentiful. Hardt et al. [8] quantified low-molecular weight peptides in parotid saliva within a day, and observed 5 categories of peptides exhibiting distinct diurnal variations. In contrast, and in accordance with our findings, Neyraud et al. [7] reported stable profiles of proteins separated by 2-D electrophoresis, i.e., in the

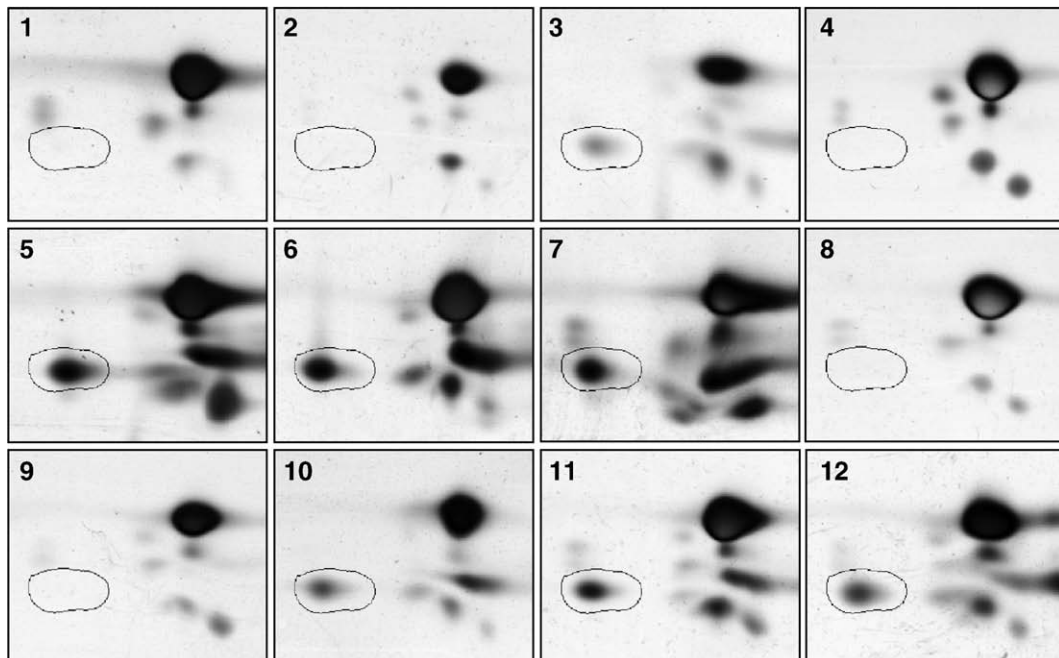


Fig. 3 – Zoom area of the gel region (approximate pI range 6.5–7.5, and MW range 15–16.5 kDa) containing spot 183, identified as cystatin D, for 12 subjects. One gel per subject was chosen.

