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REVIEW

Top-down platform for deciphering the human salivary proteome

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Proteomic platforms can be classified in bottom-up strategies, which analyze the sample after proteolytic digestion, and top-down strategies, which analyze the intact naturally occurring proteome. Bottom-up platforms are high-throughput because they can investigate a large number of proteins, regardless of their dimension. Nonetheless, information on post-translational modifications (PTMs) can be lost, especially those regarding naturally occurring cleavages and alternative splicing. Top-down platforms cannot cover vast proteomes, however, they can disclose subtle structural variations occurring during protein maturation and allow label-free relative quantifications in an unlimited number of samples. A repertoire of 256 masses belonging to naturally occurring proteins and peptides consistently detected by RP-HPLC-ESI-MS analysis of the acidic soluble fraction of human whole saliva is presented in this study. Of them, 233 have been identified, while 23 are still pending for the definitive characterization. The present review reports average and mono-isotopic masses of the peptides and proteins detected, RP-HPLC elution times, PTMs, origin and quali-quantitative variations observed in several physiological and pathological conditions. The information reported can be a reference for users of top-down RP-HPLC-ESI-MS proteomic platforms applied to the study of the human salivary proteome as well as of other human bodily fluids.

Keywords: α-defensins, β-thymosins, cystatins, histatins, human, proteome, proteomics, proline-rich proteins, saliva, statherin, S100 proteins, top-down

Introduction

Different attempts have been recently addressed towards the characterization of human salivary proteome [1–4]. Our group has been involved in this demanding task for more than twelve years utilizing a "solution based" top-down proteomic platform, focused on the detection and characterization of the intact naturally occurring proteins and peptides soluble in acidic solution by RP-HPLC-ESI-MS (reversed-phase high-performance liquid-chromatography, electrospray ionization, mass spectrometry). The general scheme of this approach is reported in Figure 1. In the first step, the average mass of intact peptides/proteins is determined by ESI-MS with a precision of at least 1:10,000 Da. For the identification purified proteins are also submitted to high resolution MS/MS experiments. Because proteins with mass higher than 3000–4000 Da do not usually provide MS/MS fragmentation spectra suitable for complete *de novo* sequencing, further experiments are required in order to obtain definitive characterization of protein structure, comprising PTMs (post-translational modifications). For this purpose, HPLC partially purified protein are submitted to different chemical and enzymatic treatments, such as removal of phosphate groups by phosphatase, reduction of disulfide bridges and protection of cysteine residues by proper reactants. For proteins without the N-terminal not blocked, automated Edman sequencing allows amino-terminal sequence to be established. Alternatively, the digestion products obtained by different proteolytic enzymes are submitted to different analyses and high resolution MS-MS identifications. Overall, collected data allowed us to hypothesize the protein structure, which was confirmed or rejected by checking the correspondence between the theoretical and the experimental (partial) MS-MS data collected on the intact naturally occurring protein.

As recently reported in the literature [5], top-down platforms nowadays cannot reach the same coverage of bottom-up platforms for different reasons: (i) the intact protein has to be soluble in the acidic solution compatible with the ESI-MS analysis; (ii) the protein should not be heterogeneous (i.e. glycosylated isoforms), because in this case the mass of the intact protein cannot be deduced by the crowded ESI spectrum; (iii) protein dimensions have to be limited because big proteins MS-MS fragmentation spectra are too complex to be interpreted. Nonetheless, top-down strategies could reveal the rich isoform and PTM diversity present in the human body.

In this paper, we report the inventory (Table I) of 256 intact protein and peptide masses detected in whole human saliva: of them, 233 have been identified while 23 are pending for definitive characterization. Together with the RP-HPLC elution time, PTMs, origin and specific quali-quantitative variations observed in several physiological and pathological conditions are reported with a particular concern to the pediatric age. The list can be used as a reference for anyone who wants to carry out RP-HPLC-ESI-MS experiments and to utilize other top-down proteomic platforms for the study of the human salivary proteome.

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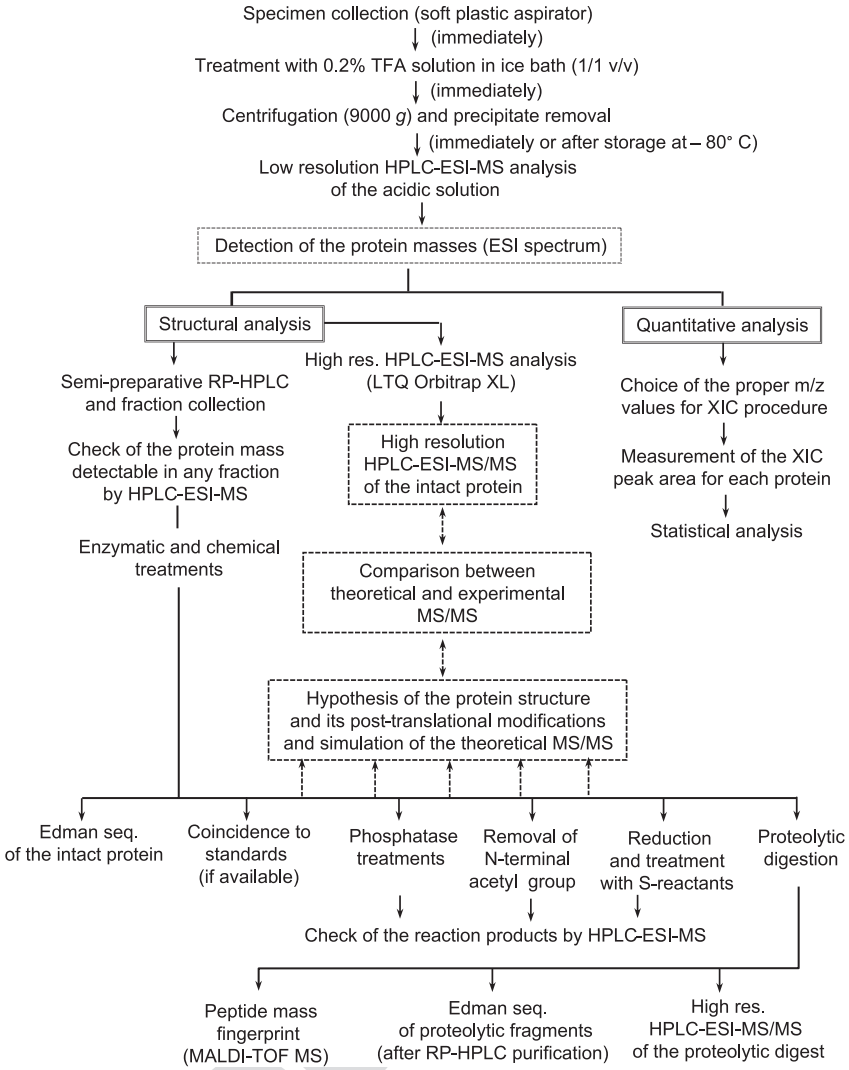

Figure 1. Flowchart of the top-down proteomic platform typically utilized for the identification of the components of whole saliva reported in Table I. (TFA: 2,2,2-trifluoroacetic acid).

Details of the top-down platforms

The basic step of the top-down approach was the determination of the mass of the intact, naturally-occurring protein/peptide with accuracy better than 1:10,000 (Figure 1). This result was typically achieved by RP-HPLC-ESI-MS experiment, utilizing a classical C-8 reversed phase column (varying according to the flow-rate and the apparatus). The chromatographic elution was performed utilizing a gradient of water/acetonitrile with 0.05% 2,2,2-trifluoroacetic acid (TFA) as ion-pairing agent. TFA is recognized as a good ion-pairing HPLC agent. The drawback is that TFA is not a good ionization agent for the electrospray process, as either acetic or formic acid are, leading to a partial suppression of the ESI signal. Moreover, sometimes some salivary protein masses were increased of about 113 Da due to formation of TFA adducts during the ESI process [3]. In our experience, the use of formic acid instead of TFA led to an overall reduction of HPLC-ESI-MS performance. Figure 2 reports typical TIC profiles of salivary samples from subjects of different ages. We have indeed demonstrated that age-dependent deep modifications of the human salivary proteome occur, especially in the pediatric age [6–8]. Figure 2 shows that different classes of salivary proteins were often detectable in well-defined chromatographic clusters. This behavior clearly reflected the similarity of the structures and polarity of the members of the different families of salivary proteins. Typically, during the first 10 min of separation many fragments of bigger proteins/peptides eluted, followed (10–20 min) by histatins, glycosylated (gPRPs) and basic-proline-rich proteins (bPRPs). Usually, the mass of gPRPs could not be established due to the crowded ESI spectra. At around 20 min, β-thymosins was detected, followed by a cluster comprising acidic-PRPs (aPRPs), and immediately after by α-defensins. In the 24–27 min range different derivatives of statherin were detectable, as well as small-proline-rich protein 3 (SPRR3), a protein present at high concentration in preterm newborn saliva. Typically, in the 27–30 min range statherin and P-B peptide eluted, followed by cystatin A and B (and their derivatives), salivary cystatins ("S"-type), different proteins of the S100 family, α-amylase, human serum albumin (showing crowded ESI spectrum) and other miscellaneous proteins.

Relative quantification of proteins and peptides reported in Table I was performed by the extracted ion current (XIC) procedure. Figure 3 shows the XIC procedure applied to the determination of the relative levels of thymosin β_4 ($T\beta_4$) in whole saliva of a preterm newborn (226 days of post-conceptional age) and of an adult. The selection of three specific *m/z* values corresponding to ions with +3/+5 charged allowed to isolate the specific peptide

Table I. List of 256 peptide and protein masses detected in whole saliva by a "solution based" top-down HPLC-ESI-MS platform.

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
1	523.2	Histatin 3 Fr. 29–32	P15516	524.235	523.543	8.3			Both major glands (↑ parotid)	[11,26]
2	556.3	Histatin 3 Fr. 1-5	P15516	557.268	556.576	5.9			Both major glands (↑ parotid)	[11,26]
3	597.3	Histatin 3 Fr. 2-6	P15516	598.342	597.675	5.5			Both major glands (↑ parotid)	[11,26]
4	640.3	Histatin 3 Fr. 7-11	P15516	641.315	640.700	5.6			Both major glands (↑ parotid)	[11,26]
5	686.3	Histatin 3 Fr. 28–32	P15516	687.298	686.719	12.5			Both major glands (↑ parotid)	[11,26]
6	710.4	bPRP fragment	Various	710.383	709.800	6.8		↓ pediatric age	Only parotid	[7,26,34]
7	712.4	Histatin 3 Fr. 1-6	P15516	713.369	712.764	6.1			Both major glands (↑ parotid)	[11,26]
8	720.4	bPRP fragment	Various	720.368	719.795	8.1		↓ pediatric age	Only parotid	[7,26,34]
9	721.4	bPRP fragment	Various	721.352	720.780	7.9		↓ pediatric age	Only parotid	[7,26,34]
10	755.4	Histatin 3 Fr. 19–24	P15516	756.354	755.791	8.7			Both major glands (↑ parotid)	[11,26]
11	796.4	Histatin 3 Fr. 6-11	P15516	797.417	796.887	7.6			Both major glands (↑ parotid)	[11,26]
12	796.4	Histatin 3 Fr. 7-12	P15516	797.417	796.887	7.8			Both major glands (↑ parotid)	[11,26]
13	800.3	Histatin 3 Fr. 27–32	P15516	801.341	800.823	14.1			Both major glands (↑ parotid)	[11,26]
14	817.5	bPRP fragment	Various	817.457	816.956	8.5		↓ pediatric age	Only parotid	[26,34]
15	874.5	bPRP fragment	Various	874.478	874.007	8.2		↓ pediatric age	Only parotid	[7,26,34]
16	887.4	Histatin 3 Fr. 26–32	P15516	888.373	887.901	13.8			Both major glands (↑ parotid)	[11,26]
17	895.4	P-C Fr. 36–44	P02810	895.427	894.940	6.9		↑ type 1 diabetes	Both major glands (↑ parotid)	[47]
18	914.5	bPRP fragment	Various	914.509	914.072	10.2		↓ pediatric age	Only parotid	[26,34]
19	924.5	Histatin 3 Fr. 5-11 (Hst 12)	P15516	925.512	925.061	7.2			Both major glands (↑ parotid)	[9,11,26]
20	924.5	Histatin 3 Fr. 7-13	P15516	925.512	925.061	6.2			Both major glands (↑ parotid)	[11,26]
21	971.5	bPRP fragment	Various	971.531	971.124	9.4		↓ pediatric age	Only parotid	[7,26,34]
22	990.5	P-C Fr. 26–35	P02810	990.512	990.087	8.2		↑ type 1 diabetes	Both major glands (↑ parotid)	[26,34,35,47]
23	1038.4	bPRP fragment	Various	1038.533	1038.128	10.7		↓ pediatric age	Only parotid	[26,34]
24	1067.6	bPRP fragment	Various	1067.559	1067.170	9.7		↓ pediatric age	Only parotid	[7,26,34]
25	1070.6	bPRP fragment	Various	1070.611	1070.260	9.5		↓ pediatric age	Only parotid	[7,26,34]
26	1071.6	bPRP fragment	Various	1071.595	1071.245	9.5		↓ pediatric age	Only parotid	[7,26,34]
27	1076.5	bPRP fragment	Various	1076.512	1076.134	7.0		↓ pediatric age	Only parotid	[7,26,34]
28	1076.5	bPRP fragment	Various	1076.548	1076.177	6.8		↓ pediatric age	Only parotid	[7,26,34]
29	1080.6	Histatin 3 Fr. 5-12 (Hst 11)	P15516	1081.613	1081.249	11.4			Both major glands (↑ parotid)	[9,11,26]
30	1080.6	Histatin 3 Fr. 6-13	P15516	1081.613	1081.249	9.1			Both major glands (↑ parotid)	[11,26]
31	1152.6	bPRP fragment	P02812	1152.576	1152.232	9.1		↓ pediatric age	Only parotid	[26,34]
32	1165.5	bPRP fragment	Various	1165.523	1165.184	7.0		↓ pediatric age	Only parotid	[7,26,34]
33	1200.4	P-B peptide Fr. 46–57	P02814	1200.641	1200.403	21.1			Both major glands	[34]
34	1208.7	Histatin 3 Fr. 5-13	P15516	1209.708	1209.423	9.5			Both major glands (↑ parotid)	[11,26]
35	1222.6	bPRP fragment	Various	1222.617	1222.323	8.2		↓ pediatric age	Only parotid	[7,26,34]
36	1224.6	aPRP Fr. 94–105	P02810	1224.623	1224.345	11.1		↑ type 1 diab.	Both major glands (↑ parotid)	[47]
37	1286.6	Histatin 3 Fr. 15–24	P15516	1287.609	1287.363	9.2			Both major glands (↑ parotid)	[11,26]
38	1334.7	Histatin 3 Fr. 1-11	P15516	1335.667	1335.448	7.8			Both major glands (↑ parotid)	[11,26]

(*Continued*)

Table I. (*Continued*).

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
39	1341.7	bPRP fragment	Various	1341.655	1341.446	10.1		↓ pediatric age	Only parotid	[26,34]
40	1433.7	Histatin 3 Fr. 14–24	P15516	1434.677	1434.540	11.6			Both major glands (↑ parotid)	[11,26]
41	1442.7	Histatin 3 Fr. 15–25	P15516	1443.710	1443.550	9.6			Both major glands (↑ parotid)	[11,26]
42	1471.7	P-C Fr. 1-14	P02810	1471.715	1471.555	8.7		↑ type 1 diabetes	Both major glands (↑ parotid)	[47]
43	1490.8	Histatin 3 Fr. 1-12	P15516	1491.768	1491.635	8.5			Both major glands (↑ parotid)	[11,26]
44	1513.6	bPRP fragment	P10163	1513.798	1513.679	8.6		↓ pediatric age	Only parotid	[26,34]
45	1561.8	Histatin 3 Fr. 13–24 (Hst 8)	P15516	1562.772	1562.714	11.5			Both major glands (↑ parotid)	[9,11,26]
46	1589.8	Histatin 3 Fr. 14–25	P15516	1590.779	1590.727	11.0			Both major glands (↑ parotid)	[11,26]
47	1618.9	Histatin 3 Fr. 1-13	P15516	1619.863	1619.809	8.4			Both major glands (↑ parotid)	[11,26]
48	1717.9	Histatin 3 Fr. 12–24 (Hst 7)	P15516	1718.873	1718.901	11.8			Both major glands (↑ parotid)	[9,11,26]
49	1717.9	Histatin 3 Fr. 13–25 (Hst 10)	P15516	1718.873	1718.901	11.8			Both major glands (↑ parotid)	[9,11,26]
50	1767.9	bPRP fragment	Various	1767.914	1767.962	9.8		↓ pediatric age	Only parotid	[7,26,34]
51	1818.0	aPRP Fr. 94–110	P02810	1818.958	1819.019	11.1		↑ type 1 diabetes	Both major glands (↑ parotid)	[47]
52	1856.9	bPRP fragment	P02812	1856.889	1856.969	10.5		↓ pediatric age	Only parotid	[26,34]
53	1867.0	P-C Fr. 26–44	P02810	1866.921	1867.011	9.5		↑ type 1 diabetes	Both major glands (↑ parotid)	[47]
54	1874.0	Histatin 3 Fr. 12–25 (Hst 9)	P15516	1874.975	1875.089	11.2			Both major glands (↑ parotid)	[9,11,26]
55	1932.2	bPRP fragment	Various	1932.009	1932.169	10.7		↓ pediatric age	Only parotid	[7,26,34]
56	2017.2	bPRP fragment	Various	2017.025	2017.232	11.5		↓ pediatric age	Only parotid	[7,26,34,35]
57	2029.2	bPRP fragment	Various	2029.025	2029.243	11.3		↓ pediatric age	Only parotid	[7,26,34,35]
58	2083.3	P-C Fr. 5-25	P02810	2083.047	2083.294	11.3		↑ type 1 diabetes	Both major glands (↑ parotid)	[35,47]
59	2521.8	P-C Fr. 1-25	P02810	2521.281	2521.781	11.8		↑ type 1 diabetes	Both major glands (↑ parotid)	[35,47]
60	2745.0	Histatin 3 Fr. 12–32 (Hst 4)	P15516	2744.330	2744.974	13.8			Both major glands (↑ parotid)	[9,11,26]
61	2917.2	P-C Fr. 15–44	P02810	2916.486	2917.236	12.6		↑ type 1 diabetes	Both major glands (↑ parotid)	[47]
62	3036.3	Histatin 3 Fr. 1-24 (Hst 5)	P15516	3035.522	3036.334	14.6			Both major glands (↑ parotid)	[9,11,26]
63	3192.5	Histatin 3 Fr. 1-25 (Hst 6)	P15516	3191.623	3192.521	14.3			Both major glands (↑ parotid)	[9,11,26]
64	3371.0	α-defensin 2	P59665/6	3369.482	3370.966	23.5	3 S-S	↓ edentulous	GCF	[30,50]
65	3442.0	α-defensin 1	P59665	3440.519	3442.045	23.5	3 S-S	↓ edentulous	GCF	[30,50]
66	3472.9	Peroxiredoxin 6 Fr. 1-32	P30041	3471.744	3472.864	31.4		↑ pre-term	?	[8]
67	3486.1	α-defensin 3	P59666	3484.509	3486.055	23.5	3 S-S	↓ edentulous	GCF	[30,50]
68	3645.0	Statherin Des_{1-13}	P02808	3643.685	3645.001	27.5			Both major glands	[24,26]
69	3707.8	α-defensin 4	P12838	3707.767	3709.414	27.2	3 S-S	↓ edentulous	GCF	[30,50]
70	3971.4	Statherin Des_{1-10}	P02808	3969.891	3971.399	28.0			Both major glands	[24,26]
71	4062.4	Histatin 3	P15516	4060.979	4062.407	17.7			Both major glands	[9,11,26]
72	4114.8	G3P dehydrogenase Fr. 1-39	P04406	4113.308	4114.807	33.2		↑ pre-term	?	[8]
73	4127.6	Statherin Des_{1-9}	P02808	4125.992	4127.587	28.5			Both major glands	[24,26]
74	4145.5	P-C peptide Des PQ_{43-44}	P02810	4144.072	4145.529	14.9			Both major glands (↑ parotid)	[20,26]
75	4242.6	P-C peptide Des Q_{44}	P02810	4241.125	4242.646	14.9			Both major glands (↑ parotid)	[20,26]
76	4370.8	P-C peptide	P02810	4369.183	4370.776	15.0			Both major glands (↑ parotid)	[20,26]
77	4392.1	G3P dehydrogenase Fr. 1-41	P04406	4390.415	4392.087	29.9		↑ pre-term	?	[8]

(*Continued*)

Table I. (*Continued*).

