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Review

The characterization of preterm newborn saliva by top-down proteomic as a stimulus for the study of human development. A review of the results obtained over the past 25 years

Tiziana Cabras[1], Barbara Manconi[1], Alessandra Olianas[1], Maria Teresa Sanna[1], Cristina Contini[2], Giulia Guadalupi[1], Antonella Fiorita[3], Lea Calò[3], Gaetano Paludetti[3], Emanuele Scarano[4], Chiara Tirone[5,6], Giovanni Vento[5,6], Simona Fattore[5,6], Nicoletta Menzella[5,6], Davide De Tomaso[5,6], Federica Iavarone[7,8], Irene Messana[9], Claudia Desiderio[9], Diana Valeria Rossetti[9], Matteo Fraschini[10], Vassilios Fanos[11], Jacopo Galli[3], Gavino Faa[2,12], Massimo Castagnola[7,8]

[1]Department of Life and Environmental Sciences, Statal University of Cagliari, Monserrato (CA), Italy
[2]Department of Medical Sciences and Public Health, Statal University of Cagliari, Monserrato (CA), Italy
[3]Otorhinolaryngology, Head and Neck Surgery, "A. Gemelli" Hospital Foundation IRCCS, Rome, Italy
[4]Otorhinolaryngology and Maxillofacial Surgery, Cardinale Giovanni Panico Hospital, Tricase (LE), Italy
[5]Neonatology Unit, Policlinico Universitario "Agostino Gemelli" Foundation IRCCS, Rome, Italy
[6]Department of Woman and Child Health and Public Health, Pediatrics Section, Catholic University of Sacred Heart, Rome, Italy
[7]Policlinico Universitario "A. Gemelli" Foundation IRCCS, Rome, Italy
[8]Department of Basic Biotechnological Sciences, Intensive and Perioperative Clinics, Catholic University of Sacred Heart, Rome, Italy
[9]Institute of Chemical Sciences and Technologies (SCITEC) "Giulio Natta", National Research Council (CNR), Rome, Italy
[10]Department of Electrical and Electronic Engineering, Statal University of Cagliari, Monserrato (CA), Italy
[11]Neonatal Intensive Care Unit, Department of Surgical Sciences, Statal University of Cagliari, Monserrato (CA), Italy
[12]Department of Biology, College of Science and Technology, Temple University, Philadelphia, PA, USA

Abstract

Around the first years of 2000, our teams, at the Cagliari and Catholic-Policlinico Gemelli (Rome) Universities, started an investigation of the intact proteins of human saliva by a top-down pipeline. Since one of the most interesting topics of our research was the study of molecular events occurring during fetal development, we carried out the top-down proteomic analysis on whole saliva of human preterm newborns, induced by the non-invasive

specimen collection. The results obtained showed a proteome profile of human preterm newborns sensibly different from that of adults, and they were a stimulus for the study of the variation of several proteins during development, with the support of immunohistochemical methods on tissues obtained at autopsy. This review describes the main results obtained over around 25 years of research. The results on the main protein families of adult saliva, i.e., acidic, basic, and glycosylated proline-rich proteins, amylase, histatins, statherin, P-B peptide, and salivary cystatins, are described in the first section. The results obtained on the salivary proteome of preterm newborns are then reported. A section was devoted to the description of the great heterogeneity of basic proline-rich proteins and of their variable structure in the salivary evolutionary pathway from *Homo Neanderthalensis* to *Homo Sapiens*. The chapter on thymosins β_4 and β_{10} peptides highlights how the results obtained on saliva from preterm newborns stimulated studies on other fetal organs, the latter suggesting some potential role for these peptides during human development. The small proline-rich proteins, cystatins, and S100 proteins are next described. The studies also showed that some enzymes, i.e., the Fam20C kinase recently characterized by another research group, are almost inactive during the last months of fetal development, while other enzymes, i.e., various proteinases and convertases, are much more active in the last fetal period of development than in adults.

Keywords

Human, saliva, top-down proteomics, proteins, peptides, preterm newborns.