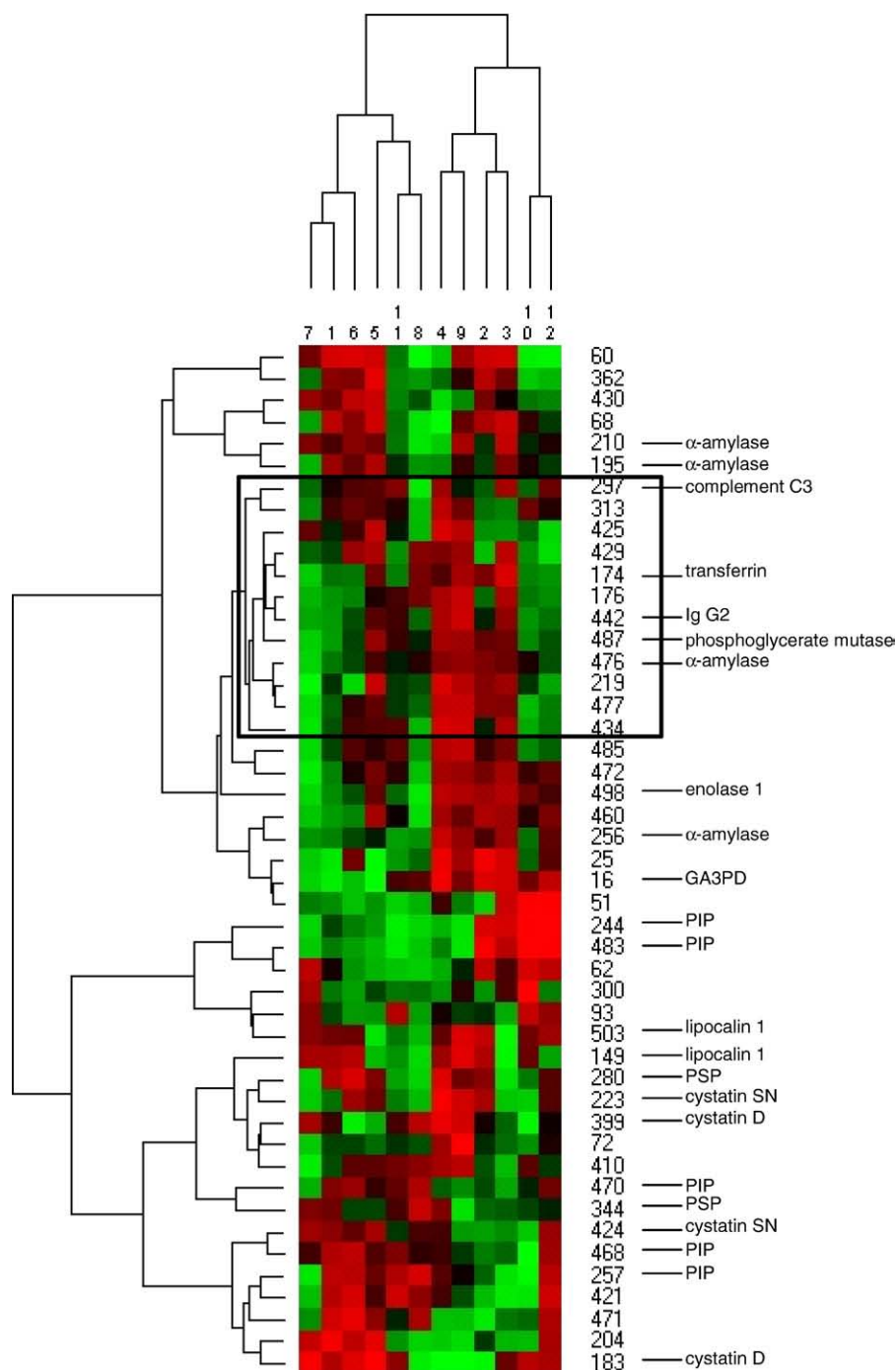


Fig. 4 – Heatmap visualising the level of 47 spots differentially represented between subjects ($p < 1\%$), for 12 subjects. Each column represents the data (average of morning and afternoon samples) for one subject. Rows represent individual proteins. Row data were mean-centered and the colour code is graduated from green (under-representation compared to the group mean) to red (over-representation compared to the group mean). Hierarchical clustering analysis was used to organise the map. Abbreviations are as in Table 1.

approximate MW range 10–100 kDa. This great stability of protein profiles within a day means that in mechanistic studies, in particular linking proteome patterns to in-mouth sensory perception, saliva sampling does not need to be performed at the exact same time of the day.

Concerning the effect of gender, it was in our study extremely limited. Sex hormonal regulation of specific sali-

vary proteins expression has been previously reported in rodents, in particular the expression of small 20–25 kDa proteins [28]. Lipocalins were subsequently described as being more abundant in male rat submandibular glands [29]. In humans, the higher prevalence of caries in women has been suggested to be partly due differences in saliva composition and flow rates induced by hormonal fluctuations. [30]. Looking

more specifically at individual proteins, levels of α -galactosidase [31] or α -amylase activity [24] were reported to be independent of gender. Conflicting results are found for secretory IgA, from over-expression in women [32], no effect of gender [33] or over-expression in men [34]. A gender effect was reported for the abundance of mucins MG1 but not MG2 [35]. Kivela et al. [36] compared the results of two independent studies and concluded that CAVI was present at lower levels in saliva of young women compared to that of young men. Finally, up-regulation of PSP (parotid secretory protein) by estradiol was demonstrated *in vitro* [37]. Our results are in accordance with the latter finding since one spot corresponding to PSP was over-represented in women. However, overall, differences in profiles between men and women were minimal, consistently with results of a study comparing 1-D electrophoretic profiles of saliva proteins and concluding that they were not influenced by gender [14].