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
78	4549.3	P-B peptide Des_{1-12}	P02814	4547.411	4549.343	27.5			Both major glands (↑ Sm/Sl)	[24,26]
79	4848.2	Histatin 1 non phosph.	P15515	4846.233	4848.172	22.0		↓ ASD	Both major glands	[9,26,46]
80	4928.2	Histatin 1	P15515	4926.200	4928.151	21.9	Phosph. (S_2)		Both major glands	[9,11,26]
81	4936.5	Thymosin β10	P63313	4934.530	4936.523	20.8	Acetyl (N-term)	↑ pre-term	GCF, both glands (in pre-term)	[8,32,40]
82	4946.5	Thymosin β4 (cyclo)	P62328	4944.467	4946.472	19.6		↑ pre-term	GCF, both glands (in pre-term)	[8,32,40]
83	4963.5	Thymosin β4	P62328	4961.494	4963.502	18.5	Acetyl (N-term)	↑ pre-term	GCF, both glands (in pre-term)	[8,32,40]
84	4979.5	Thymosin β4 sulfoxide	P62328	4977.488	4979.502	18.3	Acetyl (N-term), (Met_6) sulfoxide	↑ pre-term	GCF, both glands (in pre-term)	[8,32,40]
85	5008.2	Histatin 1 mono-sulfated	P15515	5006.156	5008.216	21.9	Phosph. (S_2), mono-sulf (Y_{27})		Only Sm/Sl	[12]
86	5060.9	P-B peptide Des_{1-7}	P02814	5058.654	5060.921	30.1			Both major glands (↑ Sm/Sl)	[24,26]
87	5088.3	Histatin 1 di-sulfated	P15515	5086.113	5088.280	21.4	Phosph. (S_2), di-sulf (Y_{27}, Y_{30})		Only Sm/Sl	[12]
88	5131.4	Statherin (di-phosph) Des $T_{42}F_{43}$	P02808	5129.334	5131.439	27.9	Di-phosph (S_2, S_3) Des $T_{42}F_{43}$		Both major glands	[24,26]
89	5152.6	Statherin mono-phosph Des F_{43}	P02808	5150.415	5152.564	27.5	Mono-phosph (S_2 or S_3)		Both major glands	[24,26]
90	5168.3	Histatin 1 tri-sulfated	P15515	5166.070	5168.344	20.8	Phosph. (S_2), tri-sulf (Y_{27}, Y_{30}, Y_{34})		Only Sm/Sl	[12]
91	5215.1	P-B peptide Des_{1-5}	P02814	5212.728	5215.090	30.3			Both major glands (↑ Sm/Sl)	[24,26]
92	5219.8	Statherin non-phosph	P02808	5217.517	5219.761	28.6			Both major glands	[24,26]
93	5232.5	Statherin (di-phosph) Des F_{43} (SV1)	P02808	5230.381	5232.544	27.8	Di-phosph (S_2, S_3) Des F_{43}		Both major glands	[24,26]
94	5248.4	Histatin 1 tetra-sulfated	P15515	5246.027	5248.408	20.5	Phosph. (S_2), tetra-sulf (Y_{27}, Y_{30}, Y_{34}, Y_{38})		Only Sm/Sl	[12]
95	5264.6	Statherin (di-phosph) Des D_1	P02808	5262.423	5264.632	28.7	Di-phosph (S_2, S_3) Des D_1		Both major glands	[24,26]
96	5299.7	Statherin mono-phosph	P02808	5297.483	5299.740	28.9	Mono-phosph (S_2 or S_3)	↑ ASD	Both major glands	[24,26,46]
97	5357.0	Cystatin B Fr. 54–98	P04080	5354.738	5357.030	29.5		↑ pre-term	?	[8]
98	5362.7	Cyclostatherin Q-37	P02808	5360.423	5362.690	29.6	Di-phosph (S_2, S_3) – cyclization K_6 – Q_{37}		Both major glands	[25,26]
99	5371.3	P-B peptide Des_{1-4}	P02814	5368.829	5371.277	30.0			Both major glands (↑ Sm/Sl)	[24,26]
100	5379.7	Statherin	P02808	5377.450	5379.720	29.2	Di-phosph (S_2, S_3)		Both major glands	[21,24,26]
101	5590.1	P-H (IB-4 – bPRP)	P02812	5587.783	5590.096	15.2		↓ pediatric age	Only parotid	[7,13,15,16]
102	5685	Non iden. (prob. bPRP)				15.0	Unknown bPRP	↓ pediatric age	Only parotid	[16]
103	5792.7	P-B peptide	P02814	5790.036	5792.734	30.0	Pyroglu (Q)		Both major glands (↑ Sm/Sl)	[24–26]
104	5842.5	P-F (IB-8c – bPRP)	P02812	5839.992	5842.493	14.7		↓ pediatric age	Only parotid	[7,13,16]
105	5842.6	Cystatin B Fr. 1-53	P04080	5839.888	5842.616	29.7	Acetyl (N-term)	↑ pre-term	?	[8]
106	5867.5	P-E Des-R_{61} (IB-9 – bPRP)	P02811	5864.987	5867.503	14.9		↓ pediatric age	Only parotid	[7,13,14,16]
107	5943.6	P-J (bPRP)		5941.003	5943.555	14.5		↓ pediatric age	Only parotid	[7,16]
108	5961.8	Cystatin B Fr. 1-53 S-cysteinyl	P04080	5958.892	5961.755	29.8	Acetyl (N-term), cysteinyl (C3)	↑ pre-term	?	
109	6023.7	P-E (IB-9 – bPRP)	P02811	6021.088	6023.690	14.9		↓ pediatric age	Only parotid	[7,13,14,16]
110	6147.9	Cystatin B Fr. 1-53 S-glutathion.	P04080	6144.956	6147.922	29.7	Acetyl (N-term), gluthationyl (C_3)	↑ pre-term	?	
111	6923.7	P-D (IB-5 – bPRP) (P_{32}→A)	P10163	6920.538	6923.692	15.9	P-D (P_{32} → A)	↓ pediatric age	Only parotid	[7,13,14,16]

(*Continued*)

Table I. (*Continued*).

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
112	6949.7	P-D (IB-5 – bPRP)	P10163	6946.554	6949.730	15.9		↓ pediatric age	Only parotid	[7,13,14,16]
113	7452.0	II-2 Des R_{75} (bPRP)	P04280	7466.621	7452.007	19.2	Phosph. (S_8), pyroglu (E) (Seq. from Ref. [3])	↓ pediatric age	Only parotid	[7,13,16]
114	7528.2	II-2 non phosph. (bPRP)	P04280	7524.746	7528.214	19.8	Pyroglu (E) (Seq. from Ref. [3])	↓ pediatric age	Only parotid	[7,13,16]
115	7608.2	II-2 (bPRP)	P04280	7604.712	7608.194	19.2	Phosph. (S_8), pyroglu (E) (Seq. from Ref. [3])	↓ pediatric age	Only parotid	[7,13,16]
116	7806	Non iden.				21.0		↑ pre-term	?	[8]
117	7867	Non iden.				21.0		↑ pre-term	?	[8]
118	9437.2	IB-1 Des R_{96} (bPRP)	P02812	9432.602	9437.196	19.4	Phosph. (S_8), pyroglu (Q)	↓ pediatric age	Only parotid	[7,13,16]
119	9513.4	IB-1 non-phosph. (bPRP)	P02812	9508.737	9513.404	19.7	Pyroglu (Q)	↓ pediatric age	Only parotid	[7,13,16]
120	9593.3	IB-1 (bPRP)	P02812	9588.703	9593.384	19.4	Phosph. (S_8), pyroglu (Q)	↓ pediatric age	Only parotid	[7,13,16]
121	9956	Non iden.				32.0		↑ pre-term	?	[8]
122	10434	Non iden. (prob. bPRP)				16.0	Unknown bPRP	↓ pediatric age	Only parotid	[16,17]
123	10444	S100A12 (calgran. C)	P80511	10438.494	10443.847	40.0	M missing (N-term)	↑ pre-term	?	[8]
124	10651	Non iden.				33.2		↑ pre-term	?	[8]
125	10765	Non iden.				31.6		↑ pre-term	?	[8]
126	10834	S100A8 (calgran. A)	P05109	10828.656	10834.511	40.4		↑ pre-term	?	[8]
127	10872	Non iden.				32.5	Cystatin A M missing (pending for characterization)	↑ pre-term	?	[8]
128	10925	aPRP (PIF-f) mono-phosph Des R_{106}	P02810	10920.008	10925.383	23.4	Pyroglu (Q), mono-phosph (S_8 or S_{22})		Both major glands (↑ parotid)	[20,26]
129	10925	aPRP (PRP-3) mono-phosph Des R_{106}	P02810	10920.008	10925.383	23.4	Pyroglu (Q), mono-phosph (S_8 or S_{22})		Both major glands (↑ parotid)	[20,26]
130	10926	aPRP (PRP-4) mono-phosph Des R_{106}	P02810	10920.992	10926.368	23.4	Pyroglu (Q), mono-phosph (S_8 or S_{22})		Both major glands (↑ parotid)	[20,26]
131	11001	aPRP (PIF-f) non-phosph	P02810	10996.142	11001.590	23.8	Pyroglu (Q)	↑ preterm	Both major glands (↑ parotid)	[6,20,26]
132	11001	aPRP (PRP-3) non-phosph	P02810	10996.142	11001.590	23.8	Pyroglu (Q)	↑ preterm	Both major glands (↑ parotid)	[6,20,26]
133	11002	aPRP (PRP-4) non-phosph	P02810	10997.126	11002.575	23.8	Pyroglu (Q)	↑ preterm	Both major glands (↑ parotid)	[6,20,26]
134	11005	aPRP (PIF-f) di-phosph Des R_{106}	P02810	10999.974	11005.363	22.8	Pyroglu (Q), di-phosph (S_8, S_{22})		Both major glands (↑ parotid)	[20,26]
135	11005	aPRP (PRP-3) di-phosph Des R_{106}	P02810	10999.974	11005.363	22.8	Pyroglu (Q), di-phosph (S_8, S_{22})		Both major glands (↑ parotid)	[20,26]
136	11006	aPRP (PRP-4) di-phosph Des R_{106}	P02810	11000.958	11006.347	22.8	Pyroglu (Q), di-phosph (S_8, S_{22})		Both major glands (↑ parotid)	[20,26]
137	11006	Cystatin A	P01040	11000.670	11006.493	31.8		↑ pre-term	?	[8,27,30]
138	11049	Cystatin A acetyl (N-term)	P01040	11042.680	11048.533	33.0	Acetyl (N-term)	↑ pre-term	?	[8,27]
139	11081	aPRP (PIF-f) mono-phosph	P02810	11076.109	11081.570	23.4	Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	Both major glands (↑ parotid)	[6,20,26,46]
140	11081	aPRP (PRP-3) mono-phosph	P02810	11076.109	11081.570	23.4	Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	Both major glands (↑ parotid)	[6,20,26,46]
141	11082	aPRP (PRP-4) mono-phosph	P02810	11077.093	11082.555	23.4	Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	Both major glands (↑ parotid)	[6,20,26,46]

(*Continued*)

Table I. (*Continued*).

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
142	11161	aPRP (PIF-f) di-phosph	P02810	11156.075	11161.550	22.8	Pyroglu (Q), di-phosph ($S_{8,}$ S_{22})	↓ preterm	Both major glands (↑ parotid)	[6,20,26]
143	11161	aPRP (PRP-3) di-phosph	P02810	11156.075	11161.550	22.8	Pyroglu (Q), di-phosph ($S_{8,}$ S_{22})	↓ preterm	Both major glands (↑ parotid)	[6,20,26]
144	11162	aPRP (PRP-4) di-phosph	P02810	11157.059	11162.535	22.8	Pyroglu (Q), di-phosph ($S_{8,}$ S_{22})	↓ preterm	Both major glands (↑ parotid)	[6,20,26]
145	11182	Cystatin B	P04080	11175.609	11181.631	33.0	Acetyl (N-term)	↑ pre-term	?	[8]
146	11241	aPRP (PIF-f) tri-phosph	P02810	11236.041	11241.530	22.4	Pyroglu (Q), tri-phosph ($S_{8,}$ S_{17}, S_{22})		Both major glands (↑ parotid)	[20,26]
147	11241	aPRP (PRP-3) tri-phosph	P02810	11236.041	11241.530	22.4	Pyroglu (Q), tri-phosph ($S_{8,}$ S_{17}, S_{22})		Both major glands (↑ parotid)	[20,26]
148	11242	aPRP (PRP-4) tri-phosph	P02810	11237.025	11242.515	22.4	Pyroglu (Q), tri-phosph ($S_{8,}$ S_{17}, S_{22})		Both major glands (↑ parotid)	[20,26]
149	11301	Cystatin B S-cysteinyl	P04080	11294.613	11300.769	32.9	Acetyl (N-term), cysteinyl (C_3)	↑ pre-term	?	[8,28]
150	11368	S100A7 (psoriasin D_{27})	P31151	11361.526	11367.798	37.0	Acetyl, M missing (N-term) $E_{27} \rightarrow D$	↑ pre-term	?	[8]
151	11382	S100A7 (psoriasin E_{27})	P31152	11375.542	11381.824	36.9	Acetyl, M missing (N-term)	↑ pre-term	?	[8]
152	11487	Cystatin B S-glutathionyl	P04080	11480.677	11486.936	32.8	Acetyl (N-term), gluthationyl (C_3)	↑ pre-term	?	[8,28]
153	11517	IB-6 (bPRP)	P04280	11510.799	11516.666	16.7	Subst $A_{63} \rightarrow S$ respect to P04280 (as rep. in 15)	↓ pediatric age	Only parotid	[13,15,16]
154	11652	S100A11 (calgizzarin)	P31949	11644.802	11651.292	42.8	Acetyl, M missing (N-term)	↑ pre-term	?	[8]
155	11710	Antileukoproteinase	P03973	11702.362	11709.804	26.2	8 S-S	↑ pre-term	?	[8]
156	11886	IB-8a Con1+ (bPRP)	P02812	11880.014	11886.120	15.6	Mistake of sequence in Swiss-Prot		Only parotid	[16,19]
157	11896	IB-8a Con1- (bPRP)	P02812	11890.035	11896.163	15.6	Mistake of sequence in Swiss-Prot	↓ pediatric age	only parotid	[16,19]
158	12689	S100A9 (calgran. B) short	P06702	12682.293	12689.228	42.2	Acetyl, MTCKM miss. (N-term)	↑ pre-term	?	[8]
159	12705	S100A9 short mono-ox	P06702	12698.288	12705.228	42.0	Acetyl, MTCKM miss. (N-term), Met_{89} sulfox.		?	
160	12769	S100A9 (calgran. B) short phosph.	P06702	12762.259	12769.208	42.2	Acetyl, MTCKM miss. (N-term), phosph (T_{108})	↑ pre-term	?	[8]
161	12785	S100A9 short phosph. mono-ox	P06702	12778.254	12785.208	42.0	Acetyl, MTCKM miss. (N-term), phosph (T_{108}), Met_{89} sulfox.		?	
162	13044	aPRP (Db-f) mono-phosph Des R_{106}	P02810	13037.048	13043.679	23.9	Pyroglu (Q), mono-phosph (S_8 or S_{22})		Both major glands (↑ parotid)	[20,26]
163	13120	aPRP (Db-f) non-phosph	P02810	13113.183	13119.886	24.1	Pyroglu (Q)		Both major glands (↑ parotid)	[20,26]
164	13124	aPRP (Db-f) di-phosph Des R_{106}	P02810	13117.014	13123.659	23.3	Pyroglu (Q), di-phosph ($S_{8,}$ S_{22})		Both major glands (↑ parotid)	[20,26]
165	13153	S100A9 (calgran. B) long	P06702	13145.485	13152.839	41.9	Acetyl, M missing (N-term)	↑ pre-term	?	[8]
166	13200	aPRP (Db-f) mono-phosph	P02810	13193.149	13199.866	23.9	Pyroglu (Q), mono-phosph (S_8 or S_{22})		Both major glands (↑ parotid)	[20,26]
167	13233	S100A9 (calgran. B) long phosph	P06702	13225.452	13232.819	41.9	Acetyl, M missing (N-term), phosph (T_{112})	↑ pre-term	?	[8]
168	13272	S100A9 (calgran. B) long cyst	P06702	13264.490	13271.977	41.6	Acetyl, M missing (N-term), cysteinyl (C_2)	↑ pre-term	?	[8]

(*Continued*)

Table I. (*Continued*).

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
169	13280	aPRP (Db-f) di-phosph	P02810	13273.115	13279.846	23.3	Pyroglu (Q), di-phosph ($S_{8,}$ S_{22})		Both major glands (↑ parotid)	[20,26]
170	13291	Ib8a (Con1+) glycosyl.	P02812	13283.521	13290	15.6	Glycosyl. (N_{98}) (see n.156–157) Fuc1Gal1Man3GlcNAc3		Only parotid	[19]
171	13343	Cystatin C (γ trace)	P01034	13335.576	13343.108	35.1	(2 S-S)		Sm/Sl (traces in parotid)	[26,27]
172	13352	S100A9 (calg. B) long cyst phosph.	P06702	13344.456	13351.957	41.6	Acetyl, M missing (N-term), cysteinyl (C_2), phosph (T_{112})	↑ pre-term	?	[8]
173	13360	aPRP (Db-f) tri-phosph	P02810	13353.081	13359.826	23.0	Pyroglu (Q), tri-phosph ($S_{8,}$ S_{17}, S_{22})		Both major glands (↑ parotid)	[20,26]
174	13458	S100A9 (calg. B) long glut	P06702	13450.553	13458.145	41.5	Acetyl, M missing (N-term), glutathionyl (C_2)	↑ pre-term	?	[8]
175	13538	S100A9 (calg. B) long glut phosph	P06702	13530.520	13538.124	41.5	Acetyl, M missing (N-term), glutathionyl (C_2), phosph (T_{112})	↑ pre-term	?	[8]
176	13656	Ib8a(Con-1+) glycosyl.	P02812	13648.653	13655.755	15.6	Glycosyl. (N_{98}) (see n.156–157) Fuc1Gal2Man3GlcNAc4		Only parotid	[19]
177	13780	Non iden.				39.3		↑ pre-term	?	[8]
178	13802	Ib8a(Con-1+) glycosyl.	P02812	13794.711	13801.898	15.6	Glycosyl. (N_{98}) (see n.156–157) Fuc2Gal2Man3GlcNAc4		Only parotid	[19]
179	13948	Ib8a(Con-1+) glycosyl.	P02812	13940.769	13948.041	15.6	Glycosyl. (N_{98}) (see n.156–157) Fuc3Gal2Man3GlcNAc4		Only parotid	[19]
180	14094	Ib8a(Con-1+) glycosyl.	P02812	14086.827	14094.184	15.6	Glycosyl. (N_{98}) (see n.156–157) Fuc4Gal2Man3GlcNAc4		Only parotid	[19]
181	14185	Cystatin S	P01036	14176.808	14184.725	35.3	2 S-S		Sm/Sl (traces in parotid)	[26,27]
182	14240	Ib8a(Con-1+) glycosyl.	P02812	14232.885	14240.327	15.6	Glycosyl. (N_{98}) (see n.156–157) Fuc5Gal2Man3GlcNAc4		Only parotid	[19]
183	14265	Cystatin S1	P01036	14256.774	14264.705	35.3	Phosph (S_3), (2 S-S)		Sm/Sl (traces in parotid)	[26,27]
184	14281	Cystatin S1 mono-ox.	P01036	14272.769	14280.704	35.2	Phosph (S_3), oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]
185	14297	Cystatin S1 di-ox.	P01036	14288.764	14296.703	35.2	Phosph (S_3), oxidation (2 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]
186	14312	Cystatin SN	P01037	14304.094	14312.038	34.6	(2 S-S)		Sm/Sl (traces in parotid)	[26,27]
187	14328	Cystatin SN mono-ox.	P01037	14320.089	14328.037	33.9	Oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[26,27]
188	14344	Cystatin SN di-ox.	P01037	14336.084	14344.036	33.7	Oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]
189	14345	Cystatin S2	P01036	14336.740	14344.684	35.3	Di-phosph (S_1,S_3), (2 S-S)		Sm/Sl (traces in parotid)	[26,27]
190	14346	Cystatin SA	P09228	14338.008	14346.018	36.8	2 S-S		Sm/Sl (traces in parotid)	[26,27]
191	14361	Cystatin S2 mono-ox.	P01036	14352.735	14360.684	35.2	Di-phosph (S_1,S_3), oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]
192	14362	Cystatin SA mono-ox	P09228	14354.003	14362.018	36.6	Oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]
193	14377	Cystatin S2 di-ox.	P01036	14368.730	14376.683	35.2	Di-phosph (S_1,S_3), oxidation (2 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]
194	14424	Cystatin SN TFA adduct	P01037	14416.072	14424.046	34.6	(2 S-S) – artifact	Artifact	Artifact	[3]

(*Continued*)

Table I. (*Continued*).

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
195	14536	Cystatin SN 2TFA adduct	P01037	14528.050	14536.055	34.6	(2 S-S) – artifact	Artifact	Artifact	[3]
196	14693	Lysozyme	P61626	14684.097	14692.604	35.6	(4 S-S)	↑ pre-term	?	[8]
197	14990	Non iden.				35.4		↑ pre-term	?	[8]
198	15079	Non iden.				34.6		↑ pre-term	?	[8]
199	15127	α-globin	P69905	15117.892	15126.853	43.0			?	
200	15354	aPRP (PIF-s) non-phosph	P02810	15346.308	15354.351	23.2	Pyroglu (Q)	↑ preterm	Both major glands (↑ parotid)	[7,20,26]
201	15354	aPRP (PRP-1) non-phosph	P02810	15346.308	15354.351	23.2	Pyroglu (Q)	↑ preterm	Both major glands (↑ parotid)	[7,20,26]
202	15355	aPRP (PRP-2) non-phosph	P02810	15347.292	15355.336	23.2	Pyroglu (Q)	↑ preterm	Both major glands (↑ parotid)	[7,20,26]
203	15381	aPRP (Pa 1-mer) mono-phosph	P02810	15373.182	15381.282	23.3	Pyroglu (Q), mono-phosph (S_8 or S_{22})		Enriched granules preparations	[20,26]
204	15434	aPRP (PIF-s) mono-phosph	P02810	15426.274	15434.331	22.9	Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	Both major glands (↑ parotid)	[7,20,26,46]
205	15434	aPRP (PRP-1) mono-phosph	P02810	15426.274	15434.331	22.9	Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	Both major glands (↑ parotid)	[7,20,26,46]
206	15435	aPRP (PRP-2) mono-phosph	P02810	15427.258	15435.316	22.9	Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	Both major glands (↑ parotid)	[7,20,26,46]
207	15461	aPRP (Pa 1-mer) di-phosph	P02810	15453.148	15461.262	23.0	Pyroglu (Q), di-phosph (S_8, S_{22})		Enriched granules preparations	[26]
208	15514	aPRP (PIF-s) di-phosph	P02810	15506.240	15514.311	22.2	Pyroglu (Q), di-phosph (S_8, S_{22})	↓ preterm	Both major glands (↑ parotid)	[7,20,26]
209	15514	aPRP (PRP-1) di-phosph	P02810	15506.240	15514.311	22.2	Pyroglu (Q), di-phosph (S_8, S_{22})	↓ preterm	Both major glands (↑ parotid)	[7,20,26]
210	15515	aPRP (PRP-2) di-phosph	P02810	15507.224	15515.296	22.2	Pyroglu (Q), di-phosph (S_8, S_{22})	↓ preterm	Both major glands (↑ parotid)	[7,20,26]
211	15541	aPRP (Pa 1-mer) tri-phosph	P02810	15533.115	15541.242	22.7	Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		Enriched granules preparations	[26]
212	15546	aPRP (PRP-1 type) mono-phos TFA adduct	P02810	15538.252	15546.340	23.9	Pyroglu (Q), mono-phosph (S_8 or S_{22}) – artifact	Artifact	Artifact	[3]
213	15594	aPRP (PIF-s) tri-phosph	P02810	15586.207	15594.291	21.6	Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		Both major glands (↑ parotid)	[20,26]
214	15594	aPRP (PRP-1) tri-phosph	P02810	15586.207	15594.291	21.6	Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		Both major glands (↑ parotid)	[20,26]
215	15595	aPRP (PRP-2) tri-phosph	P02810	15587.191	15595.276	21.6	Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		Both major glands (↑ parotid)	[20,26]
216	15626	aPRP (PRP-1 type) di-phos TFA adduct	P02810	15618.218	15626.320	23.3	Pyroglu (Q), di-phosph (S_8, S_{22}) – artifact	Artifact	Artifact	[3]
217	15867	β-globin	P68871	15858.257	15867.216	42.5			?	[8]
218	15995	γ-globin (G-γ)	P69892	15986.262	15995.248	43.7			?	[8]
219	16001	Non iden.				27.4		↑ pre-term	?	[8]
220	16009	γ-globin (A-γ)	P69891	16000.278	16009.275	43.9			?	[8]
221	17239	SPRR3 (cornifin β) 17 kDa	A5YKK8	17228.798	17238.816	27.4	Acetyl, M missing (N-term) (S-S var)	↑ pre-term	?	[8,33]
222	17358	SPRR3 17 kDa mono-cyst.	A5YKK8	17347.802	17357.955	27.8	Acetyl, M missing (N-term), mono-cyisteinyl (S-S var.)	↑ pre-term	?	[8,33]
223	17473	aPRP (Db-s) non-phosph	P02810	17463.348	17472.647	23.8	Pyroglu (Q)		Both major glands (↑ parotid)	[20,26]

(*Continued*)

Table I. (*Continued*).