Corresponding authors

- Prof. Barbara Manconi, Department of Life and Environmental Sciences, Statal University of Cagliari, Monserrato (CA), Italy; ORCID: 0000-0002-2880-9915; email: bmanconi@unica.it.
- Dr. Cristina Contini, Department of Medical Sciences and Public Health, Statal University of Cagliari, Monserrato (CA), Italy; ORCID: 0000-0002-0903-4675; email: cristina.contini93@unica.it.

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Introduction

One of the most interesting topics of our research group concerned the study of the molecular events involved in human development, with a particular focus on fetal growth. One obstacle in this type of study is represented by the choice of samples, which will allow us to obtain the most relevant information possible with specimen collection ethically acceptable, easy to perform, and painless. Among the secreted bodily fluids, saliva is particularly attractive because it can be collected easily and without stress in pediatric age [1, 2]. Pure saliva is secreted by three couples of major glands, namely, the parotid, submandibular, and sublingual; the last two are often considered together because their secretion is ensured by two closely localized ducts (Wharton and Bartholin ducts), and by a variable number of minor glands. It also contains a serum transudate that pours into the oral cavity by crossing the oral mucosa and a fluid leaking around the teeth into the dental sulcus (called gingival crevicular fluid), which can offer information on events connected to systemic diseases. This fluid is called mixed saliva or oral fluid. In preterm newborns, the salivary glands are not completely formed, and the oral fluid derives also from secretions of the oral and nasal epithelia. It can spill into the bronchi and lungs and can generate infections and breathing difficulties. Therefore, to avoid its deleterious effects, it is aspired from the newborn mouth by the staff of the Neonatal Intensive Care Unit (NICU), and it is discarded. For this reason, the Ethical Committees authorized the use of this fluid for analytical purposes. In some cases, the newborns admitted to the NICU can have less than 180 days of post-conceptional age (PCA). The specimens, when serially collected, can provide precious information to investigate the evolution of molecular events occurring in the mouth of preterm neonates in the period spanning from 25-26 weeks of PCA, up to the age equivalent to that of full-term newborns (around 38-40 weeks). More than 20 years ago, we started the study of the proteome of this oral

fluid serially collected from preterm newborns, and we compared it with the proteome observed in children and adults of different ages [3]. The differences observed with respect to adult saliva induced us to deepen the investigations on human saliva at different ages. This review reports the most relevant results obtained by our team, starting with the description of the human adult salivary proteome to evidence the differences characterized during fetal development and showing how the oral fluid of preterm newborns was an incentive for many interesting research devoted to understanding the properties of the peptides and proteins characteristic of the late fetal growth. The workflow utilized for the proteomic analyses of salivary samples is reported in **Fig. 1**. It consisted in the fast stabilization of the sample by mixing saliva in ice bath with an equal volume of an acidic solution (0.2% v/v formic acid [FA] or trifluoroacetic acid [TFA] in water). This treatment minimizes the action of enzymes present in the sample and allows us to obtain a solution compatible with the high-performance liquid chromatography (HPLC) separation and the electrospray ionization (ESI) process of the mass spectrometry (MS). The acidic soluble fraction was analyzed by HPLC coupled with high-resolution mass spectrometers of different generations (Deca XP, Orbitrap). This strategy, which does not involve the proteolytic treatment of the samples and is defined as top-down proteomics [4, 5], allowed us to obtain information on the intact proteome of the oral fluid.