In contrast to the two factors “time of sampling” and “gender” which had close to no effect at the group level on saliva proteome profiles, inter-individual variability was quite significant. Such an observation has been repeatedly reported [6,7,14,15], and variability was described as exceeding that of plasma [15]. What the present study additionally documents is the identification and classification of proteins which are discriminating subjects. First, because the majority of identified proteins appeared to be intact proteins, and not fragments, proteolysis was most probably not a predominant explanatory factor in the between-subject variations observed. This is noteworthy given the importance of proteolysis in the oral cavity but it is in agreement with reports of a high consistency of proteolytic profiles generated in whole saliva collected from different healthy subjects [38]. Second, one should note that spots which levels varied between subjects often corresponded to different isoforms of the same protein (e.g. 4 spots identified as α -amylase, 5 spots identified as PIP). It was previously described that many spots separated on 2-D gels correspond to the same accession number, which was explained by extensive *in vivo* post-translational modifications or processing of salivary proteins [3]. It is therefore unlikely that the overall abundance of one protein, which could be evaluated by immunological techniques for example, is correlated to the abundance of specific isoforms. To further support this, Fig. 4 reveals for example that the α -amylase isoforms have contrasted levels of expressions within the group. Thus, spots 256 and 476 are generally less abundant than the group average in subjects 7, 1, 6, 5, 11 and 8 but this trend does not stand for spots 195 and 210. These results illustrate that, when searching for salivary biomarkers, it is necessary to use highly resolutive techniques which allow discriminating between the many isoforms of abundant proteins.

Hierarchical cluster analysis revealed two main groups of spots, suggesting that protein expression in these two large groups is independent, i.e., probably regulated by separate mechanisms. Both clusters contain proteins secreted by salivary glands (e.g. parotid secretory protein and α -amylase), which rules out the hypothesis that protein source (secretion of glands, filtrate of serum, cellular debris etc.) is solely at the origin of protein clustering. Quite strikingly, one cluster was composed of proteins homogenous in function. Thus, cystatins and lipocalin 1 are inhibitors of cysteine proteases [39,40] and in that respect are involved in mucosal defense. Cystatins are also reported to

have antibacterial and antiviral properties [41] and lipocalin 1, PSP and PIP participate to the innate immune response [42]. This cluster of spots therefore corresponds to proteins generally involved in the non-specific protection of the oral cavity. Another common feature of these proteins is to be secreted by salivary glands. In contrast, the second cluster is more heterogeneous: it contains proteins originating from serum and involved in the innate or adaptive immune systems (complement C3, IgG2, transferrin), but also enzymes of the carbohydrates metabolism, namely amylase and enzymes of the glycolytic pathway (GA3PD, phosphoglycerate mutase and enolase 1). Several glycolytic enzymes have previously been identified as constituents of human saliva [6] and of the acquired enamel pellicle [43], but very little is said about their physiological significance in the oral cavity. Decreased levels of enolase 1 were observed in saliva of patients suffering from oral bleeding [10] or in healthy subjects after stimulation by bitterness [7]. In both cases, however, no explanation or hypothesis was provided. Whether glycolytic enzymes remain functional in an extracellular medium is unlikely: glycolysis is a highly integrated pathway relying on equilibrium between enzymes involved in the successive reactions, demanding an initial input of energy and the constant regeneration of co-factors. The presence in saliva of glycolytic enzymes could therefore merely reflect the basal level of glycolysis in mucosal and/or glandular cells of the oral cavity. In pancreatic β -cells, genes coding for glycolytic enzymes were induced by exposure to glucose [44], i.e., in conditions simulating diet-induced glucose elevation. It is plausible that this mechanism also applies to the salivary glands, especially considering the clustering of glycolytic enzymes with amylase. Salivary amylase gene copy number, correlated with expression at the protein level, has thus recently been reported to be higher in populations with traditionally high-starch diets [45]. On a different scale, namely at the individual level, induction of salivary amylase by tannin-enriched diets was observed in mice [46]. We therefore propose that dietary habits may be partly at the origin of the observed inter-individual variability in salivary enzymes.

To summarise, we observed that in the studied population, subjects could be discriminated based on the expression profiles of two clusters of spots. One was composed of secreted proteins involved in the non-specific immune system. In addition, and independently, subjects also presented a specific pattern of proteins involved in the immune response but originating from serum. A last group of spots, variable between subjects but somehow linked to those proteins of serum origin, was made of spots involved in carbohydrates metabolism which may be regulated by dietary habits. On-going research in our laboratory will further document this diet-induced modification of salivary proteins representation.

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