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
224	17544	SPRR3 17 kDa mono-glut	A5YKK8	17533.866	17544.122	27.8	Acetyl, M missing (N-term), mono-glutathionyl (S-S var.)	↑ pre-term	?	[8,33]
225	17553	aPRP (Db-s) mono-phosph	P02810	17543.314	17552.627	23.4	Pyroglu (Q), mono-phosph (S_8 or S_{22})		Both major glands (↑ parotid)	[20,26]
226	17633	aPRP (Db-s) di-phosph	P02810	17623.281	17632.607	22.9	Pyroglu (Q), di-phosph (S_8, S_{22})		Both major glands (↑ parotid)	[20,26]
227	17713	aPRP (Db-s) tri-phosph	P02810	17703.247	17712.587	22.7	Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		Both major glands (↑ parotid)	[20,26]
228	17803	Non iden.				36.4		↑ pre-term	?	[8]
229	18065	SPRR3 (cornifin β) 18 kDa	Q9UBC9	18054.203	18064.795	27.6	Acetyl, M missing (N-term) (S-S var.)	↑ pre-term	?	[8,33]
230	18184	SPRR3 18 kDa mono-cyst	Q9UBC9	18173.207	18183.933	28.0	Acetyl, M missing (N-term), mono-cysteinyl (S-S var.)	↑ pre-term	?	[8,33]
231	18303	SPRR3 18 kDa di-cyst	Q9UBC9	18292.211	18303.071	28.0	Acetyl, M missing (N-term), di-cysteinyl (S-S var.)	↑ pre-term	?	[8,33]
232	18370	SPRR3 18 kDa mono-glut	Q9UBC9	18359.271	18370.100	28.0	Acetyl, M missing (N-term), mono-gluthationyl (S-S var.)	↑ pre-term	?	[8,33]
233	18420	Non iden.				36.2		↑ pre-term	?	[8]
234	18675	SPRR3 18 kDa di-glut	Q9UBC9	18664.339	18675.406	28.1	Acetyl, M missing (N-term), di-glutathionyl (S-S var.)	↑ pre-term	?	[8,33]
235	20206	Non iden.				37.9		↑ pre-term	?	[8]
236	20930	Non iden.				33.6		↑ pre-term	?	[8]
237	22361	Cystatin B S-S dimer	P04080	22349.202	22361.246	34.3	S-S dimer (Cys_3) acetyl N-term		2	[28]
238	22365	Histone H1c	P16402	?	?	34.4	Disagreement between exp. and theor. Mass	↑ pre-term	?	[8]
239	22698	Non iden.				36.9		↑ pre-term	?	[8]
240	22778	Non iden.				36.9	Phosph isoform of 22698?	↑ pre-term	?	[8]
241	23462	Non iden. (prob. bPRP)				17.6	Unknown bPRP (Ps1?)	↓ pediatric age	Only parotid	[7,16,17]
242	24652	Non iden.				44.8		↑ pre-term	?	[8]
243	24904	Non iden.				40.0	Peroxiredoxin 6? (pending for charact.)	↑ pre-term	?	[8]
244	27050	Non iden.				26.7		↑ pre-term	?	[8]
245	29412	Non iden. (prob. bPRP)				16.8	Unknown bPRP (Ps2?)	↓ pediatric age	Only parotid	[7,16,17]
246	30712	aPRP (Pa 2-mer) tri-phosph Des Q_{150}	P02810	30696.256	30712.398	24.0	Pyroglu (Q), di-phosph (S_8, S_{22}) + mono-phos (S_8 or S_{22})		Both major glands (↑ parotid)	[20,26]
247	30761	aPRP (Pa 2-mer) di-phosph	P02810	30744.349	30760.549	24.5	Pyroglu (Q), di-phosph 2 × (S_8 or S_{22})		Both major glands (↑ parotid)	[20,26]
248	30792	aPRP (Pa 2-mer) tetra-phosph Des Q_{150}	P02810	30776.223	30792.378	23.6	Pyroglu (Q), tetra-phosph 2 × (S_8, S_{22})		Both major glands (↑ parotid)	[20,26]
249	30841	aPRP (Pa 2-mer) tri-phosph	P02810	30824.315	30840.529	24.0	Pyroglu (Q), di-phosph (S_8, S_{22}) + mono-phos (S_8 or S_{22})		Both major glands (↑ parotid)	[20,26]
250	30921	aPRP (Pa 2-mer) tetra-phosph	P02810	30904.281	30920.509	23.6	Pyroglu (Q), tetra-phosph 2 × (S_8, S_{22})		Both major glands (↑ parotid)	[20,26]

(*Continued*)

Table I. (*Continued*).

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
251	31000	aPRP (Pa 2-mer) penta-phosph	P02810	30984.247	31000.489	23.2	Pyroglu (Q), di-phosph (S_8, S_{22}) + tri-phos (S_8, S_{17}, S_{22})		Both major glands (↑ parotid)	[20,26]
252	31032	aPRP (Pa 2-mer) tetra-phosph TFA add	P02810	31016.259	31032.517	23.6	Pyroglu (Q), tetra-phosph 2 × (S_8, S_{22}) – artifact	Artifact	Artifact	[3]
253	31144	aPRP (Pa 2-mer) tetra-phosph 2 TFA add	P02810	31128.237	31144.526	23.6	Pyroglu (Q), tetra-phosph 2 × (S_8, S_{22}) – artifact	Artifact	Artifact	[3]
254	34475	SPRR3 17 kDa homo-2-mer	A5YKK8	34455.580	34475.617	28.2	Acetyl, M missing (N-term) (S-S var) S-S homo-2-mer	↑ pre-term	?	[8,33]
255	35301	SPRR3 17–18 kDa hetero-2-mer	A5YKK8; Q9UBC9	35280.985	35301.595	28.2	Acetyl, M missing (N-term) (S-S var) S-S hetero-2-mer	↑ pre-term	?	[8,33]
256	36127	SPRR3 18 kDa homo-2-mer	Q9UBC9	36106.391	36127.573	28.2	Acetyl, M missing (N-term) (S-S var) S-S homo-2-mer	↑ pre-term	?	[8,33]

[a]With an accuracy of ±1:10000.
[b]With a variation of ± 0.8 min.
ASD: autism spectrum disorder; GCF: gingival crevicular fluid.

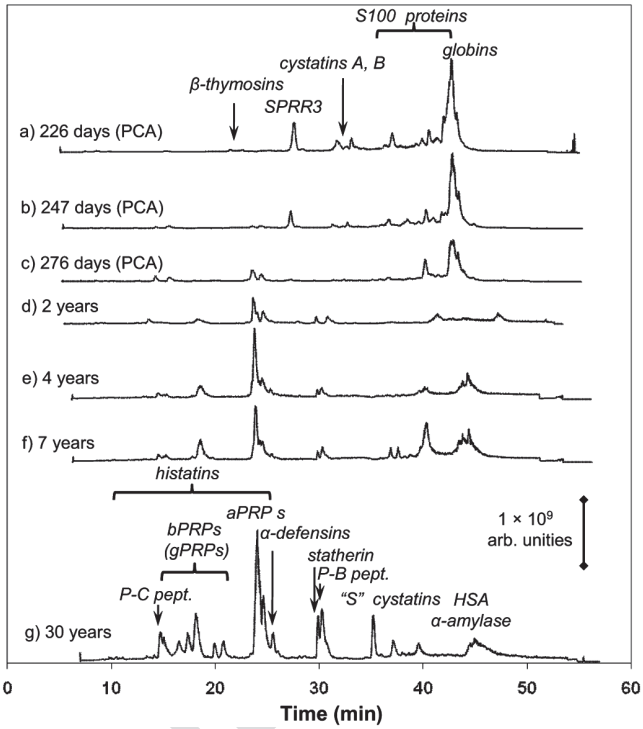

Figure 2. Typical HPLC-ESI-MS TIC (Total ion current) profiles of the acidic soluble fraction of human whole saliva of subjects with different ages. TIC profiles from (a) to (c) were from whole saliva samples of the same preterm newborn collected at different post-conceptional age (PCA). TIC profiles from (d) to (g) were from different healthy subjects. Apart from small variations linked to inter-individual differences, the seven profiles are good representative (qualitatively and quantitatively) of the profiles observed in other subjects of the same age range. The elution clusters of the most relevant salivary peptides and proteins reported in Table I (except human serum albumin (HSA) and α-amylase) are evidenced on the top of (a) and (g) profiles.

peak. The improvement of the signal-to-noise ratio reflected in enhanced sensitivity. Measured XIC peak area (MA) is proportional to the peptide amount. Thereby, under constant analytical conditions XIC peak area can be used for comparative determinations in an unlimited number of samples. Since the volume of saliva injected in the HPLC apparatus was the same, XIC peak area shows that the level of Tβ4 in whole saliva of the preterm newborn was about six fold higher than that of the adult (Figure 3).

Table I present proteins and peptides ordered according to their increasing average molecular mass measured at low-resolution MS. It is relevant to outline that often the experimental masses of the proteins/peptides of Table I did not correspond to the theoretical masses reported in international data banks. Indeed, the latter were often deduced from cDNA translation, and, consequently, did not take into account PTMs which may occur during protein maturation. In Table II, the sequence of some bPRP fragments of Table I is reported.

In the following sections, we describe some structural and genetic features of the protein families reported in Table I.

Histatins

The nice name given to these peptides by the Oppenheim group derives from the high number of histidine residues in their structure [9]. It has been shown that some of these peptides have a powerful antifungal activity against *Candida albicans* species [9]. It is widely accepted that all the members of this family arise from two parent peptides, histatin 1 and histatin 3, with a very similar sequence and are encoded by two genes (*HIS1* and *HIS2*) located

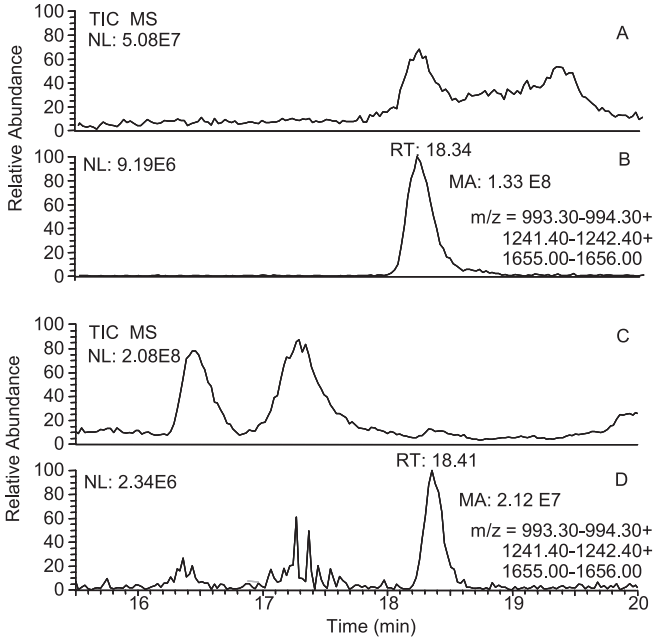

Figure 3. Extracted ion current (XIC) procedure for the detection of thymosin β_4 in a sample of whole saliva of a preterm newborn (227 days of post-conceptional age; A and B profiles) and of an adult (30 years; C and D profiles). A and C correspond to the total ion current (TIC) profiles, B and D to the XIC profiles. XIC procedure was carried out selecting three *m/z* values ($[M+3H]^{3+}$ = 1655.5 *m/z*; $[M+4H]^{4+}$ = 1241.9 *m/z* $[M+5H]^{5+}$ = 993.8 *m/z*) corresponding to characteristic ions of thymosin β_4. XIC procedure ensures an improvement of the signal to noise ratio with an enhancement of the sensitivity. NL, normalization level; MA, measured area; RT, retention time.

on chromosome 4q13 [10]. Despite the very high sequence similarity, these two peptides follow different PTM pathways. Histatin 3 is submitted to a sequential cleavage generating at first histatin 6 (histatin$_3$ Fr. 1/25), subsequently histatin 5 (histatin$_3$ Fr. 1/24) and then other fragments [11]. Before the proteomic era some of these fragments were named histatin 4-12 [9]. Recently, many other fragments have been detected, and a new nomenclature has been proposed based on the name of the parent peptide (histatin 1 or histatin 3) and the number of the fragment a.a. residues [11]. The different susceptibility to cleavage of the two histatins derives from the presence in histatin 3 of the RGYR↓ convertase consensus sequence, absent in histatin 1. Histatin 1 is phosphorylated on Ser-2 residue, but the non-phosphorylated derivative is always detectable in whole saliva, although at a low percentage. In spite of the presence of a Ser residue at position 2, histatin 3 is not phosphorylated, probably due to the absence of a +2 flanking glutamic acid residue essential for the kinase recognition. Histatin 1 is partly poly-sulfated in submandibular glands on the 4 tyrosines of the C-terminal domain, differently from histatin 3, which lacks a tyrosine equivalent to Tyr-27 of histatin 1, probably essential for the tyrosylprotein sulfotransferase recognition [12].

Basic and glycosylated (basic) proline-rich proteins

Basic and glycosylated (basic) proline-rich proteins (bPRPs) are detectable only in parotid secretion. They are the product of four loci (*PRB1-PRB4*) located on chromosome 12p13. At least four alleles (S, M, L, VL) are present at *PRB1* and *PRB3* loci, and three (S, M, L) at *PRB2* and *PRB4* loci in the western population. All the bPRPs are only detectable as multiple peptide fragments deriving from bigger pro-proteins and the connection between the most common haplotypes and salivary phenotypes is still waiting for a complete definition. The nomenclature is puzzling and complicate. The structure of 10 bPRP peptides was established by Kaufmann [13], which named the peptides according to the name of the chromatographic fractions. A different nomenclature was proposed by Isemura and coll. [14,15] that assigned to each identified salivary peptide the name P-X, where the X symbol is an alphabet letter from A to I. Nonetheless, P-B peptide, as explained in the following, cannot be considered a classical bPRP. P-A peptide is a fragment of P-B peptide, usually undetectable and generated by proteolysis during P-B purification. P-C peptide is a 44 a.a. residues fragment deriving from the C-terminal region of aPRPs. As a consequence, the authentic bPRPs peptides are P-D, P-E P-F and P-H, because the structures of P-G and P-I peptides have not been determined yet. Recently we were able to determine the structure of a new bPRP peptide that we called P-J [7,16]. Other components of this family were named Ps1 and Ps2 [17] and finally two bPRPs were named bPRP Con1+ and Con1- according to their interaction with concanavalin A [18]. A more rationale nomenclature is surely auspicable. Some protein masses pending for a definitive characterization were tentatively attributed to bPRPs family on the basis of their chromatographic properties and the absence of absorption at 270–280 nm. Some masses sporadically detected, and probably pertaining to this class of proteins, are not reported in Table I.

Broad peaks eluting in the bPRPs chromatographic cluster were attributed to gPRPs on the basis of the crowded ESI spectra, which usually did not allow deconvolution. Recently, we were able to characterized the structure of six glycoforms of IB-8a CON1+ [19]. Five of the glycoforms carry a biantennary N-linked glycan fucosylated in the innermost N-acetylglucosamine of the core and showing from zero to four additional fucoses in the antenna. The sixth glycoform carries a monoantennary mono-fucosylated oligosaccharide. Level of fucosylation showed inter-individual variability with the major relative abundance for the tri-fucosylated glycoform. Non glycosylated IB-8a CON1+ and the variant IB-8a CON1-, lacking of the glycosylation site, have been also detected in human saliva [19].

Acidic proline-rich proteins

Acidic proline-rich proteins (aPRPs) are secreted both by parotid (about 70%) and submandibular/sublingual glands (about 30%). They are the expression products of two loci, *PRH1* and *PRH2* located on chromosome 12p13, near to the cluster of bPRPs. *PRH1* codes for the PIF-s, Db-s and Pa isoforms, *PRH2* codes for the PRP-1 and PRP-2 isoforms. Therefore, five aPRP isoforms can be detected in the saliva of the Caucasian population, but considering that PRP-1 and Pif-s have the same mass and PRP-2 has 1 Da mass difference with respect to PRP-1 and Pif-s, these 3 isoforms cannot be usually discriminated in the HPLC-ESI-MS profile [20]. Moreover, TFA adducts can be detected for these isoforms [3]. All the isoforms have a pyroglutamic moiety at the N-terminus and are usually di-phosphorylated on Ser-7 and Ser-22, even though minor quantities of mono-, non-phosphorylated and tri-phosphorylated isoforms (on Ser-17) are also detectable [20]. Four of these isoforms (PRP-1, PRP-2, PIF-s and Db-s) can be partially cleaved near to the C-terminus, eventually releasing a common peptide of 44 a.a. residues (P-C peptide) and 4 truncated isoforms called PRP-3, PRP-4, PIF-f and Db-f. The Pa isoform is not cleaved, and it was usually detected in saliva as a S-S dimer due to the specific presence of a cysteine residue (Cys-103) in its structure. Minor quantities of other derivatives missing C-terminal residues from almost all isoforms were also detected [20].

Statherin and P-B peptide

Statherin is an unusual tyrosine-rich phospho-peptide (phosphorylated on Ser-2 and Ser-3) involved in oral cavity calcium ion homeostasis and teeth mineralization [21,22]. Its gene (*STATH*) is localized on chromosome 4q13.3, near to histatin genes [23]. In adult human saliva mono- and non-phosphorylated, as well as N- and C-terminal truncated isoforms are always detectable [24]. Furthermore, in adult human saliva minor amounts of a cyclic-statherin derivative (cyclo-statherin Q37) are detectable [25]. The cyclo-structure derives from an intra-molecular bridge between Lys-6 and Gln-37 generated by the action of oral transglutaminase 2 on statherin.

P-B peptide, after the determination of its structure, was (erroneously) included in the bPRPs family. However, it is the product of *PROL3* gene, localized on chromosome 4q13.3, very close to the statherin gene. Differently from classical bPRPs, P-B peptide is not a fragment of a bigger pro-protein, it is secreted both from parotid and Sm/Sl glands [26] and it displays three Tyr residues in the sequence. Statherin and P-B peptide elute closely in the chromatographic profile suggesting a similar polarity. For these reasons, P-B peptide could be functionally connected to statherin. However, while the statherin role on the modulation of oral calcium ion was recognized, none specific function for P-B peptide has been proposed to date.

Salivary ("S"-type) and other cystatins

Cystatins are detected in the RP-HPLC-ESI-MS chromatographic profile after the peaks of statherin and P-B peptides [27]. "S-type" cystatins comprise cystatin S, SN and SA which belong to family 2 of cystatins, inhibitors of cysteine-proteinases and are mainly secreted by Sm/Sl glands. Recent studies suggested that their secretion is not granule-mediated [26]. Cystatin S may be mono-phosphorylated on Ser-3 (cystatin S1; about 65%) or di-phosphorylated on Ser-1 and Ser-3 (cystatin S2; about 25%). Cystatin C was frequently detectable in human saliva, while, until now, no protein mass detected in saliva could be attributed to cystatins D and M. Cystatin A and B (called also stefins) belong to family 1 of cystatins, differing from type 2 cystatins for size and phosphorylation. Cystatin A was detectable in 2 isoforms (acetylated and non-acetylated on its N-terminal) [8]. Cystatin B was N-terminally acetylated and usually it was usually not detected as unmodified protein in adult whole saliva, because of the reactivity of Cys-3 residue. Cystatin B, indeed was present in whole saliva as S-glutathionylated (about 55%) S-cysteinylated (about 15%) derivatives or as S-S dimer (about 30%) [28].

Proteins of the S100 family

Proteins of the S100 family elute in the terminal part of the chromatographic profile. We were able to identify S100A7 (D_{27} and E_{27} isoforms), S100A8, S100A9 (8 isoforms: short, long, long S-glutathionylated, long S-cysteinylated and their phosphorylated counterparts), S100A11 and S100A12 [8]. The difficulty in the characterization of these proteins relied in the mismatch between the theoretical masses reported in data banks and the experimental masses of mature proteins [8]. For this reason, some of the masses pending for characterization could pertain to S100 B, S100A5 and S100A16. The source of these proteins in whole human saliva is unknown.

α-defensins and β-thymosins

α-Defensins 1-3 elute as a unique chromatographic peak after the aPRPs cluster. α-Defensin 4 eluted separately about 2 min before the statherin peak. α-Defensins belong to a family of broad-spectrum antimicrobial peptides, identified originally in human and rabbit leucocytes. 6 α-defensins (cryptidins) have been identified in humans to date. α-Defensins 1-4 are expressed in neutrophils, whereas α-defensins 5-6 are expressed in epithelial cells of the intestinal and reproductive tracts [29]. The name of β-thymosins derives from their first characterization from calf thymus extracts. Different studies have evidenced their ubiquitous presence in many organs and tissues as well as in various bodily fluids. Thymosin β_4, the most abundant, and thymosin β_{10} are typical in humans [30]. Gingival crevicular fluid is one of the main source of these two peptide classes [31,32].

Miscellaneous proteins and peptides

Some other proteins have been characterized along the HPLC-ESI-MS profile. SPRR3 (small-proline-rich protein 3) was mainly detectable in preterm newborn saliva in two variants, one with a M_{av} of 17239 ± 3 Da and the other with a M_{av} of 18065 ± 3 Da [33]. Therefore, 3 different phenotypes (2 homozygous and 1 heterozygous) can be found in the western population. Antileucoproteinase, also mainly detectable in preterm newborn saliva, elutes between α-defensins 1-3 and SPRR3 [8]. Lysozyme elutes near to the S-type cystatins. The protein with a M_{av} 22365 ± 4 Da was identified as the histone H1c, but the structure is pending for unambiguous characterization [8]. At the end of the chromatographic profile α, β and γ (both Gγ and Aγ) globins were sometimes detectable. In the terminal region of the HPLC profile other masses probably pertaining to α-amylase were sometimes detectable. However, the ESI spectrum was often crowded by multiple charge values, suggesting heterogeneity for this and other proteins which are not reported in Table I.

Fragments of bigger proteins and peptides

The most relevant PTM of salivary proteins is the proteolytic cleavage by a complex and not well known set of endo- and exo-proteinases that generates a multitude of small peptides. On the basis of the RXXR↓ consensus sequence, some of the endoproteinases probably belong to the furin-like convertase family, work before granule storage and are responsible for the generation of bPRPs, truncated isoforms of aPRPs, P-C peptide and histatin 6 [11]. The convertase acting on bPRPs is responsible for a complete digestion of the pro-protein, while aPRPs convertase, more active in Sm/Sl gland, is responsible for a partial cleavage of the proteins. Also the proteinase responsible for the first cleavage of histatin 3 can be included in the convertase family, because 2 Arg separated by 2 a.a. residues seems a mandatory requirement for the enzyme recognition. In fact, histatin 1, lacking the Arg-25 residue present in histatin 3, is less prone to proteolysis [11,26].

The removal of a C-terminal residue by specific carboxypeptidases following the convertase cleavage is a widespread event in many secretion processes and it can be observed also in many salivary peptides. One of the most relevant a.a. removal, in terms of relative abundance, involves the C-terminal Arg-25 of histatin 6 generating histatin 5 [11].