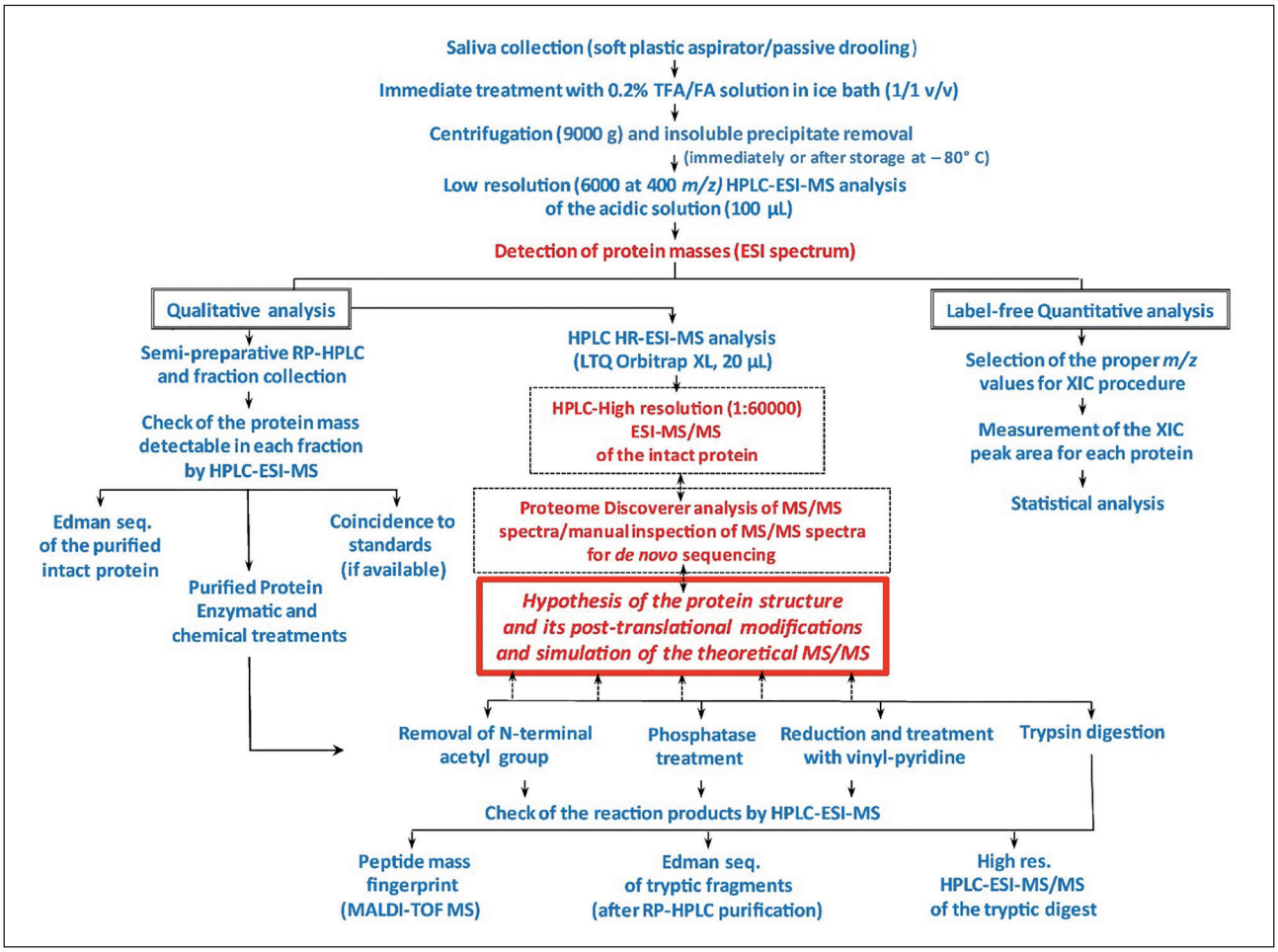

Figure 1. A scheme of the analytical workflow utilized in our top-down proteomic analysis of human salivary samples (modified from: Castagnola et al., 2011 [3]).

It consisted in the fast stabilization of saliva collected either by suction with a soft plastic pipette from the floor under the tongue or by passive drooling, by mixing it in ice bath with an equal volume of an acidic solution (0.2% FA or TFA in water). This treatment minimizes the action of enzymes present in the sample and allows us to obtain a solution compatible with the HPLC separation and the ESI process of the high-resolution Orbitrap MS. All the other steps were carried out to define the primary structure of the protein and to compare its theoretical high-resolution MS/MS fragmentation spectrum with that obtained experimentally.

ESI: electrospray ionization; FA: formic acid; HPLC: high-performance liquid chromatography; HR: high resolution; MALDI-TOF: matrix assisted laser desorption ionization time of flight; MS: mass spectrometry; RP: reversed-phase; TFA: trifluoroacetic acid; XIC: extracted ion current.