Other peptidases of endogen or exogen origin are active after granule secretion and in the oral cavity. Some salivary proteins, such as bPRPs, P-C and P-B peptides, statherin, histatin 3 and histatin 5 are more prone to proteolysis than other salivary proteins. Some fragments of glyceraldehyde 3-phosphate dehydrogenase were often detected, especially in saliva of preterm newborns, but the intact protein was not found in human saliva till now. Other authors have studied the naturally occurring peptides generated by oral proteolytic activity [34,35]. Table I

reports some of the fragments identified by Huq and coll. [34] that we were able to consistently detect by HPLC-ESI-MS in different samples of whole saliva. The aim of a study of Helmerhorst and coll. [35] was the detection of specific proteinase activities in the oral cavity. They found a lot of salivary protein fragments which allow revealing, in the oral cavity, the activity of a specific glutamine endoproteinase that recognizes KPQ↓ as the main consensus sequence. This proteinase probably derives from dental plaque and it is likely microbial in origin. Table I reports only some fragments of this list, because their presence in saliva immediately after specimen collection has to be confirmed. Some of the bPRP fragments detected by us and other researchers have been also reported by Vitorino et al. as recurrent non-covalent bound components of the enamel surface. This finding might provide a functional significance to this final bPRP maturation [36].

Other post-translational modifications

Beyond cleavage, salivary proteins are submitted to many other PTMs before, during and after secretion. Glycosylation is a relevant PTM of salivary proteins, but as previously mentioned, salivary glycosylated proteins are not easily evidenced by top-down proteomic analysis because of the high heterogeneity. Many salivary proteins are phosphorylated. Top-down platform evidenced 2 major (Ser-8 and Ser-22) and 1 minor site (Ser-17) on all aPRPs, 1 site (Ser-8) on II-2 and IB-1 (bPRPs), 2 sites (Ser-2 and Ser-3) on statherin, 1 site (Ser-2) on histatin 1 and 2 sites on cystatin S (Ser-2 and Ser-3). Due to the similar SX (E/Sp) consensus sequence, the kinase responsible for the phosphorylation of these sites should be an elusive Golgi casein kinase acting before granule storage [37]. About 40% of all the S100A9 isoforms were found to be phosphorylated on the penultimate Thr of the sequence by p38 MAP kinase [38].

Until now O-tyrosine sulfation of salivary proteins has been detected only on histatin 1 [12]. This modification was found to be specific of Sm/Sl glands, where not more than 10% of total histatin 1 could be detected as a mixture of isoforms carrying a different number of sulfate moieties (from 1 to 4). This PTM was probably hierarchical, being Tyr-27 the first residue submitted to sulfation, followed by Tyr-30, Tyr-34 and Tyr-36 [12].

Specific cysteine residues of cystatin B, S100A9 and SPRR3 appeared prone to S-glutathionylation and S-cysteinylation. These PTMs were particularly extensive for cystatin B, because the S-unmodified protein was often undetectable in adult whole saliva [28]. Cysteine residues were also responsible for several covalent dimerization. Only Pa S-S 2-mer (Pa is one of the 5 isoforms of aPRPs) was detectable in whole saliva. About 30% of cystatin B and small amounts of SPRR3 were also detectable as S-S 2-mer. Because SPRR3 exists in 2 isoforms, both homo- and hetero-dimers were detectable in heterozygosis.

Methionine residues are often prone to the oxidation (sulfoxide and sulfone). Met-sulfoxide derivatives of thymosin β_4 (Met-6), S100A9 short (Met-89) and "S-type" cystatins were detected in whole saliva. These derivatives could be indicative of oxidative stress in the oral cavity.

In the presence of a glutamine or a glutamic acid, the N-terminal residue of many salivary proteins was typically cyclized in pyroglutamic acid, precluding Edman sequencing. Identified examples were the entire and truncated isoforms of all aPRPs, the bPRPs named IB-1 and II-2, as well as the P-B peptide.

N-terminal acetylation was another PTM largely found in many identified proteins. It was usually reported that this PTM was subsequent to the removal of the N-terminal Met residue [39]. S100A7 (both D_{27} and E_{27} isoforms), S100A11, SPRR3 and β-thymosins followed this rule. PTMs of S100A9 are particular. Two phosphorylated and non-phosphorylated isoforms, called long isoforms, derive from the removal of the N-terminal Met residue followed by acetylation. These 2 isoforms can be also partly S-glutathionylated and S-cysteinylated (on Cys-2 residue) bringing to 6 the total number of S100A9 long isoforms detectable in whole saliva. On the other hand, other 2 S100A9 isoforms, called short isoforms (phosphorylated and non-phosphorylated) derive from acetylation after removal of the N-terminal pentapeptide (MTCKM). Because this removal eliminates the Cys-2 residue from the S100A9 sequence, the S100A9 short isoforms does not originate S-modified derivatives. S100A12 loose the N-terminal Met residue, but it is not acetylated, cystatin [redacted] does not loose the N-terminal Met residue, but it is not found acetylated and S100A8 does not loose the N-terminal Met residue and it is not acetylated. Cystatin A was detected in 3 different isoforms, 1 with the N-terminal Met residue, 1 acetylated on the N-terminal Met and 1 (M_{av} 10872 ± 2 Da) without the N-terminal Met and non-acetylated (this last pending for definitive characterization). This puzzling situation outlines that probably each protein has specific structural requirements on its N-terminal residues. However, the understanding of the molecular signals that any protein utilizes to drive its correct N-terminal modification and the functional significance of the different N-terminal modifications occurring in the same protein is surely a challenging task.

Source of salivary proteins

Most of the information reported in Table I on the source of salivary proteins was obtained by a top-down proteomic study that compared the natural occurring proteome of adult whole saliva with the proteins detectable in glandular (parotid and Sm/Sl) saliva and enriched granule preparation from parotid and submandibular glands [26]. This study confirmed that bPRPs were exclusively secreted by the parotid gland. Approximately 70% of aPRPs isoforms was found to be secreted by parotid glands, the remaining 30% by Sm/Sl glands. Also, histatin 3 was secreted more by parotid than by Sm/Sl glands. Histatin 1, statherin and P-B peptide were roughly secreted in similar amounts by both major glands, while the main sources of "S type" cystatins were Sm/Sl glands. Gingival crevicular fluid was a relevant source of cystatin A, α-defensins and β-thymosins [30,32]. Conversely, in preterm saliva the high amount of β-thymosins derived from major salivary glands [40] even though the secretion pathway of this leaderless peptide [31] is still unknown. The sources and the secretion pathways of other proteins reported in Table I are not still well defined too.

Surprising differences of salivary proteome in pre-term newborns and in the pediatric age

Figure 2 shows the striking differences present in human salivary proteome as a function of age, with a particular concern to the pediatric age [7]. Given to the non-invasive collection of the sample the salivary proteome of preterm newborns was investigated from about 195 days of post-conceptional age (PCA) [6,8]. In preterm newborns of about 195–220 days of PCA noticeable amounts of more than 40 proteins were detectable. Cystatin A (2 isoforms), cystatin B (3 isoforms), S100A7 (2 isoforms), S100A8, S100A9 (8 isoforms), S100A11, S100A12, small proline-rich protein 3 (2 isoforms), lysozyme C, thymosins β_4 and β_{10}, antileukoproteinase, histone H1c, and α and γ globins [8] have been identified. The concentration of these proteins decreased quickly, even though with different trends, as a function of PCA reaching values detectable in adult at a PCA corresponding to the

normal term of delivery (about 270 days). At the same time, the level of salivary proteins characteristic of the adult increased, also in this case with different trends.

Many of the proteins identified in saliva of pre-term newborns are considered tumor markers in the adults. This observation led to suppose that these proteins might contribute to the molecular events that regulate cell growth, proliferation, or death during fetal development. The abnormal expression in the adults might be at the basis of anomalous cellular growth and to the development of different tumors with embryonic etiology. The recognition of tumor stem cells in many solid cancers has reinvigorated the hypothesis of a pluripotent stem cell as the cell of origin for cancer [41]. Data from our group on Tβ4 expression in salivary glands' tumors and in colon cancer [43] evidenced Tβ4 reactivity in tumor cells undergoing epithelial-mesenchymal transition, a highly conserved cellular program typical of several stages of embryonic development as well as of cancer invasion and metastasis [44].

A study carried out on 67 subjects aged between 3 and 44 years evidenced several qualitative and quantitative age-dependent modifications of the salivary proteome. It was found that the concentration of salivary acidic proline-rich phosphoproteins, histatin-5, histatin-6, and monophosphorylated and diphosphorylated cystatin S showed a minimum between 6 and 9 years of age. Interestingly, bPRPs, almost absent in saliva of children, reached adult levels only after puberty [7], suggesting a potential role of these peptides in the modulation of taste perception. Indeed a recent study of our group showed for the first time that responsiveness to 6-n-propylthiouracil bitter taste is associated with salivary levels of II-2 peptide and Ps-1 protein, which are products of the PRB1 gene [45].

It is relevant to outline that the striking age-dependent modification of the human salivary proteome has to be carefully considered for the choice of the proper control group in the characterization of new disease biomarkers, relevant aim of any proteomic platform.

Pathological modification of the human salivary proteome detected by the top-down approaches

A conclusive aim of a proteomic dataset is its use for diagnostic and prognostic purposes. Due to the non invasive specimen collection saliva is very attractive in this regard. The protein list reported in this review could be a helpful reference for the detection of potential early biomarker of disease by using quantitation based on XIC area determination, as shown in Figure 3. Top-down analyses for the detection of the variations of the salivary proteome in other local and systemic diseases are in progress upon our laboratories.

Studies carried out on different patients with autism spectrum disorders (ASD) evidenced the hypo-phosphorylation of several salivary peptides and proteins (histatin 1, statherin and both the entire and truncated isoforms of aPRPs) in a subset of about 60% of ASD subjects, the majority comprised in the normal to border-line cognitive development [46]. Hypo-phosphorylation of salivary peptides suggested potential asynchronies in the phosphorylation of other secretory proteins, which could be relevant in central nervous system during either embryonic development or early infancy.

A study carried out on whole saliva of 31 children affected by type 1 diabetes revealed a lower concentration of statherin, proline-rich peptides, P-B, P-C peptides, and histatins, and higher concentration of α-defensins 1, 2 and 4 and S100A9 short isoforms with respect to an age and sex matched control group [47]. The lower concentration of P-C peptide was paralleled by higher levels of some of its fragments. On the whole, the study highlighted the severe impairment of the repertoire of peptides involved in the safeguard of the oral cavity in diabetic children. A study carried out on 9 patients with primary Sjögren syndrome (SS) evidenced that pilocarpine treatment restored the protein levels and partially restored the protein numbers that were found to be decreased in primary SS patients, with the parotid gland proteins showing the best response to the drug [48,49]. Finally, a study carried out on 11 totally edentulous patients evidenced reduced levels of α-defensins respect to two groups of controls (one matched for age and gender, the second of younger subjects) [50]. Since these peptides have mainly crevicular origin, most likely the low levels measured resulted from the absence of the gingival sulcus in the edentulous subjects.

Concluding remarks

A limit of the "solution-based" top-down platform utilized was that many masses attributable to well know salivary proteins, such as carbonic anhydrase, immunoglobulin, peroxidases, mieloperoxidases, mucins were never detected in the HPLC-ESI-MS profile, likely due to their insolubility in acidic solution. It should be taken into account that the limit of sensitivity of the HPLC-ESI-MS apparatus in use upon in laboratories on saliva samples was, at the best, in the range of 10–50 nanomoles/L and that increase of instrumental sensitivity can disclose a lot of other protein and peptide masses that might be added to the list in the future. Table I is a dynamic table that anyone can implement with new attributions. It can help not only researchers that would experience top-down platforms on human saliva, but also anyone who is involved in top-down attributions in other bodily fluid, because many proteins reported are not specific of saliva.

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References

1. Helmerhorst EJ, Oppenheim FG. Saliva: a dynamic proteome. J Dent Res 2007;86:680–693.
2. Amado F, Lobo MJ, Domingues P, Duarte JA, Vitorino R. Salivary peptidomics. Expert Rev Proteomics 2010;7:709–721.
3. Messana I, Inzitari R, Fanali C, Cabras T, Castagnola M. Facts and artifacts in proteomics of body fluids. What proteomics of saliva is telling us? J Sep Sci 2008;31:1948–1963.
4. Al-Tarawneh SK, Border MB, Dibble CF, Bencharit S. Defining salivary biomarkers using mass spectrometry-based proteomics: a systematic review. OMICS 2011;15:353–361.
5. Tipton JD, Tran JC, Catherman AD, Ahlf DR, Durbin KR, Kelleher NL. Analysis of intact protein isoforms by mass spectrometry. J Biol Chem 2011;286:25451–25458.
6. Inzitari R, Vento G, Capoluongo E, Boccacci S, Fanali C, Cabras T, Romagnoli C, et al. Proteomic analysis of salivary acidic proline-rich proteins in human preterm and at-term newborns. J Proteome Res 2007;6:1371–1377.
7. Cabras T, Pisano E, Boi R, Olianas A, Manconi B, Inzitari R, Fanali C, et al. Age-dependent modifications of the human salivary secretory protein complex. J Proteome Res 2009;8:4126–4134.
8. Castagnola M, Inzitari R, Fanali C, Iavarone F, Vitali A, Desiderio C, Vento G, et al. The surprising composition of the salivary proteome of preterm human newborn. Mol Cell Proteomics 2011;10:M110.003467.
9. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, Troxler RF. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary

structure, and fungistatic effects on Candida albicans. J Biol Chem 1988;263:7472–7477.
10. Sabatini LM, Azen EA. Histatins, a family of salivary histidine-rich proteins, are encoded by at least two loci (HIS1 and HIS2). Biochem Biophys Res Commun 1989;160:495–502.
11. Castagnola M, Inzitari R, Rossetti DV, Olmi C, Cabras T, Piras V, Nicolussi P, et al. A cascade of 24 histatins (histatin 3 fragments) in human saliva. Suggestions for a pre-secretory sequential cleavage pathway. J Biol Chem 2004;279:41436–41443.
12. Cabras T, Fanali C, Monteiro JA, Amado F, Inzitari R, Desiderio C, Scarano E, et al. Tyrosine polysulfation of human salivary histatin 1. A post-translational modification specific of the submandibular gland. J Proteome Res 2007;6:2472–2480.
13. Kauffman DL, Bennick A, Blum M, Keller PJ. Basic proline-rich proteins from human parotid saliva: relationships of the covalent structures of ten proteins from a single individual. Biochemistry 1991;30:3351–3356.
14. Isemura S, Saitoh E, Sanada K. Fractionation and characterization of basic proline-rich peptides of human parotid saliva and the amino acid sequence of proline-rich peptide P-E. J Biochem 1982;91:2067–2075.
15. Isemura S, Saitoh E, Sanada K. Further fractionation of basic proline-rich peptides from human parotid saliva and complete amino acid sequence of basic proline-rich peptide P-H. J Biochem 1983;94:1991–1999.
16. Messana I, Cabras T, Inzitari R, Lupi A, Zuppi C, Olmi C, Fadda MB, et al. Characterization of the human salivary basic proline-rich protein complex by a proteomic approach. J Proteome Res 2004;3:792–800.
17. Azen EA, Latreille P, Niece RL. PRBI gene variants coding for length and null polymorphisms among human salivary Ps, PmF, PmS, and Pe proline-rich proteins (PRPs). Am J Hum Genet 1993;53:264–278.
18. Azen EA, Amberger E, Fisher S, Prakobphol A, Niece RL. PRB1, PRB2, and PRB4 coded polymorphisms among human salivary concanavalin-A binding, II-1, and Po proline-rich proteins. Am J Hum Genet 1996;58:143–153.
19. Cabras T, Boi R, Pisano E, Iavarone F, Fanali C, Nemolato S, Faa G, et al. HPLC-ESI-MS and MS/MS structural characterization of multifucosylated N-glycoforms of the basic proline-rich protein IB-8a CON1+ in human saliva. J Sep Sci 2012;35:1079–1086.
20. Inzitari R, Cabras T, Onnis G, Olmi C, Mastinu A, Sanna MT, Pellegrini MG, et al. Different isoforms and post-translational modifications of human salivary acidic proline-rich proteins. Proteomics 2005;5:805–815.
21. Schlesinger DH, Hay DI. Complete covalent structure of statherin, a tyrosine-rich acidic peptide which inhibits calcium phosphate precipitation from human parotid saliva. J Biol Chem 1977;252:1689–1695.
22. Schwartz SS, Hay DI, Schluckebier SK. Inhibition of calcium phosphate precipitation by human salivary statherin: structure-activity relationships. Calcif Tissue Int 1992;50:511–517.
23. Sabatini LM, Carlock LR, Johnson GW, Azen EA. cDNA cloning and chromosomal localization (4q11-13) of a gene for statherin, a regulator of calcium in saliva. Am J Hum Genet 1987;41:1048–1060.
24. Inzitari R, Cabras T, Rossetti DV, Fanali C, Vitali A, Pellegrini M, Paludetti G, et al. Detection in human saliva of different statherin and P-B fragments and derivatives. Proteomics 2006;6:6370–6379.
25. Cabras T, Inzitari R, Fanali C, Scarano E, Patamia M, Sanna MT, Pisano E, et al. HPLC-MS characterization of cyclo-statherin Q-37, a specific cyclization product of human salivary statherin generated by transglutaminase 2. J Sep Sci 2006;29:2600–2608.
26. Messana I, Cabras T, Pisano E, Sanna MT, Olianas A, Manconi B, Pellegrini M, et al. Trafficking and postsecretory events responsible for the formation of secreted human salivary peptides: a proteomics approach. Mol Cell Proteomics 2008;7:911–926.
27. Lupi A, Messana I, Denotti G, Schininà ME, Gambarini G, Fadda MB, Vitali A, et al. Identification of the human salivary cystatin complex by the coupling of high-performance liquid chromatography and ion-trap mass spectrometry. Proteomics 2003;3:461–467.
28. Cabras T, Manconi B, Iavarone F, Fanali C, Nemolato S, Fiorita A, Scarano E, et al. RP-HPLC-ESI-MS evidenced that salivary cystatin B is detectable in adult human whole saliva mostly as S-modified derivatives: S-Glutathionyl, S-cysteinyl and S-S 2-mer. J Proteomics 2012;75:908–913.
29. Ganz T, Lehrer RI. Defensins. Curr Opin Immunol 1994;6:584–589.
30. Hannappel E. β-Thymosins. Ann N Y Acad Sci 2007;1112:21–37.
31. Pisano E, Cabras T, Montaldo C, Piras V, Inzitari R, Olmi C, Castagnola M, Messana I. Peptides of human gingival crevicular fluid determined by HPLC-ESI-MS. Eur J Oral Sci 2005;113:462–468.
32. Inzitari R, Cabras T, Pisano E, Fanali C, Manconi B, Scarano E, Fiorita A, et al. HPLC-ESI-MS analysis of oral human fluids reveals that gingival crevicular fluid is the main source of oral thymosins β(4) and β(10). J Sep Sci 2009;32:57–63.
33. Manconi B, Cabras T, Pisano E, Nemolato S, Inzitari R, Iavarone F, Fanali C, et al. Characterization of two isoforms of human SPRR3 from saliva of preterm human newborn and autoptic fetal oral mucosa, parotid and submandibular gland samples. Biochem Biophys Res Commun 2010;398:477–481.
34. Huq NL, Cross KJ, Ung M, Myroforidis H, Veith PD, Chen D, Stanton D, et al. A review of the salivary proteome and peptidome and saliva-derived therapeutics. Int J Pept Res Ther 2007;13:547–564.
35. Helmerhorst EJ, Sun X, Salih E, Oppenheim FG. Identification of Lys-Pro-Gln as a novel cleavage site specificity of saliva-associated proteases. J Biol Chem 2008;283:19957–19966.
36. Vitorino R, Calheiros-Lobo MJ, Williams J, Ferrer-Correia AJ, Tomer KB, Duarte JA, Domingues PM, Amado FM. Peptidomic analysis of human acquired enamel pellicle. Biomed Chromatogr 2007;21:1107–1117.
37. Tibaldi E, Arrigoni G, Brunati AM, James P, Pinna LA. Analysis of a sub-proteome which co-purifies with and is phosphorylated by the Golgi casein kinase. Cell Mol Life Sci 2006;63:378–389.
38. Lominadze G, Rane MJ, Merchant M, Cai J, Ward RA, McLeish KR. Myeloid-related protein-14 is a p38 MAPK substrate in human neutrophils. J Immunol 2005;174:7257–7267.
39. Arnesen T, Van Damme P, Polevoda B, Helsens K, Evjenth R, Colaert N, Varhaug JE, et al. Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proc Natl Acad Sci USA 2009;106:8157–8162.
40. Nemolato S, Messana I, Cabras T, Manconi B, Inzitari R, Fanali C, Vento G, et al. Thymosin β(4) and β(10) levels in pre-term newborn oral cavity and foetal salivary glands evidence a switch of secretion during foetal development. PLoS ONE 2009;4:e5109.
41. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–111.
42. Grichnik JM. Melanoma, nevogenesis, and stem cell biology. J Invest Dermatol 2008;128:2365–2380.
43. Nemolato S, Restivo A, Cabras T, Coni P, Zorcolo L, Orrù G, Fanari M, et al. Thymosin ß 4 in colorectal cancer is localized predominantly at the invasion front in tumor cells undergoing epithelial mesenchymal transition. Cancer Biol Ther 2012;13:191–197.
44. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 2008;14:818–829.
45. Cabras T, Melis M, Castagnola M, Padiglia A, Tepper BJ, Messana I, Tomassini Barbarossa I. Responsiveness to 6-n-propylthiouracil (PROP) is associated with salivary levels of two specific basic proline-rich proteins in humans. PLoS ONE 2012;7:e30962.
46. Castagnola M, Messana I, Inzitari R, Fanali C, Cabras T, Morelli A, Pecoraro AM, et al. Hypo-phosphorylation of salivary peptidome as a clue to the molecular pathogenesis of autism spectrum disorders. J Proteome Res 2008;7:5327–5332.
47. Cabras T, Pisano E, Mastinu A, Denotti G, Pusceddu PP, Inzitari R, Fanali C, et al. Alterations of the salivary secretory peptidome profile in children affected by type 1 diabetes. Mol Cell Proteomics 2010;9:2099–2108.
48. Peluso G, De Santis M, Inzitari R, Fanali C, Cabras T, Messana I, Castagnola M, Ferraccioli GF. Proteomic study of salivary peptides and proteins in patients with Sjögren's syndrome before and after pilocarpine treatment. Arthritis Rheum 2007;56:2216–2222.
49. Rigante D, Inzitari R, Carone M, Fanali C, Stabile A, Cabras T, Capoluongo E, et al. Correspondence between clinical improvement and proteomic changes of the salivary peptide complex in a child with primary Sjögren syndrome. Rheumatol Int 2008;28:801–806.
50. Fanali C, Inzitari R, Cabras T, Pisano E, Castagnola M, Celletti R, Manni A, Messana I. alpha-Defensin levels in whole saliva of totally edentulous subjects. Int J Immunopathol Pharmacol 2008;21:845–849.

Review

Top-down proteomics of human salivary proteome

M. Castagnola et al.

Top-down platform for deciphering the human salivary proteome

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~~Abstract~~

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Proteomic platforms can be classified in bottom-up strategies, which analyze the sample after proteolytic digestion, and top-down strategies, which analyze the intact naturally occurring proteome. Bottom-up platforms are high-throughput because they can investigate a large number of proteins, regardless of their dimension. Nonetheless, information on post-translational modifications (PTMs) can be lost, especially those regarding naturally occurring cleavages and alternative splicing. Top-down platforms cannot cover vast proteomes, however, they can disclose subtle structural variations occurring during protein maturation and allow label-free relative quantifications in an unlimited number of samples. A repertoire of 256 masses belonging to naturally occurring proteins and peptides consistently detected by RP-HPLC-ESI-MS analysis of the acidic soluble fraction of human whole saliva is presented in this study. Of them, 233 have been identified, while 23 are still pending for the definitive characterization. The present review reports average and mono-isotopic masses of the peptides and proteins detected, RP-HPLC elution times, PTMs, origin and quali-quantitative variations observed in several physiological and pathological conditions. The information reported can be a reference for users of top-down RP-HPLC-ESI-MS proteomic platforms applied to the study of the human salivary proteome as well as of other human bodily fluids.

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Keywords: α~~alpha~~-defensins, β~~beta~~-thymosins, cystatins, histatins, human, proteome, proteomics, proline-rich proteins, saliva, statherin, S100 proteins, top-down

Introduction

Different attempts have been recently addressed towards the characterization of human salivary proteome [1–4]. Our group has been involved in this demanding task for more than twelve years utilizing a “solution based” top-down proteomic platform, focused on the detection and characterization of the intact naturally occurring proteins and peptides soluble in acidic solution by RP-HPLC-ESI-MS (reversed-phase high-performance liquid-chromatography, electrospray ionization, mass spectrometry). The general scheme of this

approach is reported in Figure 1. In the first step, the average mass of intact peptides/proteins is determined by ESI-MS with a precision of at least 1:10,000 Da. For the identification purified proteins are also submitted to high resolution MS/MS experiments. Because proteins with mass higher than 3000–4000 Da do not usually provide MS/MS fragmentation spectra suitable for complete *de novo* sequencing, further experiments are required in order to obtain definitive characterization of protein structure, comprising PTMs (post-translational modifications). For this purpose, HPLC partially purified protein are submitted to different chemical and enzymatic treatments, such as removal of phosphate groups by phosphatase, reduction of disulfide bridges and protection of cysteine residues by proper reactants. For proteins without the N-terminal not blocked, automated Edman sequencing allows amino-terminal sequence to be established. Alternatively, the digestion products obtained by different proteolytic enzymes are submitted to different analyses and high resolution MS-MS identifications. Overall, collected data allowed us to hypothesize the protein structure, which was confirmed or rejected by checking the correspondence between the theoretical and the experimental (partial) MS-MS data collected on the intact naturally occurring protein.

As recently reported in the literature [5], top-down platforms nowadays cannot reach the same coverage of bottom-up platforms for different reasons: (i) the intact protein has to be soluble in the acidic solution compatible with the ESI-MS analysis; (ii) the protein should not be heterogeneous (i.e. glycosylated isoforms), because in this case the mass of the intact protein cannot be deduced by the crowded ESI spectrum; (iii) protein dimensions have to be limited because big proteins MS-MS fragmentation spectra are too complex to be interpreted. Nonetheless, top-down strategies could reveal the rich isoform and PTM diversity present in the human body.

In this paper, we report the inventory (Table ~~1~~I) of 256 intact protein and peptide masses detected in whole human saliva: of them, 233 have been identified while 23 are

pending for definitive characterization. Together with the RP-HPLC elution time, PTMs, origin and specific quali-quantitative variations observed in several physiological and pathological conditions are reported with a particular concern to the p~~a~~ediatric age. The list can be used as a reference for anyone who wants to carry out RP-HPLC-ESI-MS experiments and to utilize other top-down proteomic platforms for the study of the human salivary proteome.

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Details of the top-down platforms

The basic step of the top-down approach was the determination of the mass of the intact, naturally-occurring protein/peptide with accuracy better than 1:10,000 (Fig~~-~~ure 1). This result was typically achieved by RP-HPLC-ESI-MS experiment, utilizing a classical C-8 reversed phase column (varying according to the flow-rate and the apparatus). The chromatographic elution was performed utilizing a gradient of water/acetonitrile with 0.05% 2,2,2-trifluoroacetic acid (TFA) as ion-pairing agent. TFA is recognized as a good ion-pairing HPLC agent. The drawback is that TFA is not a good ionization agent for the electrospray process, as either acetic or formic acid are, leading to a partial suppression of the ESI signal. Moreover, sometimes some salivary protein masses were increased of about 113 Da due to formation of TFA adducts during the ESI process [3]. In our experience, the use of formic acid instead of TFA led to an overall reduction of HPLC-ESI-MS performance. Figure~~Fig.~~ 2 reports typical TIC profiles of salivary samples from subjects of different ages. We have indeed demonstrated that age-dependent deep modifications of the human salivary proteome occur, especially in the pediatric age [6–8]~~[6-8]~~. Figure~~Fig.~~ 2 shows that different classes of salivary proteins were often detectable in well-defined chromatographic clusters. This behavior clearly reflected the similarity of the structures and polarity of the members of the different families of salivary proteins. Typically, during the first 10 min of separation many fragments of bigger proteins/peptides eluted, followed (10–20 min) by histatins, glycosylated

(gPRPs) and basic-proline-rich proteins (bPRPs). Usually, the mass of gPRPs could not be established due to the crowded ESI spectra. At around 20 min, β-thymosins was detected, followed by a cluster comprising acidic-PRPs (aPRPs), and immediately after by α-defensins. In the 24–27 min range different derivatives of statherin were detectable, as well as small-proline-rich protein 3 (SPRR3), a protein present at high concentration in preterm newborn saliva. Typically, in the 27–30 min range statherin and P-B peptide eluted, followed by cystatin A and B (and their derivatives), salivary cystatins ("S"-type), different proteins of the S100 family, α-amylase, human serum albumin (showing crowded ESI spectrum) and other miscellaneous proteins.

Relative quantification of proteins and peptides reported in Table *I* was performed by the extracted ion current (XIC) procedure. Figure~~Fig.~~ 3 shows the XIC procedure applied to the determination of the relative levels of thymosin β_4 ($T\beta_4$) in whole saliva of a preterm newborn (226 days of post-conceptional age) and of an adult. The selection of three specific *m/z* values corresponding to ions with +3/+5 charged allowed to isolate the specific peptide peak. The improvement of the signal-to-noise ratio reflected in enhanced sensitivity. Measured XIC peak area (MA) is proportional to the peptide amount. Thereby, under constant analytical conditions XIC peak area can be used for comparative determinations in an unlimited number of samples. Since the volume of saliva injected in the HPLC apparatus was the same, XIC peak area shows that the level of Tβ4 in whole saliva of the preterm newborn was about six fold higher than that of the adult (Figure~~Fig.~~ 3).

Table ~~1~~ I present proteins and peptides ordered according to their increasing average molecular mass measured at low-resolution MS. It is relevant to outline that often the experimental masses of the proteins/peptides of Table ~~1~~ I did not correspond to the theoretical masses reported in international data banks. Indeed, the latter were often deduced from cDNA translation, and, consequently, did not take into account PTMs which may occur

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during protein maturation. In Table ~~2~~II[AU: Please note that the article contains a citation for Table II. However, Table II is missing in the article. Please provide Table II and a caption for it.], the sequence of some bPRP fragments of Table ~~1~~I is reported.

In the following sections, we describe some structural and genetic features of the protein families reported in Table~~s 1~~I.

Histatins

The nice name given to these peptides by the Oppenheim group derives from the high number of histidine residues in their structure [9]. It has been shown that some of these peptides have a powerful antifungal activity against *Candida albicans* species [9]. It is widely accepted that all the members of this family arise from two parent peptides, histatin 1 and histatin 3, with a very similar sequence and are encoded by two genes (*HIS1* and *HIS2*) located on chromosome 4q13 [10]. Despite the very high sequence similarity, these two peptides follow different PTM pathways. Histatin 3 is submitted to a sequential cleavage generating at first histatin 6 (histatin$_3$ Fr. 1/25), subsequently histatin 5 (histatin$_3$ Fr. 1/24) and then other fragments [11]. Before the proteomic era some of these fragments were named histatin 4-12 [9]. Recently, many other fragments have been detected, and a new nomenclature has been proposed based on the name of the parent peptide (histatin 1 or histatin 3) and the number of the fragment a.a. residues [11]. The different susceptibility to cleavage of the two histatins derives from the presence in histatin 3 of the RGYR~~↓~~↓ convertase consensus sequence, absent in histatin 1. Histatin 1 is phosphorylated on Ser-2 residue, but the non-phosphorylated derivative is always detectable in whole saliva, although at a low percentage. In spite of the presence of a Ser residue at position 2, histatin 3 is not phosphorylated, probably due to the absence of a +2 flanking glutamic acid residue essential for the kinase recognition. Histatin 1 is partly poly-sulfated in submandibular glands on the 4

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tyrosines of the C-terminal domain, differently from histatin 3, which lacks a tyrosine equivalent to Tyr-27 of histatin 1, probably essential for the tyrosylprotein sulfotransferase recognition [12].

Basic and glycosylated (basic) proline-rich proteins ~~(bPRPs)~~

Basic and glycosylated (basic) proline-rich proteins (bPRPs) are detectable only in parotid secretion. They are the product of four loci (*PRB1-PRB4*) located on chromosome 12p13. At least four alleles (S, M, L, VL) are present at *PRB1* and *PRB3* loci, and three (S, M~~-~~, L) at *PRB2* and *PRB4* loci in the western population. All the bPRPs are only detectable as multiple peptide fragments deriving from bigger pro-proteins and the connection between the most common haplotypes and salivary phenotypes is still waiting for a complete definition. The nomenclature is puzzling and complicate. The structure of 10 bPRP peptides was established by Kaufmann [13], which named the peptides according to the name of the chromatographic fractions. A different nomenclature was proposed by Isemura and coll. [14,15]~~-~~ that assigned to each identified salivary peptide the name P-X, where the X symbol is an alphabet letter from A to I. Nonetheless, P-B peptide, as explained in the following, cannot be considered a classical bPRP. P-A peptide is a fragment of P-B peptide, usually undetectable and generated by proteolysis during P-B purification. P-C peptide is a 44 a.a. residues fragment deriving from the C-terminal region of aPRPs. As a consequence, the authentic bPRPs peptides are P-D, P-E P-F and P-H, because the structures of P-G and P-I peptides have not been determined yet. Recently we were able to determine the structure of a new bPRP peptide that we called P-J [7,16]. Other components of this family were named Ps1 and Ps2 [17] and finally two bPRPs were named bPRP Con1+ and Con1- according to their interaction with concanavalin A [18]. A more rationale nomenclature is surely auspicable. Some protein masses pending for a definitive characterization were tentatively attributed to bPRPs family on the basis of their chromatographic properties and the absence of absorption at 270–280 nm. Some masses

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sporadically detected, and probably pertaining to this class of proteins, are not reported in Table ~~I~~I.

Broad peaks eluting in the bPRPs chromatographic cluster were attributed to gPRPs on the basis of the crowded ESI spectra, which usually did not allow deconvolution. Recently, we were able to characterized the structure of six glycoforms of IB-8a CON1+ [19]. Five of the glycoforms carry a biantennary N-linked glycan fucosylated in the innermost N-acetylglucosamine of the core and showing from zero to four additional fucoses in the antenna. The sixth glycoform carries a monoantennary mono-fucosylated oligosaccharide. Level of fucosylation showed inter-individual variability with the major relative abundance for the tri-fucosylated glycoform. Non glycosylated IB-8a CON1+ and the variant IB-8a CON1-, lacking of the glycosylation site, have been also detected in human saliva [19].

Acidic proline-rich proteins ~~(aPRPs)~~

Acidic proline-rich proteins (aPRPs) are secreted both by parotid (about 70-%) and submandibular/sublingual glands (about 30%). They are the expression products of two loci, *PRH1* and *PRH2* located on chromosome 12p13, near to the cluster of bPRPs. *PRH1* codes for the PIF-s, Db-s and Pa isoforms, *PRH2* codes for the PRP-1 and PRP-2 isoforms. Therefore, five aPRP isoforms can be detected in the saliva of the Caucasian population, but considering that PRP-1 and Pif-s have the same mass and PRP-2 has 1 Da mass difference with respect to PRP-1 and Pif-s, these 3 isoforms cannot be usually discriminated in the HPLC-ESI-MS profile [20]. Moreover, TFA adducts can be detected for these isoforms [3]. All the isoforms have a pyroglutamic moiety at the N-terminus and are usually di-phosphorylated on Ser-7 and Ser-22, even though minor quantities of mono-, non-phosphorylated and tri-phosphorylated isoforms (on Ser-17) are also detectable [20]. Four of these isoforms (PRP-1, PRP-2, PIF-s and Db-s) can be partially cleaved near to the C-

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terminus, eventually releasing a common peptide of 44 a.a. residues (P-C peptide) and 4 truncated isoforms called PRP-3, PRP-4, PIF-f and Db-f. The Pa isoform is not cleaved, and it was usually detected in saliva as a S-S dimer due to the specific presence of a cysteine residue (Cys-103) in its structure. Minor quantities of other derivatives missing C-terminal residues from almost all isoforms were also detected [20].

Statherin and P-B peptide

Statherin is an unusual tyrosine-rich phospho-peptide (phosphorylated on Ser-2 and Ser-3) involved in oral cavity calcium ion homeostasis and teeth mineralization [21,22]. Its gene (*STATH*) is localized on chromosome 4q13.3, near to histatin genes [23]. In adult human saliva mono- and non-phosphorylated, as well as N- and C-terminal truncated isoforms are always detectable [24]. Furthermore, in adult human saliva minor amounts of a cyclic-statherin derivative (cyclo-statherin Q37) are detectable [25]. The cyclo-structure derives from an intra-molecular bridge between Lys-6 and Gln-37 generated by the action of oral transglutaminase 2 on statherin.

P-B peptide, after the determination of its structure, was (erroneously) included in the bPRPs family. However, it is the product of *PROL3* gene, ~~localised~~ localized on chromosome 4q13.3, very close to the statherin gene. Differently from classical bPRPs, P-B peptide is not a fragment of a bigger pro-protein, it is secreted both from parotid and Sm/Sl glands [26] and it displays three Tyr residues in the sequence. Statherin and P-B peptide elute closely in the chromatographic profile suggesting a similar polarity. For these reasons, P-B peptide could be functionally connected to statherin. However, while the statherin role on the modulation of oral calcium ion was recognized, none specific function for P-B peptide has been proposed to date.

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Salivary (“S”-type) and other cystatins

Cystatins are detected in the RP-HPLC-ESI-MS chromatographic profile after the peaks of statherin and P-B peptides [27]. "S-type" cystatins comprise cystatin S, SN and SA which belong to family 2 of cystatins, inhibitors of cysteine-proteinases and are mainly secreted by Sm/Sl glands. Recent studies suggested that their secretion is not granule-mediated [26]. Cystatin S may be mono-phosphorylated on Ser-3 (cystatin S1; about 65-%) or di-phosphorylated on Ser-1 and Ser-3 (cystatin S2; about 25%). Cystatin C was frequently detectable in human saliva, while, until now, no protein mass detected in saliva could be attributed to cystatins D and M. Cystatin A and B (called also stefins) belong to family 1 of cystatins, differing from type 2 cystatins for size and phosphorylation. Cystatin A was detectable in 2 isoforms (acetylated and non-acetylated on its N-terminal ~~[8]~~) [8]. Cystatin B was N-terminally acetylated and usually it was usually not detected as unmodified protein in adult whole saliva, because of the reactivity of Cys-3 residue. Cystatin B, indeed was present in whole saliva as S-glutathionylated (about 55-%) S-cysteinylated (about 15-%) derivatives or as S-S dimer (about 30-% ~~[28]~~) [28].

Proteins of the S100 family

Proteins of the S100 family elute in the terminal part of the chromatographic profile. We were able to identify S100A7 (D_{27} and E_{27} isoforms), S100A8, S100A9 (8 isoforms: short, long, long S-glutathionylated, long S-cysteinylated and their phosphorylated counterparts), S100A11 and S100A12 [8]. The difficulty in the characterization of these proteins relied in the mismatch between the theoretical masses reported in data banks and the experimental masses of mature proteins [8]. For this reason, some of the masses pending for characterization could pertain to S100 B, S100A5 and S100A16. The source of these proteins in whole human saliva is unknown.

α-defensins and β-thymosins

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α-Defensins 1-3 elute as a unique chromatographic peak after the aPRPs cluster. α-Defensin 4 eluted separately about 2 min before the statherin peak. α-Defensins belong to a family of broad-spectrum antimicrobial peptides, identified originally in human and rabbit leucocytes. 6 α-defensins (cryptidins) have been identified in humans to date. α-Defensins 1-4 are expressed in neutrophils, whereas α-defensins 5-6 are expressed in epithelial cells of the intestinal and reproductive tracts [29]. The name of β-thymosins derives from their first characterization from calf thymus extracts. Different studies have evidenced their ubiquitous presence in many organs and tissues as well as in various bodily fluids. Thymosin β_4, the most abundant, and thymosin β_{10} are typical in humans [30]. Gingival crevicular fluid is one of the main source of these two peptide classes [31,32].

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Miscellaneous proteins and peptides

Some other proteins have been characterized along the HPLC-ESI-MS profile. SPRR3 (small-proline-rich protein 3) was mainly detectable in preterm newborn saliva in two variants, one with a M_{av} of 17239 ± 3 Da and the other with a M_{av} of 18065 ± 3 Da [33]. Therefore, 3 different phenotypes (2 homozygous and 1 heterozygous) can be found in the western population. Antileucoproteinase, also mainly detectable in preterm newborn saliva, elutes between α-defensins 1-3 and SPRR3 [8]. Lysozyme elutes near to the S-type cystatins. The protein with a M_{av} 22365 ± 4 Da was identified as the histone H1c, but the structure is pending for unambiguous characterization [8]. At the end of the chromatographic profile α, β and γ (both Gγ and Aγ) globins were sometimes detectable. In the terminal region of the HPLC profile other masses probably pertaining to α-amylase were sometimes detectable. However, the ESI spectrum was often crowded by multiple charge values, suggesting heterogeneity for this and other proteins which are not reported in Table ~~1~~I.

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Fragments of bigger proteins and peptides

The most relevant PTM of salivary proteins is the proteolytic cleavage by a complex and not well known set of endo- and exo-proteinases that generates a multitude of small peptides. On the basis of the RXXR↓ consensus sequence, some of the endo-proteinases probably belong to the furin-like convertase family, work before granule storage and are responsible for the generation of bPRPs, truncated isoforms of aPRPs, P-C peptide and histatin 6 [11]. The convertase acting on bPRPs is responsible for a complete digestion of the pro-protein, while aPRPs convertase, more active in Sm/Sl gland, is responsible for a partial cleavage of the proteins. Also the proteinase responsible for the first cleavage of histatin 3 can be included in the convertase family, because 2 Arg separated by 2 a.a. residues seems a mandatory requirement for the enzyme recognition. In fact, histatin 1, lacking the Arg-25 residue present in histatin 3, is less prone to proteolysis [11,26].

The removal of a C-terminal residue by specific carboxypeptidases following the convertase cleavage is a widespread event in many secretion processes and it can be observed also in many salivary peptides. One of the most relevant a.a. removal, in terms of relative abundance, involves the C-terminal Arg-25 of histatin 6 generating histatin 5 [11].

Other peptidases of endogen or exogen origin are active after granule secretion and in the oral cavity. Some salivary proteins, such as bPRPs, P-C and P-B peptides, statherin, histatin 3 and histatin 5 are more prone to proteolysis than other salivary proteins. Some fragments of glyceraldehyde 3-phosphate dehydrogenase were often detected, especially in saliva of preterm newborns, but the intact protein was not found in human saliva till now. Other authors have studied the naturally occurring peptides generated by oral proteolytic activity [34,35]. Table 1 [reports some of the fragments identified by Huq and coll. [34] that we were able to consistently detect by HPLC-ESI-MS in different samples of whole saliva. The aim of a study of Helmerhorst and coll. [35] was the detection of specific proteinase activities in the oral cavity. They found a lot of salivary protein fragments which

allow revealing, in the oral cavity, the activity of a specific glutamine endoproteinase that recognizes KPQ↓ as the main consensus sequence. This proteinase probably derives from dental plaque and it is likely microbial in origin. Table 1 reports only some fragments of this list, because their presence in saliva immediately after specimen collection has to be confirmed. Some of the bPRP fragments detected by us and other researchers have been also reported by Vitorino et al. as recurrent non-covalent bound components of the enamel surface. This finding might provide a functional significance to this final bPRP maturation [36].

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Other post-translational modifications

Beyond cleavage, salivary proteins are submitted to many other PTMs before, during and after-secretion. Glycosylation is a relevant PTM of salivary proteins, but as previously mentioned, salivary glycosylated proteins are not easily evidenced by top-down proteomic analysis because of the high heterogeneity. Many salivary proteins are phosphorylated. Top-down platform evidenced 2 major (Ser-8 and Ser-22) and 1 minor site (Ser-17) on all aPRPs, 1 site (Ser-8) on II-2 and IB-1 (bPRPs), 2 sites (Ser-2 and Ser-3) on statherin, 1 site (Ser-2) on histatin 1 and 2 sites on cystatin S (Ser-2 and Ser-3). Due to the similar SX (E/Sp) consensus sequence, the kinase responsible for the phosphorylation of these sites should be an elusive Golgi casein kinase acting before granule storage [37]. About 40% of all the S100A9 isoforms were found to be phosphorylated on the penultimate Thr of the sequence by p38 MAP kinase [38].

Until now O-tyrosine sulfation of salivary proteins has been detected only on histatin 1 [12]. This modification was found to be specific of Sm/Sl glands, where not more than 10% of total histatin 1 could be detected as a mixture of isoforms carrying a different number of sulfate moieties (from 1 to 4). This PTM was probably hierarchical, being Tyr-27 the first residue submitted to sulfation, followed by Tyr-30, Tyr-34 and Tyr-36 [12].

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Specific cysteine residues of cystatin B, S100A9 and SPRR3 appeared prone to S-glutathionylation and S-cysteinylation. These PTMs were particularly extensive for cystatin B, because the S-unmodified protein was often undetectable in adult whole saliva [28]. Cysteine residues were also responsible for several covalent dimerization. Only Pa S-S 2-mer (Pa is one of the 5 isoforms of aPRPs) was detectable in whole saliva. About 30% of cystatin B and small amounts of SPRR3 were also detectable as S-S 2-mer. Because SPRR3 exists in 2 isoforms, both homo- and hetero-dimers were detectable in heterozygosis.

Methionine residues are often prone to the oxidation (sulfoxide and sulfone). Met-sulfoxide derivatives of thymosin β_4 (Met-6), S100A9 short (Met-89) and "S-type" cystatins were detected in whole saliva. These derivatives could be indicative of oxidative stress in the oral cavity.

In the presence of a glutamine or a glutamic acid, the N-terminal residue of many salivary proteins was typically cyclized in pyroglutamic acid, precluding Edman sequencing. Identified examples were the entire and truncated isoforms of all aPRPs, the bPRPs named IB-1 and II-2, as well as the P-B peptide.