The adult human salivary proteome

Adult human saliva contains various families of specific and non-specific proteins [6, 7], summarized in **Fig. 2**, many of them characterized before the proteomic era. It is worthwhile to outline that gender differences have not been found in human saliva so far [2, 6, 7].

The protein families specific to human saliva are presented below.

1. Proline-rich proteins (PRPs), which are further subdivided into basic (bPRPs), acidic (aPRPs), and glycosylated basic (gPRPs) [8]. Globally, this family accounts for about 40% of the proteins detectable in human saliva. Two loci, *PRH1* and *PRH2*, encode for aPRPs. *PRH2* locus is biallelic and encodes for PRP-1 and PRP-2, two proteoforms of 150 amino acid residues differing only at position 50 (Asn_{50}/Asp_{50}, respectively). Three different alleles of *PRH1* locus give rise to the parotid isoelectric-focusing variant slow (PIF-s), the parotid acidic protein (Pa), both 150 residues long, and the double band isoform slow (Db-s), 171 amino acid residues long for the insertion of a 21 amino acid repeat after position 81 [8]. PRP-1, PRP-2, PIF-s, and Db-s undergo a partial cleavage at Arg_{106} residue (Arg_{127} in Db-s) by the action of a convertase recognizing the $R_{103}PPR_{106}\downarrow$ consensus sequence, originating a common 44-amino acid residue peptide (named P-C) and four truncated derivatives, called PRP-3, PRP-4, PIF-f and Db-f (f stands for fast). The cleavage is only partial, leading to the presence in saliva of both full-length and truncated aPRPs [9]. Due to the lack of the $R_{103}PPR_{106}\downarrow$ consensus sequence, resulting from the substitution $Arg_{103}\rightarrow Cys$, Pa does not undergo the cleavage. In whole saliva, it is detectable only as Pa