N-terminal acetylation was another PTM largely found in many identified proteins. It was usually reported that this PTM was subsequent to the removal of the N-terminal Met residue [39]. S100A7 (both D_{27} and E_{27} isoforms), S100A11, SPRR3 and β-thymosins followed this rule. PTMs of S100A9 are particular. Two phosphorylated and non-phosphorylated isoforms, called long isoforms, derive from the removal of the N-terminal Met residue followed by acetylation. These 2 isoforms can be also partly S-glutathionylated and S-cysteinylated (on Cys-2 residue) bringing to 6 the total number of S100A9 long isoforms detectable in whole saliva. On the other hand, other 2 S100A9 isoforms, called short isoforms (phosphorylated and non-phosphorylated) derive from acetylation after removal of the N-terminal pentapeptide (MTCKM). Because this removal eliminates the Cys-2 residue

from the S100A9 sequence, the S100A9 short isoforms does not originate S-modified derivatives. S100A12 loose the N-terminal Met residue, but it is not acetylated, cystatin B does not loose the N-terminal Met residue, but it is not found acetylated and S100A8 does not loose the N-terminal Met residue and it is not acetylated. Cystatin A was detected in 3 different isoforms, 1 with the N-terminal Met residue, 1 acetylated on the N-terminal Met and 1 (M_{av} 10872 ± 2 Da) without the N-terminal Met and non-acetylated (this last pending for definitive characterization). This puzzling situation outlines that probably each protein has specific structural requirements on its N-terminal residues. However, the understanding of the molecular signals that any protein utilizes to drive its correct N-terminal modification and the functional significance of the different N-terminal modifications occurring in the same protein is surely a challenging task.

Source of salivary proteins

Most of the information reported in Table 1 on the source of salivary proteins was obtained by a top-down proteomic study that compared the natural occurring proteome of adult whole saliva with the proteins detectable in glandular (parotid and Sm/Sl) saliva and enriched granule preparation from parotid and submandibular glands [26]. This study confirmed that bPRPs were exclusively secreted by the parotid gland. Approximately 70% of aPRPs isoforms was found to be secreted by parotid glands, the remaining 30% by Sm/Sl glands. Also, histatin 3 was secreted more by parotid than by Sm/Sl glands. Histatin 1, statherin and P-B peptide were roughly secreted in similar amounts by both major glands, while the main sources of “S type” cystatins were Sm/Sl glands. Gingival crevicular fluid was a relevant source of cystatin A, α-defensins and β-thymosins [30,32]. Conversely, in preterm saliva the high amount of β-thymosins derived from major salivary glands [40] even though the secretion pathway of this leaderless peptide [31] is still unknown. The sources and the secretion pathways of other proteins reported in Table 1 are not still well defined too.

~~The s~~Surprising differences of salivary proteome in pre-term newborns and in the pediatric age~~.~~

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Fig~~-~~ure 2 shows the striking differences present in human salivary proteome as a function of age, with a particular concern to the pediatric age [7]. Given to the non-invasive collection of the sample the salivary proteome of preterm newborns was investigated from about 195 days of post-conceptional age (PCA~~[6,8]~~) [6,8]. In preterm newborns of about 195–220 days of PCA noticeable amounts of more than 40 proteins were detectable. Cystatin A (2 isoforms), cystatin B (3 isoforms), S100A7 (2 isoforms), S100A8, S100A9 (8 isoforms), S100A11, S100A12, small proline-rich protein 3 (2 isoforms), lysozyme C, thymosins β_4 and β_{10}, antileukoproteinase, histone H1c, and α and γ globins [8] have been identified. The concentration of these proteins decreased quickly, even though with different trends, as a function of PCA reaching values detectable in adult at a PCA corresponding to the normal term of delivery (about 270 days). At the same time, the level of salivary proteins characteristic of the adult increased, also in this case with different trends.

Many of the proteins identified in saliva of pre-term newborns are considered tumor markers in the adults. This observation led to suppose that these proteins might contribute to the molecular events that regulate cell growth, proliferation, or death during fetal development. The abnormal expression in the adults might be at the basis of anomalous cellular growth and to the development of different tumors with embryonic etiology. The recognition of tumor stem cells in many solid cancers has reinvigorated the hypothesis of a pluripotent stem cell as the cell of origin for cancer [41]. Data from our group on Tβ4 expression in salivary glands' tumors and in colon cancer [43] evidenced Tβ4 reactivity in tumor cells undergoing epithelial-mesenchymal transition, a highly conserved cellular

program typical of several stages of embryonic development as well as of cancer invasion and metastasis [44].

A study carried out on 67 subjects aged between 3 and 44 years evidenced several qualitative and quantitative age-dependent modifications of the salivary proteome. It was found that the concentration of salivary acidic proline-rich phosphoproteins, histatin-5, histatin-6, and monophosphorylated and diphosphorylated cystatin S showed a minimum between 6 and 9 years of age. Interestingly, bPRPs, almost absent in saliva of children, reached adult levels only after puberty [7], suggesting a potential role of these peptides in the modulation of taste perception. Indeed a recent study of our group showed for the first time that responsiveness to 6-n-propylthiouracil bitter taste is associated with salivary levels of II-2 peptide and Ps-1 protein, which are products of the PRB1 gene [45].

It is relevant to outline that the striking age-dependent modification of the human salivary proteome has to be carefully considered for the choice of the proper control group in the characterization of new disease biomarkers, relevant aim of any proteomic platform.

Pathological modification of the human salivary proteome detected by the top-down approaches.

A conclusive aim of a proteomic dataset is its use for diagnostic and prognostic purposes. Due to the non invasive specimen collection saliva is very attractive in this regard. The protein list reported in this review could be a helpful reference for the detection of potential early biomarker of disease by using quantitation based on XIC area determination, as shown in Figure~~Fig.~~ 3. Top-down analyses for the detection of the variations of the salivary proteome in other local and systemic diseases are in progress upon our laboratories.

Studies carried out on different patients with autism spectrum disorders (ASD) evidenced the hypo-phosphorylation of several salivary peptides and proteins (histatin 1,

statherin and both the entire and truncated isoforms of aPRPs) in a subset of about 60% of ASD subjects, the majority comprised in the normal to border-line cognitive development [46]. Hypo-phosphorylation of salivary peptides suggested potential asynchronies in the phosphorylation of other secretory proteins, which could be relevant in central nervous system during either embryonic development or early infancy.

A study carried out on whole saliva of 31 children affected by type 1 diabetes revealed a lower concentration of statherin, proline-rich peptides, P-B, P-C peptides, and histatins, and higher concentration of α-defensins 1, 2 and 4 and S100A9 short isoforms with respect to an age and sex matched control group [47]. The lower concentration of P-C peptide was paralleled by higher levels of some of its fragments. On the whole, the study highlighted the severe impairment of the repertoire of peptides involved in the safeguard of the oral cavity in diabetic children. A study carried out on 9 patients with primary Sjögren syndrome (SS) evidenced that pilocarpine treatment restored the protein levels and partially restored the protein numbers that were found to be decreased in primary SS patients, with the parotid gland proteins showing the best response to the drug [48,49]. Finally, a study carried out on 11 totally edentulous patients evidenced reduced levels of α-defensins respect to two groups of controls (one matched for age and gender, the second of younger subjects~~[50]~~) [50]. Since these peptides have mainly crevicular origin, most likely the low levels measured resulted from the absence of the gingival sulcus in the edentulous subjects.

Concluding remarks

A limit of the "solution-based" top-down platform utilized was that many masses attributable to well know salivary proteins, such as carbonic anhydrase, immunoglobulin, peroxidases, mieloperoxidases, mucins were never detected in the HPLC-ESI-MS profile, likely due to their insolubility in acidic solution. It should be taken into account that the limit of sensitivity

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of the HPLC-ESI-MS apparatus in use upon in laboratories on saliva samples was, at the best, in the range of 10–50 nanomoles/L and that increase of instrumental sensitivity can disclose a lot of other protein and peptide masses that might be added to the list in the future. Table ~~1~~ I is a dynamic table that anyone can implement with new attributions. It can help not only researchers that would experience top-down platforms on human saliva, but also anyone who is involved in top-down attributions in other bodily fluid, because many proteins reported are not specific of saliva.

Declaration of Interest: ~~Acknowledgements~~

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References

1. Helmerhorst EJ, Oppenheim FG. Saliva: a dynamic proteome. J Dent Res 2007;86:680–693.
2. Amado F, Lobo MJ, Domingues P, Duarte JA, Vitorino R. Salivary peptidomics. Expert Rev Proteomics 2010;7:709–721.
3. Messana I, Inzitari R, Fanali C, Cabras T, Castagnola M. Facts and artifacts in proteomics of body fluids. What proteomics of saliva is telling us? J Sep Sci 2008;31:1948–1963.
4. Al-Tarawneh SK, Border MB, Dibble CF, Bencharit S. Defining salivary biomarkers using mass spectrometry-based proteomics: a systematic review. OMICS 2011;15:353–361.

5. Tipton JD, Tran JC, Catherman AD, Ahlf DR, Durbin KR, Kelleher NL. Analysis of intact protein isoforms by mass spectrometry. J Biol Chem 2011;286:25451–25458.

6. Inzitari R, Vento G, Capoluongo E, Boccacci S, Fanali C, Cabras T, Romagnoli C, et al. Proteomic analysis of salivary acidic proline-rich proteins in human preterm and at-term newborns. J Proteome Res 2007;6:1371–1377.

7. Cabras T, Pisano E, Boi R, Olianas A, Manconi B, Inzitari R, Fanali C, et al. Age-dependent modifications of the human salivary secretory protein complex. J Proteome Res 2009;8:4126–4134.

8. Castagnola M, Inzitari R, Fanali C, Iavarone F, Vitali A, Desiderio C, Vento G, et al. The surprising composition of the salivary proteome of preterm human newborn. Mol Cell Proteomics 2011;10:M110.003467.

9. Oppenheim FG, Xu T, McMillian FM, Levitz SM, Diamond RD, Offner GD, Troxler RF. Histatins, a novel family of histidine-rich proteins in human parotid secretion. Isolation, characterization, primary structure, and fungistatic effects on Candida albicans. J Biol Chem 1988;263:7472–7477.

10. Sabatini LM, Azen EA. Histatins, a family of salivary histidine-rich proteins, are encoded by at least two loci (HIS1 and HIS2). Biochem Biophys Res Commun 1989;160:495–502.

11. Castagnola M, Inzitari R, Rossetti DV, Olmi C, Cabras T, Piras V, Nicolussi P, et al. A cascade of 24 histatins (histatin 3 fragments) in human saliva. Suggestions for a pre-secretory sequential cleavage pathway. J Biol Chem 2004;279:41436–41443.

12. Cabras T, Fanali C, Monteiro JA, Amado F, Inzitari R, Desiderio C, Scarano E, et al. Tyrosine polysulfation of human salivary histatin 1. A post-translational modification specific of the submandibular gland. J Proteome Res 2007;6:2472–2480.

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13. Kauffman DL, Bennick A, Blum M, Keller PJ. Basic proline-rich proteins from human parotid saliva: relationships of the covalent structures of ten proteins from a single individual. Biochemistry 1991;30:3351–3356.

14. Isemura S, Saitoh E, Sanada K. Fractionation and characterization of basic proline-rich peptides of human parotid saliva and the amino acid sequence of proline-rich peptide P-E. J Biochem 1982;91:2067–2075.

15. Isemura S, Saitoh E, Sanada K. Further fractionation of basic proline-rich peptides from human parotid saliva and complete amino acid sequence of basic proline-rich peptide P-H. J Biochem 1983;94:1991–1999.

16. Messana I, Cabras T, Inzitari R, Lupi A, Zuppi C, Olmi C, Fadda MB, et al. Characterization of the human salivary basic proline-rich protein complex by a proteomic approach. J Proteome Res 2004;3:792–800.

17. Azen EA, Latreille P, Niece RL. PRBI gene variants coding for length and null polymorphisms among human salivary Ps, PmF, PmS, and Pe proline-rich proteins (PRPs). Am J Hum Genet 1993;53:264–278.

18. Azen EA, Amberger E, Fisher S, Prakobphol A, Niece RL. PRB1, PRB2, and PRB4 coded polymorphisms among human salivary concanavalin-A binding, II-1, and Po proline-rich proteins. Am J Hum Genet 1996;58:143–153.

19. Cabras T, Boi R, Pisano E, Iavarone F, Fanali C, Nemolato S, Faa G, et al. HPLC-ESI-MS and MS/MS structural characterization of multifucosylated N-glycoforms of the basic proline-rich protein IB-8a CON1+ in human saliva. J Sep Sci 2012;35:1079–1086.

20. Inzitari R, Cabras T, Onnis G, Olmi C, Mastinu A, Sanna MT, Pellegrini MG, et al. Different isoforms and post-translational modifications of human salivary acidic proline-rich proteins. Proteomics 2005;5:805–815.

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21. Schlesinger DH, Hay DI. Complete covalent structure of statherin, a tyrosine-rich acidic peptide which inhibits calcium phosphate precipitation from human parotid saliva. J Biol Chem 1977;252:1689–1695.

22. Schwartz SS, Hay DI, Schluckebier SK. Inhibition of calcium phosphate precipitation by human salivary statherin: structure-activity relationships. Calcif Tissue Int 1992;50:511–517.

23. Sabatini LM, Carlock LR, Johnson GW, Azen EA. cDNA cloning and chromosomal localization (4q11-13) of a gene for statherin, a regulator of calcium in saliva. Am J Hum Genet 1987;41:1048–1060.

24. Inzitari R, Cabras T, Rossetti DV, Fanali C, Vitali A, Pellegrini M, Paludetti G, et al. Detection in human saliva of different statherin and P-B fragments and derivatives. Proteomics 2006;6:6370–6379.

25. Cabras T, Inzitari R, Fanali C, Scarano E, Patamia M, Sanna MT, Pisano E, et al. HPLC-MS characterization of cyclo-statherin Q-37, a specific cyclization product of human salivary statherin generated by transglutaminase 2. J Sep Sci 2006;29:2600–2608.

26. Messana I, Cabras T, Pisano E, Sanna MT, Olianas A, Manconi B, Pellegrini M, et al. Trafficking and postsecretory events responsible for the formation of secreted human salivary peptides: a proteomics approach. Mol Cell Proteomics 2008;7:911–926.

27. Lupi A, Messana I, Denotti G, Schininà ME, Gambarini G, Fadda MB, Vitali A, et al. Identification of the human salivary cystatin complex by the coupling of high-performance liquid chromatography and ion-trap mass spectrometry. Proteomics 2003;3:461–467.

28. Cabras T, Manconi B, Iavarone F, Fanali C, Nemolato S, Fiorita A, Scarano E, et al. RP-HPLC-ESI-MS evidenced that salivary cystatin B is detectable in adult human

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whole saliva mostly as S-modified derivatives: S-Glutathionyl, S-cysteinyl and S-S 2-mer. J Proteomics 2012;75:908–913.

29. Ganz T, Lehrer RI. Defensins. Curr Opin Immunol 1994;6:584–589.

30. Hannappel E. β~~beta~~-Thymosins. Ann N Y Acad Sci 2007;1112:21–37.

31. Pisano E, Cabras T, Montaldo C, Piras V, Inzitari R, Olmi C, Castagnola M, Messana I. Peptides of human gingival crevicular fluid determined by HPLC-ESI-MS. Eur J Oral Sci 2005;113:462–468.

32. Inzitari R, Cabras T, Pisano E, Fanali C, Manconi B, Scarano E, Fiorita A, et al. HPLC-ESI-MS analysis of oral human fluids reveals that gingival crevicular fluid is the main source of oral thymosins β~~beta~~(4) and β~~beta~~(10). J Sep Sci 2009;32:57–63.

33. Manconi B, Cabras T, Pisano E, Nemolato S, Inzitari R, Iavarone F, Fanali C, et al. Characterization of two isoforms of human SPRR3 from saliva of preterm human newborn and autoptic fetal oral mucosa, parotid and submandibular gland samples. Biochem Biophys Res Commun 2010;398:477–481.

34. Huq NL, Cross KJ, Ung M, Myroforidis H, Veith PD, Chen D, Stanton D, ~~He H, Ward BR, Reynolds EC~~et al. A review of the salivary proteome and peptidome and saliva-derived therapeutics. Int J Pept Res Ther 2007;13:547–564.

35. Helmerhorst EJ, Sun X, Salih E, Oppenheim FG. Identification of Lys-Pro-Gln as a novel cleavage site specificity of saliva-associated proteases. J Biol Chem 2008;283:19957–19966.

36. Vitorino R, Calheiros-Lobo MJ, Williams J, Ferrer-Correia AJ, Tomer KB, Duarte JA, Domingues PM, Amado FM. Peptidomic analysis of human acquired enamel pellicle. Biomed Chromatogr 2007;21:1107–1117.

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37. Tibaldi E, Arrigoni G, Brunati AM, James P, Pinna LA. Analysis of a sub-proteome which co-purifies with and is phosphorylated by the Golgi casein kinase. Cell Mol Life Sci 2006;63:378–389.

38. Lominadze G, Rane MJ, Merchant M, Cai J, Ward RA, McLeish KR. Myeloid-related protein-14 is a p38 MAPK substrate in human neutrophils. J Immunol 2005;174:7257–7267.

39. Arnesen T, Van Damme P, Polevoda B, Helsens K, Evjenth R, Colaert N, Varhaug JE, et al. Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proc Natl Acad Sci USA 2009;106:8157–8162.

40. Nemolato S, Messana I, Cabras T, Manconi B, Inzitari R, Fanali C, Vento G, et al. Thymosin βbeta(4) and βbeta(10) levels in pre-term newborn oral cavity and foetal salivary glands evidence a switch of secretion during foetal development. PLoS ONE 2009;4:e5109.

41. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–111.

42. Grichnik JM. Melanoma, nevogenesis, and stem cell biology. J Invest Dermatol 2008;128:2365–2380.

43. Nemolato S, Restivo A, Cabras T, Coni P, Zorcolo L, Orrù G, Fanari M, et al. Thymosin ß 4 in colorectal cancer is localized predominantly at the invasion front in tumor cells undergoing epithelial mesenchymal transition. Cancer Biol Ther 2012;13:191–197.

44. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Dev Cell 2008;14:818–829.

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45. Cabras T, Melis M, Castagnola M, Padiglia A, Tepper BJ, Messana I, Tomassini Barbarossa I. Responsiveness to 6-n-propylthiouracil (PROP) is associated with salivary levels of two specific basic proline-rich proteins in humans. PLoS ONE 2012;7:e30962.

46. Castagnola M, Messana I, Inzitari R, Fanali C, Cabras T, Morelli A, Pecoraro AM, et al. Hypo-phosphorylation of salivary peptidome as a clue to the molecular pathogenesis of autism spectrum disorders. J Proteome Res 2008;7:5327–5332.

47. Cabras T, Pisano E, Mastinu A, Denotti G, Pusceddu PP, Inzitari R, Fanali C, et al. Alterations of the salivary secretory peptidome profile in children affected by type 1 diabetes. Mol Cell Proteomics 2010;9:2099–2108.

48. Peluso G, De Santis M, Inzitari R, Fanali C, Cabras T, Messana I, Castagnola M, Ferraccioli GF. Proteomic study of salivary peptides and proteins in patients with Sjögren's syndrome before and after pilocarpine treatment. Arthritis Rheum 2007;56:2216–2222.

49. Rigante D, Inzitari R, Carone M, Fanali C, Stabile A, Cabras T, Capoluongo E, et al. Correspondence between clinical improvement and proteomic changes of the salivary peptide complex in a child with primary Sjögren syndrome. Rheumatol Int 2008;28:801–806.

50. Fanali C, Inzitari R, Cabras T, Pisano E, Castagnola M, Celletti R, Manni A, Messana I. alpha-Defensin levels in whole saliva of totally edentulous subjects. Int J Immunopathol Pharmacol 2008;21:845–849.

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Table ~~1~~I. -List of 256 peptide and protein masses detected in whole saliva by a "solution based" top-down HPLC-ESI-MS platform.

	M aver exper.[a]	Protein identification	Swiss Prot n.	M+H+ theor.	M aver theor.	Elut. time[b]	PTM modification	Increase-decrease	Origin	Reference
1	523.2	~~histatin~~ Histatin 3 Fr. 29–32	P15516	524.235	523.543	8.3			~~both~~ Both major glands (↑ parotid)	[11,26]
2	556.3	~~histatin~~ Histatin 3 Fr. 1-5	P15516	557.268	556.576	5.9			~~both~~ Both major glands (↑ parotid)	[11,26]
3	597.3	~~histatin~~ Histatin 3 Fr. 2-6	P15516	598.342	597.675	5.5			~~both~~ Both major glands (↑ parotid)	[11,26]
4	640.3	~~histatin~~ Histatin 3 Fr. 7-11	P15516	641.315	640.700	5.6			~~both~~ Both major glands (↑ parotid)	[11,26]
5	686.3	~~histatin~~ Histatin 3 Fr. 28–32	P15516	687.298	686.719	12.5			~~both~~ Both major glands (↑ parotid)	[11,26]
6	710.4	bPRP fragment	~~various~~Various	710.383	709.800	6.8		↓ paediatric age	~~only~~ Only parotid	[7,26,34]
7	712.4	~~histatin~~ Histatin 3 Fr. 1-6	P15516	713.369	712.764	6.1			~~both~~ Both major glands (↑ parotid)	[11,26]
8	720.4	bPRP fragment	~~various~~Various	720.368	719.795	8.1		↓ paediatric age	~~only~~ Only parotid	[7,26,34]
9	721.4	bPRP fragment	~~various~~Various	721.352	720.780	7.9		↓ paediatric age	~~only~~ Only parotid	[7,26,34]
10	755.4	~~histatin~~ Histatin 3 Fr. 19–24	P15516	756.354	755.791	8.7			~~both~~ Both major glands (↑ parotid)	[11,26]

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11	796.4	~~histatin~~ Histatin 3 Fr. 6-11	P15516	797.417	796.887	7.6			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
12	796.4	~~histatin~~ Histatin 3 Fr. 7-12	P15516	797.417	796.887	7.8			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
13	800.3	~~histatin~~ Histatin 3 Fr. 27–32	P15516	801.341	800.823	14.1			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
14	817.5	bPRP fragment	~~various~~Various	817.457	816.956	8.5		↓ paediatric age	~~only~~ Only parotid	[26,34]	**Formatted:** English (U.S.)
15	874.5	bPRP fragment	~~various~~Various	874.478	874.007	8.2		↓ paediatric age	~~only~~ Only parotid	[7,26,34]	**Formatted:** English (U.S.)
16	887.4	~~histatin~~ Histatin 3 Fr. 26–32	P15516	888.373	887.901	13.8			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
17	895.4	P-C Fr. 36–44	P02810	895.427	894.940	6.9		↑ type 1 diabetes	~~both~~ Both major glands (↑ parotid)	[47]	**Formatted:** English (U.S.)
18	914.5	bPRP fragment	~~various~~Various	914.509	914.072	10.2		↓ paediatric age	~~only~~ Only parotid	[26,34]	**Formatted:** English (U.S.)
19	924.5	~~histatin~~ Histatin 3 Fr. 5-11 (Hst 12)	P15516	925.512	925.061	7.2			~~both~~ Both major glands (↑ parotid)	[9,11,26]	**Formatted:** English (U.S.)
20	924.5	~~histatin~~ Histatin 3 Fr. 7-13	P15516	925.512	925.061	6.2			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
21	971.5	bPRP fragment	~~various~~Various	971.531	971.124	9.4		↓ paediatric age	~~only~~ Only parotid	[7,26,34]	**Formatted:** English (U.S.)
22	990.5	P-C Fr. 26–35	P02810	990.512	990.087	8.2		↑ type 1 diabetes	~~both~~ Both major glands (↑ parotid)	[26,34,35,47]	**Formatted:** English (U.S.)
23	1038.4	bPRP fragment	~~various~~Various	1038.533	1038.128	10.7		↓ paediatric age	~~only~~ Only parotid	[26,34]	**Formatted:** English (U.S.)