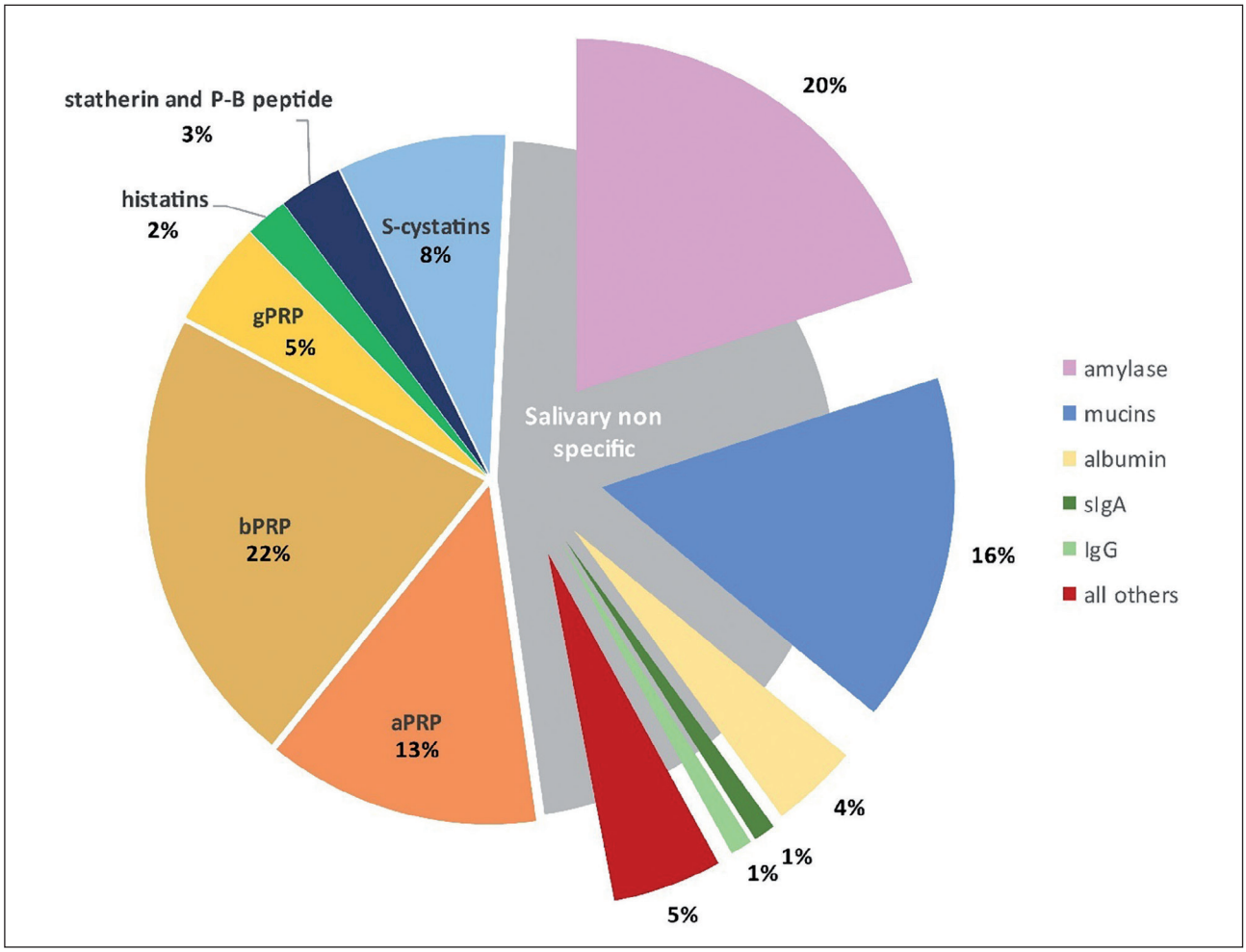

Figure 2. Approximate composition of the proteome of adult human saliva considering a comparable contribution of parotid and submandibular/sublingual glands (modified from: Messana et al., 2008 [6]).

All saliva-specific protein families shown on the left in the figure and amylase are of secretory origin.

aPRPs: acidic proline-rich proteins; bPRPs: basic proline-rich proteins; gPRPs: glycosylated basic proline-rich proteins; IgG: immunoglobulin G; sIgA: secretory immunoglobulin A.

2-mer, generated via a disulfide bridge. bPRPs, constituting about 40% of the proteins secreted by the human parotid glands [6, 10, 11], are the most complex family of salivary proteins. The cluster of genes encoding for bPRPs includes *PRB1–PRB4*, each one existing in several allelic forms: at least four alleles (*S*, small; *M*, medium; *L*, large; and *VL*, very large) are present in the western population at *PRB1* and *PRB3* loci, and three (*S*, *M*, *L*) at *PRB2* and *PRB4* loci. These alleles exhibit tandem repeat length variations and single nucleotide polymorphisms (SNPs) in the coding region, polymorphic cleavage sites and stop codons. Moreover, alternative splicing generates multiple transcript variants encoding distinct protein species. Genetic variability and post-translational modifications (PTMs) are implicated in the pre-secretory maturation processes, contributing to the heterogeneity of bPRPs. Except for the protein encoded by the *PRB3* locus that originates gPRPs, the proteolytic cleavage is the main post-translational event occurring on bPRPs; indeed, the pre-proproteins encoded by each allele, after peptide-signal removal, are completely cleaved by proprotein convertases in smaller peptides before secretion [10]. Despite the high abundance of PRPs in human saliva, their role is still not well understood. aPRPs show a high affinity for hydroxyapatite and are involved in the formation of acquired enamel pellicle. They probably protect dental enamel, too, and are implicated in the modulation of oral microbioma. bPRPs could have a role in taste perception, while gPRPs probably have lubricating properties and a defensive role against some pathogens [8, 10].