24	1067.6	bPRP fragment	~~various~~Various	1067.559	1067.170	9.7		↓ ~~p~~aediatric age	~~only~~ Only parotid	[7,26,34]	**Formatted:** English (U.S.)
25	1070.6	bPRP fragment	~~various~~Various	1070.611	1070.260	9.5		↓ ~~p~~aediatric age	~~only~~ Only parotid	[7,26,34]	**Formatted:** English (U.S.)
26	1071.6	bPRP fragment	~~various~~Various	1071.595	1071.245	9.5		↓ ~~p~~aediatric age	~~only~~ Only parotid	[7,26,34]	**Formatted:** English (U.S.)
27	1076.5	bPRP fragment	~~various~~Various	1076.512	1076.134	7.0		↓ ~~p~~aediatric age	~~only~~ Only parotid	[7,26,34]	**Formatted:** English (U.S.)
28	1076.5	bPRP fragment	~~various~~Various	1076.548	1076.177	6.8		↓ ~~p~~aediatric age	~~only~~ Only parotid	[7,26,34]	**Formatted:** English (U.S.)
29	1080.6	~~histatin~~ Histatin 3 Fr. 5-12 (Hst 11)	P15516	1081.613	1081.249	11.4			~~b~~Both major glands (↑ parotid)	[9,11,26]	**Formatted:** English (U.S.)
30	1080.6	~~histatin~~ Histatin 3 Fr. 6-13	P15516	1081.613	1081.249	9.1			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
31	1152.6	bPRP fragment	P02812	1152.576	1152.232	9.1		↓ ~~p~~aediatric age	~~only~~ Only parotid	[26,34]	**Formatted:** English (U.S.)
32	1165.5	bPRP fragment	~~various~~Various	1165.523	1165.184	7.0		↓ ~~p~~aediatric age	~~only~~ Only parotid	[7,26,34]	**Formatted:** English (U.S.)
33	1200.4	P-B peptide Fr. 46–57	P02814	1200.641	1200.403	21.1			~~both~~ Both major glands	[34]	**Formatted:** English (U.S.)
34	1208.7	~~histatin~~ Histatin 3 Fr. 5-13	P15516	1209.708	1209.423	9.5			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
35	1222.6	bPRP fragment	~~various~~Various	1222.617	1222.323	8.2		↓ ~~p~~aediatric age	~~only~~ Only parotid	[7,26,34]	**Formatted:** English (U.S.)
36	1224.6	aPRP Fr. 94–105	P02810	1224.623	1224.345	11.1		↑ type 1 diab.	~~both~~ Both major glands (↑ parotid)	[47]	**Formatted:** English (U.S.)

37	1286.6	~~histatin~~ Histatin 3 Fr. 15–24	P15516	1287.609	1287.363	9.2			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
38	1334.7	~~histatin~~ Histatin 3 Fr. 1-11	P15516	1335.667	1335.448	7.8			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
39	1341.7	bPRP fragment	~~various~~Various	1341.655	1341.446	10.1		↓ ~~pa~~ediatric age	~~only~~ Only parotid	[26,34]	**Formatted:** English (U.S.)
40	1433.7	~~histatin~~ Histatin 3 Fr. 14–24	P15516	1434.677	1434.540	11.6			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
41	1442.7	~~histatin~~ Histatin 3 Fr. 15–25	P15516	1443.710	1443.550	9.6			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
42	1471.7	P-C Fr. 1-14	P02810	1471.715	1471.555	8.7		↑ type 1 diabetes	~~both~~ Both major glands (↑ parotid)	[47]	**Formatted:** English (U.S.)
43	1490.8	~~histatin~~ Histatin 3 Fr. 1-12	P15516	1491.768	1491.635	8.5			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
44	1513.6	bPRP fragment	P10163	1513.798	1513.679	8.6		↓ ~~pa~~ediatric age	~~only~~ Only parotid	[26,34]	**Formatted:** English (U.S.)
45	1561.8	~~histatin~~ Histatin 3 Fr. 13–24 (Hst 8)	P15516	1562.772	1562.714	11.5			~~both~~ Both major glands (↑ parotid)	[9,11,26]	**Formatted:** English (U.S.)
46	1589.8	~~histatin~~ Histatin 3 Fr. 14–25	P15516	1590.779	1590.727	11.0			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
47	1618.9	~~histatin~~ Histatin 3 Fr. 1-13	P15516	1619.863	1619.809	8.4			~~both~~ Both major glands (↑ parotid)	[11,26]	**Formatted:** English (U.S.)
48	1717.9	~~histatin~~ Histatin 3 Fr. 12–24 (Hst 7)	P15516	1718.873	1718.901	11.8			~~both~~ Both major glands (↑ parotid)	[9,11,26]	**Formatted:** English (U.S.)
49	1717.9	~~histatin~~ Histatin 3 Fr. 13–25	P15516	1718.873	1718.901	11.8			~~both~~ Both major	[9,11,26]	**Formatted:** English (U.S.)

		(Hst 10)							glands (↑ parotid)	
50	1767.9	bPRP fragment	~~various~~Various	1767.914	1767.962	9.8		↓ paediatric age	~~only~~ Only parotid	[7,26,34]
51	1818.0	aPRP Fr. 94–110	P02810	1818.958	1819.019	11.1		↑ type 1 diabetes	~~both~~ Both major glands (↑ parotid)	[47]
52	1856.9	bPRP fragment	P02812	1856.889	1856.969	10.5		↓ paediatric age	~~only~~ Only parotid	[26,34]
53	1867.0	P-C Fr. 26–44	P02810	1866.921	1867.011	9.5		↑ type 1 diabetes	~~both~~ Both major glands (↑ parotid)	[47]
54	1874.0	~~histatin~~ Histatin 3 Fr. 12–25	P15516	1874.975	1875.089	11.2			~~both~~ Both major	[9,11,26]
		(Hst 9)							glands (↑ parotid)	
55	1932.2	bPRP fragment	~~various~~Various	1932.009	1932.169	10.7		↓ paediatric age	~~only~~ Only parotid	[7,26,34]
56	2017.2	bPRP fragment	~~various~~Various	2017.025	2017.232	11.5		↓ paediatric age	~~only~~ Only parotid	[7,26,34,35]
57	2029.2	bPRP fragment	~~various~~Various	2029.025	2029.243	11.3		↓ paediatric age	~~only~~ Only parotid	[7,26,34,35]
58	2083.3	P-C Fr. 5-25	P02810	2083.047	2083.294	11.3		↑ type 1 diabetes	~~both~~ Both major glands (↑ parotid)	[35,47]
59	2521.8	P-C Fr. 1-25	P02810	2521.281	2521.781	11.8		↑ type 1 diabetes	~~both~~ Both major glands (↑ parotid)	[35,47]
60	2745.0	~~histatin~~ Histatin 3 Fr. 12–32	P15516	2744.330	2744.974	13.8			~~both~~ Both major	[9,11,26]
		(Hst 4)							glands (↑ parotid)	
61	2917.2	P-C Fr. 15–44	P02810	2916.486	2917.236	12.6		↑ type 1 diabetes	~~both~~ Both major glands (↑ parotid)	[47]

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62	3036.3	~~histatin~~ Histatin 3 Fr. 1-24 (Hst 5)	P15516	3035.522	3036.334	14.6			~~both~~ Both major glands (↑ parotid)	[9,11,26]
63	3192.5	~~histatin~~ Histatin 3 Fr. 1-25 (Hst 6)	P15516	3191.623	3192.521	14.3			~~both~~ Both major glands (↑ parotid)	[9,11,26]
64	3371.0	α-defensin 2	P59665/6	3369.482	3370.966	23.5	3 S-S	↓ edentulous	GCF	[30,50]
65	3442.0	α-defensin 1	P59665	3440.519	3442.045	23.5	3 S-S	↓ edentulous	GCF	[30,50]
66	3472.9	~~peroxiredoxin~~ Peroxiredoxin 6 Fr. 1-32	P30041	3471.744	3472.864	31.4		↑ pre-term	?	[8]
67	3486.1	α-defensin 3	P59666	3484.509	3486.055	23.5	3 S-S	↓ edentulous	GCF	[30,50]
68	3645.0	~~statherin~~ Statherin Des_{1-13}	P02808	3643.685	3645.001	27.5			~~both~~ Both major glands	[24,26]
69	3707.8	α-defensin 4	P12838	3707.767	3709.414	27.2	3 S-S	↓ edentulous	GCF	[30,50]
70	3971.4	~~statherin~~ Statherin Des_{1-10}	P02808	3969.891	3971.399	28.0			~~both~~ Both major glands	[24,26]
71	4062.4	~~histatin~~ Histatin 3	P15516	4060.979	4062.407	17.7			~~both~~ Both major glands	[9,11,26]
72	4114.8	G3P dehydrogenase Fr. 1-39	P04406	4113.308	4114.807	33.2		↑ pre-term	?	[8]
73	4127.6	~~statherin~~ Statherin Des_{1-9}	P02808	4125.992	4127.587	28.5			~~both~~ Both major glands	[24,26]
74	4145.5	P-C peptide Des PQ_{43-44}	P02810	4144.072	4145.529	14.9			~~both~~ Both major glands (↑ parotid)	[20,26]

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75	4242.6	P-C peptide Des Q_{44}	P02810	4241.125	4242.646	14.9			~~both~~ Both major glands (↑ parotid)	[20,26]	**Formatted:** English (U.S.)
76	4370.8	P-C peptide	P02810	4369.183	4370.776	15.0			~~both~~ Both major glands (↑ parotid)	[20,26]	**Formatted:** English (U.S.)
77	4392.1	G3P dehydrogenase Fr. 1-41	P04406	4390.415	4392.087	29.9		↑ pre-term	?	[8]	**Formatted:** English (U.S.)
78	4549.3	P-B peptide Des_{1-12}	P02814	4547.411	4549.343	27.5			~~both~~ Both major glands (↑ Sm/Sl)	[24,26]	**Formatted:** English (U.S.)
79	4848.2	~~histatin~~ Histatin 1 non phosph.	P15515	4846.233	4848.172	22.0		↓ ASD	~~both~~ Both major glands	[9,26,46]	**Formatted:** English (U.S.)
80	4928.2	~~histatin~~ Histatin 1	P15515	4926.200	4928.151	21.9	~~phosph~~Phosph. (S_2)		~~both~~ Both major glands	[9,11,26]	**Formatted:** English (U.S.)
81	4936.5	~~thymosin~~ Thymosin β10	P63313	4934.530	4936.523	20.8	~~acetyl~~ Acetyl (N-term)	↑ pre-term	GCF, both glands (in pre-term)	[8,32,40]	**Formatted:** English (U.S.)
82	4946.5	~~thymosin~~ Thymosin β4 (cyclo)	P62328	4944.467	4946.472	19.6		↑ pre-term	GCF, both glands (in pre-term)	[8,32,40]	**Formatted:** English (U.S.)
83	4963.5	~~thymosin~~ Thymosin β4	P62328	4961.494	4963.502	18.5	~~acetyl~~ Acetyl (N-term)	↑ pre-term	GCF, both glands (in pre-term)	[8,32,40]	**Formatted:** English (U.S.)
84	4979.5	~~thymosin~~ Thymosin β4 sulfoxide	P62328	4977.488	4979.502	18.3	~~acetyl~~ Acetyl (N-term), (Met_6) sulfoxide	↑ pre-term	GCF, both glands (in pre-term)	[8,32,40]	**Formatted:** English (U.S.)
85	5008.2	~~histatin~~ Histatin 1 mono-sulfated	P15515	5006.156	5008.216	21.9	~~phosph~~Phosph. (S_2), mono-sulf (Y_{27})		~~only~~ Only Sm/Sl	[12]	**Formatted:** English (U.S.)
86	5060.9	P-B peptide Des_{1-7}	P02814	5058.654	5060.921	30.1			~~both~~ Both major glands (↑ Sm/Sl)	[24,26]	**Formatted:** English (U.S.)
87	5088.3	~~histatin~~ Histatin 1 di-sulfated	P15515	5086.113	5088.280	21.4	~~phosph~~Phosph. (S_2), di-sulf (Y_{27}, Y_{30})		~~only~~ Only Sm/Sl	[12]	**Formatted:** English (U.S.)

88	5131.4	~~statherin~~ Statherin (di-phosph) Des $T_{42}F_{43}$	P02808	5129.334	5131.439	27.9	~~di~~Di-phosph (S_2, S_3) Des $T_{42}F_{43}$		~~both~~ Both major glands	[24,26]	
89	5152.6	~~statherin~~ Statherin mono-phosph Des F_{43}	P02808	5150.415	5152.564	27.5	~~mono~~Mono-phosph (S_2 or S_3)		~~both~~ Both major glands	[24,26]	
90	5168.3	~~histatin~~ Histatin 1 tri-sulfated	P15515	5166.070	5168.344	20.8	~~phosph~~Phosph. (S_2), tri-sulf (Y_{27}, Y_{30}, Y_{34})		~~only~~ Only Sm/Sl	[12]	
91	5215.1	P-B peptide Des_{1-5}	P02814	5212.728	5215.090	30.3			~~both~~ Both major glands (↑ Sm/Sl)	[24,26]	
92	5219.8	~~statherin~~ Statherin non-phosph	P02808	5217.517	5219.761	28.6			~~both~~ Both major glands	[24,26]	
93	5232.5	~~statherin~~ Statherin (di-phosph) Des F_{43} (SV1)	P02808	5230.381	5232.544	27.8	~~di~~Di-phosph (S_2, S_3) Des F_{43}		~~both~~ Both major glands	[24,26]	
94	5248.4	~~histatin~~ Histatin 1 tetra-sulfated	P15515	5246.027	5248.408	20.5	~~phosph~~Phosph. (S_2), tetra-sulf (Y_{27}, Y_{30}, Y_{34}, Y_{38})		~~only~~ Only Sm/Sl	[12]	
95	5264.6	~~statherin~~ Statherin (di-phosph) Des D_1	P02808	5262.423	5264.632	28.7	~~di~~Di-phosph (S_2, S_3) Des D_1		~~both~~ Both major glands	[24,26]	
96	5299.7	~~statherin~~ Statherin mono-phosph	P02808	5297.483	5299.740	28.9	~~mono~~Mono-phosph (S_2 or S_3)	↑ ASD	~~both~~ Both major glands	[24,26,46]	
97	5357.0	~~cystatin~~ Cystatin B Fr. 54–98	P04080	5354.738	5357.030	29.5		↑ pre-term	?	[8]	
98	5362.7	~~cyclostatherin~~ Cyclostatherin Q-37	P02808	5360.423	5362.690	29.6	~~di~~Di-phosph (S_2, S_3) – cyclization $K_6 – Q_{37}$		~~both~~ Both major glands	[25,26]	
99	5371.3	P-B peptide Des_{1-4}	P02814	5368.829	5371.277	30.0			~~both~~ Both major glands (↑ Sm/Sl)	[24,26]	

100	5379.7	~~statherin~~Statherin	P02808	5377.450	5379.720	29.2	~~di~~Di-phosph (S_2, S_3)		~~both~~ Both major glands	[21,24,26]
101	5590.1	P-H (IB-4 – bPRP)	P02812	5587.783	5590.096	15.2		↓ paediatric age	~~only~~ Only parotid	[7,13,15,16]
102	5685	~~non~~ Non iden. (prob. bPRP)				15.0	~~unknown~~ Unknown bPRP	↓ paediatric age	~~only~~ Only parotid	[16]
103	5792.7	P-B peptide	P02814	5790.036	5792.734	30.0	~~pyroglu~~ Pyroglu (Q)		~~both~~ Both major glands (↑ Sm/Sl)	[24–26]
104	5842.5	P-F (IB-8c – bPRP)	P02812	5839.992	5842.493	14.7		↓ paediatric age	~~only~~ Only parotid	[7,13,16]
105	5842.6	~~cystatin~~ Cystatin B Fr. 1-53	P04080	5839.888	5842.616	29.7	~~acetyl~~ Acetyl (N-term)	↑ pre-term	?	[8]
106	5867.5	P-E Des-R_{61} (IB-9 – bPRP)	P02811	5864.987	5867.503	14.9		↓ paediatric age	~~only~~ Only parotid	[7,13,14,16]
107	5943.6	P-J (bPRP)		5941.003	5943.555	14.5		↓ paediatric ~~A~~age	~~only~~ Only parotid	[7,16]
108	5961.8	~~cystatin~~ Cystatin B Fr. 1-53 S-cysteinyl	P04080	5958.892	5961.755	29.8	~~acetyl~~ Acetyl (N-term), cysteinyl (C3)	↑ pre-term	?	
109	6023.7	P-E (IB-9 – bPRP)	P02811	6021.088	6023.690	14.9		↓ paediatric age	~~only~~ Only parotid	[7,13,14,16]
110	6147.9	~~cystatin~~ Cystatin B Fr. 1-53 S-glutathion.	P04080	6144.956	6147.922	29.7	~~acetyl~~ Acetyl (N-term), gluthationyl (C_3)	↑ pre-term	?	
111	6923.7	P-D (IB-5 – bPRP) (P_{32}→A)	P10163	6920.538	6923.692	15.9	P-D (P_{32} → A)	↓ paediatric age	~~only~~ Only parotid	[7,13,14,16]
112	6949.7	P-D (IB-5 – bPRP)	P10163	6946.554	6949.730	15.9		↓ paediatric age	~~only~~ Only parotid	[7,13,14,16]

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113	7452.0	II-2 Des R_{75} (bPRP)	P04280	7466.621	7452.007	19.2	~~phosph~~Phosph. (S_8), pyroglu (E) (Seq. from Ref. [3])	↓ paediatric age	~~only~~ Only parotid	[7,13,16]
114	7528.2	II-2 non phosph. (bPRP)	P04280	7524.746	7528.214	19.8	~~pyroglu~~ Pyroglu (E) (Seq. from Ref. [3])	↓ paediatric age	~~only~~ Only parotid	[7,13,16]
115	7608.2	II-2 (bPRP)	P04280	7604.712	7608.194	19.2	~~phosph~~Phosph. (S_8), pyroglu (E) (Seq. from Ref. [3])	↓ paediatric age	~~only~~ Only parotid	[7,13,16]
116	7806	~~non~~ Non iden.				21.0		↑ pre-term	?	[8]
117	7867	~~non~~ Non iden.				21.0		↑ pre-term	?	[8]
118	9437.2	IB-1 Des R_{96} (bPRP)	P02812	9432.602	9437.196	19.4	~~phosph~~Phosph. (S_8), pyroglu (Q)	↓ paediatric age	~~only~~ Only parotid	[7,13,16]
119	9513.4	IB-1 non-phosph. (bPRP)	P02812	9508.737	9513.404	19.7	~~pyroglu~~ Pyroglu (Q)	↓ paediatric age	~~only~~ Only parotid	[7,13,16]
120	9593.3	IB-1 (bPRP)	P02812	9588.703	9593.384	19.4	~~phosph~~Phosph. (S_8), pyroglu (Q)	↓ paediatric age	~~only~~ Only parotid	[7,13,16]
121	9956	~~non~~ Non iden.				32.0		↑ pre-term	?	[8]
122	10434	~~non~~ Non iden. (prob. bPRP)				16.0	~~unknown~~ Unknown bPRP	↓ paediatric age	~~only~~ Only parotid	[16,17]
123	10444	S100A12 (calgran. C)	P80511	10438.494	10443.847	40.0	M missing (N-term)	↑ pre-term	?	[8]
124	10651	~~non~~ Non iden.				33.2		↑ pre-term	?	[8]

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125	10765	~~non~~ Non iden.				31.6		↑ pre-term	?	[8]
126	10834	S100A8 (calgran. A)	P05109	10828.656	10834.511	40.4		↑ pre-term	?	[8]
127	10872	~~non~~ Non iden.				32.5	~~cystatin~~ Cystatin A M missing (pending for characterization)	↑ pre-term	?	[8]
128	10925	aPRP (PIF-f) mono-phosph Des R_{106}	P02810	10920.008	10925.383	23.4	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
129	10925	aPRP (PRP-3) mono-phosph Des R_{106}	P02810	10920.008	10925.383	23.4	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
130	10926	aPRP (PRP-4) mono-phosph Des R_{106}	P02810	10920.992	10926.368	23.4	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
131	11001	aPRP (PIF-f) non-phosph	P02810	10996.142	11001.590	23.8	~~pyroglu~~ Pyroglu (Q)	↑ preterm	~~both~~ Both major glands (↑ parotid)	[6,20,26]
132	11001	aPRP (PRP-3) non-phosph	P02810	10996.142	11001.590	23.8	~~pyroglu~~ Pyroglu (Q)	↑ preterm	~~both~~ Both major glands (↑ parotid)	[6,20,26]
133	11002	aPRP (PRP-4) non-phosph	P02810	10997.126	11002.575	23.8	~~pyroglu~~ Pyroglu (Q)	↑ preterm	~~both~~ Both major glands (↑ parotid)	[6,20,26]
134	11005	aPRP (PIF-f) di-phosph Des R_{106}	P02810	10999.974	11005.363	22.8	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
135	11005	aPRP (PRP-3) di-phosph Des R_{106}	P02810	10999.974	11005.363	22.8	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
136	11006	aPRP (PRP-4) di-phosph Des R_{106}	P02810	11000.958	11006.347	22.8	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
137	11006	~~cystatin~~ Cystatin A	P01040	11000.670	11006.493	31.8		↑ pre-term	?	[8,27,30]

138	11049	~~cystatin~~ Cystatin A acetyl (N-term)	P01040	11042.680	11048.533	33.0	~~acetyl~~ Acetyl (N-term)	↑ pre-term	?	[8,27]
139	11081	aPRP (PIF-f) mono-phosph	P02810	11076.109	11081.570	23.4	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	~~both~~ Both major glands (↑ parotid)	[6,20,26,46]
140	11081	aPRP (PRP-3) mono-phosph	P02810	11076.109	11081.570	23.4	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	~~both~~ Both major glands (↑ parotid)	[6,20,26,46]
141	11082	aPRP (PRP-4) mono-phosph	P02810	11077.093	11082.555	23.4	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	~~both~~ Both major glands (↑ parotid)	[6,20,26,46]
142	11161	aPRP (PIF-f) di-phosph	P02810	11156.075	11161.550	22.8	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})	↓ preterm	~~both~~ Both major glands (↑ parotid)	[6,20,26]
143	11161	aPRP (PRP-3) di-phosph	P02810	11156.075	11161.550	22.8	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})	↓ preterm	~~both~~ Both major glands (↑ parotid)	[6,20,26]
144	11162	aPRP (PRP-4) di-phosph	P02810	11157.059	11162.535	22.8	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})	↓ preterm	~~both~~ Both major glands (↑ parotid)	[6,20,26]
145	11182	~~cystatin~~ Cystatin B	P04080	11175.609	11181.631	33.0	~~acetyl~~ Acetyl (N-term)	↑ pre-term	?	[8]
146	11241	aPRP (PIF-f) tri-phosph	P02810	11236.041	11241.530	22.4	~~pyroglu~~ Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
147	11241	aPRP (PRP-3) tri-phosph	P02810	11236.041	11241.530	22.4	~~pyroglu~~ Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
148	11242	aPRP (PRP-4) tri-phosph	P02810	11237.025	11242.515	22.4	~~pyroglu~~ Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
149	11301	~~cystatin~~ Cystatin B S-cysteinyl	P04080	11294.613	11300.769	32.9	~~acetyl~~ Acetyl (N-term), cysteinyl (C_3)	↑ pre-term	?	[8,28]
150	11368	S100A7 (psoriasin D_{27})	P31151	11361.526	11367.798	37.0	~~acetyl~~Acetyl, M missing (N-term) $E_{27} \rightarrow D$	↑ pre-term	?	[8]

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151	11382	S100A7 (psoriasin E_{27})	P31152	11375.542	11381.824	36.9	~~acetyl~~Acetyl, M missing (N-term)	↑ pre-term	?	[8]
152	11487	~~cystatin~~ Cystatin B S-glutathionyl	P04080	11480.677	11486.936	32.8	~~acetyl~~ Acetyl (N-term), gluthationyl (C_3)	↑ pre-term	?	[8,28]
153	11517	IB-6 (bPRP)	P04280	11510.799	11516.666	16.7	Subst A_{63} → S respect to P04280 (as rep. in 15)	↓ p~~a~~ediatric age	~~only~~ Only parotid	[13,15,16]
154	11652	S100A11 (calgizzarin)	P31949	11644.802	11651.292	42.8	~~acetyl~~Acetyl, M missing (N-term)	↑ pre-term	?	[8]
155	11710	~~antileukoproteinase~~Antileukoproteinase	P03973	11702.362	11709.804	26.2	8 S-S	↑ pre-term	?	[8]
156	11886	IB-8a Con1+ (bPRP)	P02812	11880.014	11886.120	15.6	Mistake of sequence in Swiss-Prot		~~only~~ Only parotid	[16,19]
157	11896	IB-8a Con1- (bPRP)	P02812	11890.035	11896.163	15.6	Mistake of sequence in Swiss-Prot	↓ p~~a~~ediatric age	only parotid	[16,19]
158	12689	S100A9 (calgran. B) short	P06702	12682.293	12689.228	42.2	~~acetyl~~Acetyl, MTCKM miss. (N-term)	↑ pre-term	?	[8]
159	12705	S100A9 short mono-ox	P06702	12698.288	12705.228	42.0	~~acetyl~~Acetyl, MTCKM miss. (N-term), Met_{89} sulfox.		?	
160	12769	S100A9 (calgran. B) short phosph.	P06702	12762.259	12769.208	42.2	~~acetyl~~Acetyl, MTCKM miss. (N-term), phosph (T_{108})	↑ pre-term	?	[8]
161	12785	S100A9 short phosph. mono-ox	P06702	12778.254	12785.208	42.0	~~acetyl~~Acetyl, MTCKM miss. (N-term), phosph (T_{108}), Met_{89} sulfox.		?	

162	13044	aPRP (Db-f) mono-phosph Des R_{106}	P02810	13037.048	13043.679	23.9	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]	
163	13120	aPRP (Db-f) non-phosph	P02810	13113.183	13119.886	24.1	~~pyroglu~~ Pyroglu (Q)		~~both~~ Both major glands (↑ parotid)	[20,26]	
164	13124	aPRP (Db-f) di-phosph Des R_{106}	P02810	13117.014	13123.659	23.3	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]	
165	13153	S100A9 (calgran. B) long	P06702	13145.485	13152.839	41.9	~~acetyl~~Acetyl, M missing (N-term)	↑ pre-term	?	[8]	
166	13200	aPRP (Db-f) mono-phosph	P02810	13193.149	13199.866	23.9	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]	
167	13233	S100A9 (calgran. B) long phosph	P06702	13225.452	13232.819	41.9	~~acetyl~~Acetyl, M missing (N-term), phosph (T_{112})	↑ pre-term	?	[8]	
168	13272	S100A9 (calgran. B) long cyst	P06702	13264.490	13271.977	41.6	~~acetyl~~Acetyl, M missing (N-term), cysteinyl (C_2)	↑ pre-term	?	[8]	
169	13280	aPRP (Db-f) di-phosph	P02810	13273.115	13279.846	23.3	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]	
170	13291	Ib8a (Con1+) glycosyl.	P02812	13283.521	13290417	15.6	~~glycosyl~~Glycosyl. (N_{98}) (see n.156–157) Fuc1Gal1Man3GlcNAc3		~~only~~ Only parotid	[19]	
171	13343	~~cystatin~~ Cystatin C (γ trace)	P01034	13335.576	13343.108	35.1	(2 S-S)		Sm/Sl (traces in parotid)	[26,27]	
172	13352	S100A9 (calg. B) long cyst phosph.	P06702	13344.456	13351.957	41.6	~~acetyl~~Acetyl, M missing (N-term), cysteinyl (C_2), phosph (T_{112})	↑ pre-term	?	[8]	
173	13360	aPRP (Db-f) tri-phosph	P02810	13353.081	13359.826	23.0	~~pyroglu~~ Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]	

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174	13458	S100A9 (calg. B) long glut	P06702	13450.553	13458.145	41.5	~~acetyl~~Acetyl, M missing (N-term), glutathionyl (C_2)	↑ pre-term	?	[8]
175	13538	S100A9 (calg. B) long glut phosph	P06702	13530.520	13538.124	41.5	~~acetyl~~Acetyl, M missing (N-term), glutathionyl (C_2), phosph (T_{112})	↑ pre-term	?	[8]
176	13656	Ib8a(Con-1+) glycosyl.	P02812	13648.653	13655.755	15.6	~~glycosyl~~Glycosyl. (N_{98}) (see n.156–157) Fuc1Gal2Man3GlcNAc4		~~only~~ Only parotid	[19]
177	13780	~~non~~ Non iden.				39.3		↑ pre-term	?	[8]
178	13802	Ib8a(Con-1+) glycosyl.	P02812	13794.711	13801.898	15.6	~~glycosyl~~Glycosyl. (N_{98}) (see n.156–157) Fuc2Gal2Man3GlcNAc4		~~only~~ Only parotid	[19]
179	13948	Ib8a(Con-1+) glycosyl.	P02812	13940.769	13948.041	15.6	~~glycosyl~~Glycosyl. (N_{98}) (see n.156–157) Fuc3Gal2Man3GlcNAc4		~~only~~ Only parotid	[19]
180	14094	Ib8a(Con-1+) glycosyl.	P02812	14086.827	14094.184	15.6	~~glycosyl~~Glycosyl. (N_{98}) (see n.156–157) Fuc4Gal2Man3GlcNAc4		~~only~~ Only parotid	[19]
181	14185	~~cystatin~~ Cystatin S	P01036	14176.808	14184.725	35.3	2 S-S		Sm/Sl (traces in parotid)	[26,27]
182	14240	Ib8a(Con-1+) glycosyl.	P02812	14232.885	14240.327	15.6	~~glycosyl~~Glycosyl. (N_{98}) (see n.156–157) Fuc5Gal2Man3GlcNAc4		~~only~~ Only parotid	[19]

183	14265	~~cystatin~~ Cystatin S1	P01036	14256.774	14264.705	35.3	~~phosph~~ Phosph (S_3), (2 S-S)		Sm/Sl (traces in parotid)	[26,27]	**Formatted:** English (U.S.)
184	14281	~~cystatin~~ Cystatin S1 mono-ox.	P01036	14272.769	14280.704	35.2	~~phosph~~ Phosph (S_3), oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]	**Formatted:** English (U.S.)
185	14297	~~cystatin~~ Cystatin S1 di-ox.	P01036	14288.764	14296.703	35.2	~~phosph~~ Phosph (S_3), oxidation (2 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]	**Formatted:** English (U.S.)
186	14312	~~cystatin~~ Cystatin SN	P01037	14304.094	14312.038	34.6	(2 S-S)		Sm/Sl (traces in parotid)	[26,27]	**Formatted:** English (U.S.)
187	14328	~~cystatin~~ Cystatin SN mono-ox.	P01037	14320.089	14328.037	33.9	~~oxidation~~ Oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[26,27]	**Formatted:** English (U.S.)
188	14344	~~cystatin~~ Cystatin SN di-ox.	P01037	14336.084	14344.036	33.7	~~oxidation~~ Oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]	**Formatted:** English (U.S.)
189	14345	~~cystatin~~ Cystatin S2	P01036	14336.740	14344.684	35.3	~~di~~Di-phosph (S_1,S_3), (2 S-S)		Sm/Sl (traces in parotid)	[26,27]	**Formatted:** English (U.S.)
190	14346	~~cystatin~~ Cystatin SA	P09228	14338.008	14346.018	36.8	2 S-S		Sm/Sl (traces in parotid)	[26,27]	**Formatted:** English (U.S.)
191	14361	~~cystatin~~ Cystatin S2 mono-ox.	P01036	14352.735	14360.684	35.2	~~di~~Di-phosph (S_1,S_3), oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]	**Formatted:** English (U.S.)
192	14362	~~cystatin~~ Cystatin SA mono-ox	P09228	14354.003	14362.018	36.6	~~oxidation~~ Oxidation (1 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]	**Formatted:** English (U.S.)
193	14377	~~cystatin~~ Cystatin S2 di-ox.	P01036	14368.730	14376.683	35.2	~~di~~Di-phosph (S_1,S_3), oxidation (2 O) unknown res., (2 S-S)		Sm/Sl (traces in parotid)	[27]	**Formatted:** English (U.S.)

194	14424	~~cystatin~~ Cystatin SN TFA adduct	P01037	14416.072	14424.046	34.6	(2 S-S) – artifact	~~artifact~~Artifact	~~artifact~~Artifact	[3]
195	14536	~~cystatin~~ Cystatin SN 2TFA adduct	P01037	14528.050	14536.055	34.6	(2 S-S) – artifact	~~artifact~~Artifact	~~artifact~~Artifact	[3]
196	14693	Lysozyme	P61626	14684.097	14692.604	35.6	(4 S-S)	↑ pre-term	?	[8]
197	14990	~~non~~ Non iden.				35.4		↑ pre-term	?	[8]
198	15079	~~non~~ Non iden.				34.6		↑ pre-term	?	[8]
199	15127	α-globin	P69905	15117.892	15126.853	43.0			?	
200	15354	aPRP (PIF-s) non-phosph	P02810	15346.308	15354.351	23.2	~~pyroglu~~ Pyroglu (Q)	↑ preterm	~~both~~ Both major glands (↑ parotid)	[7,20,26]
201	15354	aPRP (PRP-1) non-phosph	P02810	15346.308	15354.351	23.2	~~pyroglu~~ Pyroglu (Q)	↑ preterm	~~both~~ Both major glands (↑ parotid)	[7,20,26]
202	15355	aPRP (PRP-2) non-phosph	P02810	15347.292	15355.336	23.2	~~pyroglu~~ Pyroglu (Q)	↑ preterm	~~both~~ Both major glands (↑ parotid)	[7,20,26]
203	15381	aPRP (Pa 1-mer) mono-phosph	P02810	15373.182	15381.282	23.3	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})		~~enriched~~ Enriched granules preparations	[20,26]
204	15434	aPRP (PIF-s) mono-phosph	P02810	15426.274	15434.331	22.9	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	~~both~~ Both major glands (↑ parotid)	[7,20,26,46]
205	15434	aPRP (PRP-1) mono-phosph	P02810	15426.274	15434.331	22.9	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	~~both~~ Both major glands (↑ parotid)	[7,20,26,46]
206	15435	aPRP (PRP-2) mono-phosph	P02810	15427.258	15435.316	22.9	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})	↑ ASD and preterm	~~both~~ Both major glands (↑ parotid)	[7,20,26,46]

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207	15461	aPRP (Pa 1-mer) di-phosph	P02810	15453.148	15461.262	23.0	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})		~~enriched~~ Enriched granules preparations	[26]
208	15514	aPRP (PIF-s) di-phosph	P02810	15506.240	15514.311	22.2	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})	↓ preterm	~~both~~ Both major glands (↑ parotid)	[7,20,26]
209	15514	aPRP (PRP-1) di-phosph	P02810	15506.240	15514.311	22.2	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})	↓ preterm	~~both~~ Both major glands (↑ parotid)	[7,20,26]
210	15515	aPRP (PRP-2) di-phosph	P02810	15507.224	15515.296	22.2	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})	↓ preterm	~~both~~ Both major glands (↑ parotid)	[7,20,26]
211	15541	aPRP (Pa 1-mer) tri-phosph	P02810	15533.115	15541.242	22.7	~~pyroglu~~ Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		~~enriched~~ Enriched granules preparations	[26]
212	15546	aPRP (PRP-1 type) mono-phos TFA adduct	P02810	15538.252	15546.340	23.9	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22}) – artifact	~~artifact~~Artifact	~~artifact~~Artifact	[3]
213	15594	aPRP (PIF-s) tri-phosph	P02810	15586.207	15594.291	21.6	~~pyroglu~~ Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
214	15594	aPRP (PRP-1) tri-phosph	P02810	15586.207	15594.291	21.6	~~pyroglu~~ Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
215	15595	aPRP (PRP-2) tri-phosph	P02810	15587.191	15595.276	21.6	~~pyroglu~~ Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
216	15626	aPRP (PRP-1 type) di-phos TFA adduct	P02810	15618.218	15626.320	23.3	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22}) – artifact	~~artifact~~Artifact	~~artifact~~Artifact	[3]
217	15867	β-globin	P68871	15858.257	15867.216	42.5			?	[8]

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218	15995	γ-globin (G-γ~~gamma~~)	P69892	15986.262	15995.248	43.7			?	[8]
219	16001	~~non~~ Non iden.				27.4		↑ pre-term	?	[8]
220	16009	γγ-globin (A-γ~~gamma~~)	P69891	16000.278	16009.275	43.9			?	[8]
221	17239	SPRR3 (cornifin β) 17 kDa	A5YKK8	17228.798	17238.816	27.4	~~acetyl~~Acetyl, M missing (N-term) (S-S var)	↑ pre-term	?	[8,33]
222	17358	SPRR3 17 kDa mono-cyst.	A5YKK8	17347.802	17357.955	27.8	~~acetyl~~Acetyl, M missing (N-term), mono-cysteinyl (S-S var.)	↑ pre-term	?	[8,33]
223	17473	aPRP (Db-s) non-phosph	P02810	17463.348	17472.647	23.8	~~pyroglu~~ Pyroglu (Q)		~~both~~ Both major glands (↑ parotid)	[20,26]
224	17544	SPRR3 17 kDa mono-glut	A5YKK8	17533.866	17544.122	27.8	~~acetyl~~Acetyl, M missing (N-term), mono-glutathionyl (S-S var.)	↑ pre-term	?	[8,33]
225	17553	aPRP (Db-s) mono-phosph	P02810	17543.314	17552.627	23.4	~~pyroglu~~ Pyroglu (Q), mono-phosph (S_8 or S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
226	17633	aPRP (Db-s) di-phosph	P02810	17623.281	17632.607	22.9	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
227	17713	aPRP (Db-s) tri-phosph	P02810	17703.247	17712.587	22.7	~~pyroglu~~ Pyroglu (Q), tri-phosph (S_8, S_{17}, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
228	17803	Non iden.				36.4		↑ pre-term	?	[8]
229	18065	SPRR3 (cornifin β) 18 kDa	Q9UBC9	18054.203	18064.795	27.6	~~acetyl~~Acetyl, M missing (N-term) (S-S var.)	↑ pre-term	?	[8,33]

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230	18184	SPRR3 18 kDa mono-cyst	Q9UBC9	18173.207	18183.933	28.0	~~acetyl~~Acetyl, M missing (N-term), mono-cysteinyl (S-S var.)	↑ pre-term	?	[8,33]
231	18303	SPRR3 18 kDa di-cyst	Q9UBC9	18292.211	18303.071	28.0	~~acetyl~~Acetyl, M missing (N-term), di-cysteinyl (S-S var.)	↑ pre-term	?	[8,33]
232	18370	SPRR3 18 kDa mono-glut	Q9UBC9	18359.271	18370.100	28.0	~~acetyl~~Acetyl, M missing (N-term), mono-gluthationyl (S-S var.)	↑ pre-term	?	[8,33]
233	18420	~~non~~ Non iden.				36.2		↑ pre-term	?	[8]
234	18675	SPRR3 18 kDa di-glut	Q9UBC9	18664.339	18675.406	28.1	~~acetyl~~Acetyl, M missing (N-term), di-glutathionyl (S-S var.)	↑ pre-term	?	[8,33]
235	20206	~~non~~ Non iden.				37.9		↑ pre-term	?	[8]
236	20930	~~non~~ Non iden.				33.6		↑ pre-term	?	[8]
237	22361	~~cystatin~~ Cystatin B S-S dimer	P04080	22349.202	22361.246	34.3	S-S dimer (Cys_3) acetyl N-term		2	[28]
238	22365	~~histone~~ Histone H1c	P16402	?	?	34.4	~~disagrement~~ Disagreement between exp. and theor. Mass	↑ pre-term	?	[8]
239	22698	~~non~~ Non iden.				36.9		↑ pre-term	?	[8]
240	22778	~~non~~ Non iden.				36.9	~~phosph~~ Phosph isoform of 22698?	↑ pre-term	?	[8]

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241	23462	~~non~~ Non iden. (prob. bPRP)				17.6	~~unknown~~ Unknown bPRP (Ps1?)	↓ p~~a~~ediatric age	~~only~~ Only parotid	[7,16,17]
242	24652	~~non~~ Non iden.				44.8		↑ pre-term	?	[8]
243	24904	~~non~~ Non iden.				40.0	~~peroxiredoxin~~ Peroxiredoxin 6? (pending for charact.)	↑ pre-term	?	[8]
244	27050	~~non~~ Non iden.				26.7		↑ pre-term	?	[8]
245	29412	~~non~~ Non iden. (prob. bPRP)				16.8	~~unknown~~ Unknown bPRP (Ps2?)	↓ p~~a~~ediatric age	~~only~~ Only parotid	[7,16,17]
246	30712	aPRP (Pa 2-mer) tri-phosph Des Q_{150}	P02810	30696.256	30712.398	24.0	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22}) + mono-phos (S_8 or S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
247	30761	aPRP (Pa 2-mer) di-phosph	P02810	30744.349	30760.549	24.5	~~pyroglu~~ Pyroglu (Q), di-phosph 2 × (S_8 or S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
248	30792	aPRP (Pa 2-mer) tetra-phosph Des Q_{150}	P02810	30776.223	30792.378	23.6	~~pyroglu~~ Pyroglu (Q), tetra-phosph 2 × ~~×~~(S_8, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
249	30841	aPRP (Pa 2-mer) tri-phosph	P02810	30824.315	30840.529	24.0	~~pyroglu~~ Pyroglu (Q), di-phosph (S_8, S_{22}) + mono-phos (S_8 or S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
250	30921	aPRP (Pa 2-mer) tetra-phosph	P02810	30904.281	30920.509	23.6	~~pyroglu~~ Pyroglu (Q), tetra-phosph 2 × ~~×~~(S_8, S_{22})		~~both~~ Both major glands (↑ parotid)	[20,26]
251	31000	aPRP (Pa 2-mer) penta-	P02810	30984.247	31000.489	23.2	~~pyroglu~~ Pyroglu (Q), di-		~~both~~ Both major	[20,26]

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		phosph					phosph (S_8, S_{22}) + tri-phos (S_8, S_{17}, S_{22})		glands (↑ parotid)	
252	31032	aPRP (Pa 2-mer) tetra-phosph TFA add	P02810	31016.259	31032.517	23.6	~~pyroglu~~ Pyroglu (Q), tetra-phosph 2 × ~~×~~(S_8, S_{22}) – artifact	~~artifact~~Artifact	~~artifact~~Artifact	[3]
253	31144	aPRP (Pa 2-mer) tetra-phosph 2 TFA add	P02810	31128.237	31144.526	23.6	~~pyroglu~~ Pyroglu (Q), tetra-phosph 2 × ~~×~~(S_8, S_{22}) – artifact	~~artifact~~Artifact	~~artifact~~Artifact	[3]
254	34475	SPRR3 17 kDa homo-2-mer	A5YKK8	34455.580	34475.617	28.2	~~acetyl~~Acetyl, M missing (N-term) (S-S var) S-S homo-2-mer	↑ pre-term	?	[8,33]
255	35301	SPRR3 17–18 kDa hetero-2-mer	A5YKK8 ; Q9UBC9	35280.985	35301.595	28.2	~~acetyl~~Acetyl, M missing (N-term) (S-S var) S-S hetero-2-mer	↑ pre-term	?	[8,33]
256	36127	SPRR3 18 kDa homo-2-mer	Q9UBC9	36106.391	36127.573	28.2	~~acetyl~~Acetyl, M missing (N-term) (S-S var) S-S homo-2-mer	↑ pre-term	?	[8,33]

[a]With an error of ±1:10000.

[b]With a variation of ± 0.8 min.

ASD: autism spectrum disorder; GCF: gingival crevicular fluid.

Figure 1. Flowchart of the top-down proteomic platform typically utilized for the identification of the components of whole saliva reported in Table ~~1~~I. (TFA: 2,2,2-trifluoroacetic acid).

Figure 2. Typical HPLC-ESI-MS TIC (Total ion current) profiles of the acidic soluble fraction of human whole saliva of subjects with different ages. TIC profiles from (a) to (c) were from whole saliva samples of the same preterm newborn collected at different post-conceptional age (PCA). TIC profiles from (d) to (g) were from different healthy subjects. Apart from small variations linked to inter-individual differences, the seven profiles are good representative (qualitatively and quantitatively) of the profiles observed in other subjects of the same age range. The elution clusters of the most relevant salivary peptides and proteins reported in Table ~~1~~ I (except human serum albumin (HSA) and α-amylase) are evidenced on the top of (a) and (g) profiles.

Figure 3. Extracted ion current (XIC) procedure for the detection of thymosin β_4 in a sample of whole saliva of a preterm newborn (227 days of post-conceptional age; A and B profiles) and of an adult (30 years; C and D profiles). A and C correspond to the total ion current (TIC) profiles, B and D to the XIC profiles. XIC procedure was carried out selecting three *m/z* values ($[M+3H]^{3+}$ = 1655.5 *m/z*; $[M+4H]^{4+}$ = 1241.9 *m/z* $[M+5H]^{5+}$ = 993.8 *m/z*) corresponding to characteristic ions of thymosin β_4. XIC procedure ensures an improvement of the signal to noise ratio with an enhancement of the sensitivity. NL~~:~~, normalization level; MA~~:~~, measured area; RT~~:~~, retention time.

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