2. Salivary amylase is the most abundant protein in human saliva (about 15-20%). It is mainly secreted by the parotid gland, slightly differing from the pancreatic one [6, 7, 12].
3. Histatins: a series of small histidine-rich peptides [13, 14], deriving their name from the high number of histidine residues in their structure, some of them (i.e., histatin 3 fr. 1/24) having powerful anti-fungal activity [13]. All the members of this family arise from two parent peptides, histatin 1 and histatin 3, characterized by similar sequences and encoded by two genes (*HIS1* and *HIS2*) located on chromosome 4q13 [15]. Despite the high sequence similarity, these two peptides follow different PTMs pathways. Differently from histatin 1, histatin 3 is submitted during granule maturation 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 [9, 14]. The different susceptibility to cleavage derives from the presence of the $R_{22}GYR_{25}\downarrow$ convertase consensus sequence in histatin 3, absent in histatin 1. Histatin 1 is phosphorylated on Ser_2 residue, while histatin 3 is not, due to the absence of a +2-flanking glutamic acid residue to Ser_2, essential for the FAm20C kinase recognition. In the submandibular glands, a part of histatin 1 undergoes a poly-sulfation process at the level of the four terminal tyrosine residues (Tyr_{27}, Tyr_{30}, Tyr_{34}, Tyr_{36}). This modification has not been observed for histatin 3, because it does not have a tyrosine equivalent to Tyr_{27} of histatin 1, essential for the recognition by the sulfotransferase and the initiation of the hierarchical sulfation process [16]. Histatin 1 is the lonely phospho-sulfo-peptide detected in human saliva till now.
4. Statherin and P-B peptide. Statherin is a multifunctional peptide specific to human saliva rich in tyrosine and glutamine residues involved in oral calcium homeostasis, phosphate buffering and formation of protein networks [17]. P-B peptide is usually included in the bPRP family, but it shows some similarities with statherin: it is rich in proline residues, and it has two tyrosine residues, while bPRPs do not have aromatic amino acid. The origin of its alternative name "submaxillary gland androgen-regulated protein" is not clear (reported at the Swiss-Prot code P02814). The biological role of P-B peptide is still unknown [18].
5. Salivary cystatins (S-type) comprise five major proteins (S, S1, S2, SA, SN) composed of ~115–120 amino acid residues with a molecular mass ranging from 13.5 to 14.5 kDa. Cystatins S may be either monophosphorylated on Ser_3 (cystatin S1) or diphosphorylated on Ser_1 and Ser_3 (cystatin S2) [19, 20].

Globally, the proteins pertaining to all the above families account for about 90% w/w of the salivary proteome (**Fig. 2**). The remaining 10% w/w of the adult salivary proteome is represented by a multitude of non-specific peptides and proteins like mucins, α-defensins, β-thymosins, human serum albumin (HSA), prolactin inducible protein (PIP), S100 proteins, other cystatins, lysozyme, histones, keratins, lipocalins to cite the most relevant. The current proteomic inventories

report more than 2,400 protein identifications [21]. Human saliva also contains a myriad of fragments derived from bigger proteins, many of which may be called cryptides, having biological properties proper to, and different from, the parent proteins [22]. The characterization of these fragments provides clues to the presence of endogenous and exogenous proteinases, the latter deriving from the microbiota, acting in the oral cavity. Peptide fragments naturally present in saliva can only be detected using a top-down strategy, i.e., a proteomic pipeline that does not involve treatment of the sample with proteolytic enzymes before the analysis [4]. However, the protocol we adopted involving the treatment of the sample with an acidic solution precludes the possibility of studying proteins insoluble in acid solution and proteins with a mass not resolvable by the MS apparatus (for instance, complex glycoproteins and multiprotein complexes with quaternary structures). Nonetheless, it provides precious information on the PTMs occurring *in vivo*. A recent review has widely described the PTMs of the human salivary proteome characterized up to 2023 [23].

The salivary proteome during human development

The first total ion current (TIC)-HPLC profile of preterm newborn saliva obtained by our group around 20 years ago using the top-down pipeline described in **Fig. 1** surprisingly evidenced a protein pattern different from that of adults (**Fig. 3**).

The Deca XP MS apparatus available in our laboratories at that time did not allow us to obtain easily interpretable MS/MS fragmentation spectra of large proteins. Therefore, many masses detected in the TIC profiles remained uncharacterized [3].

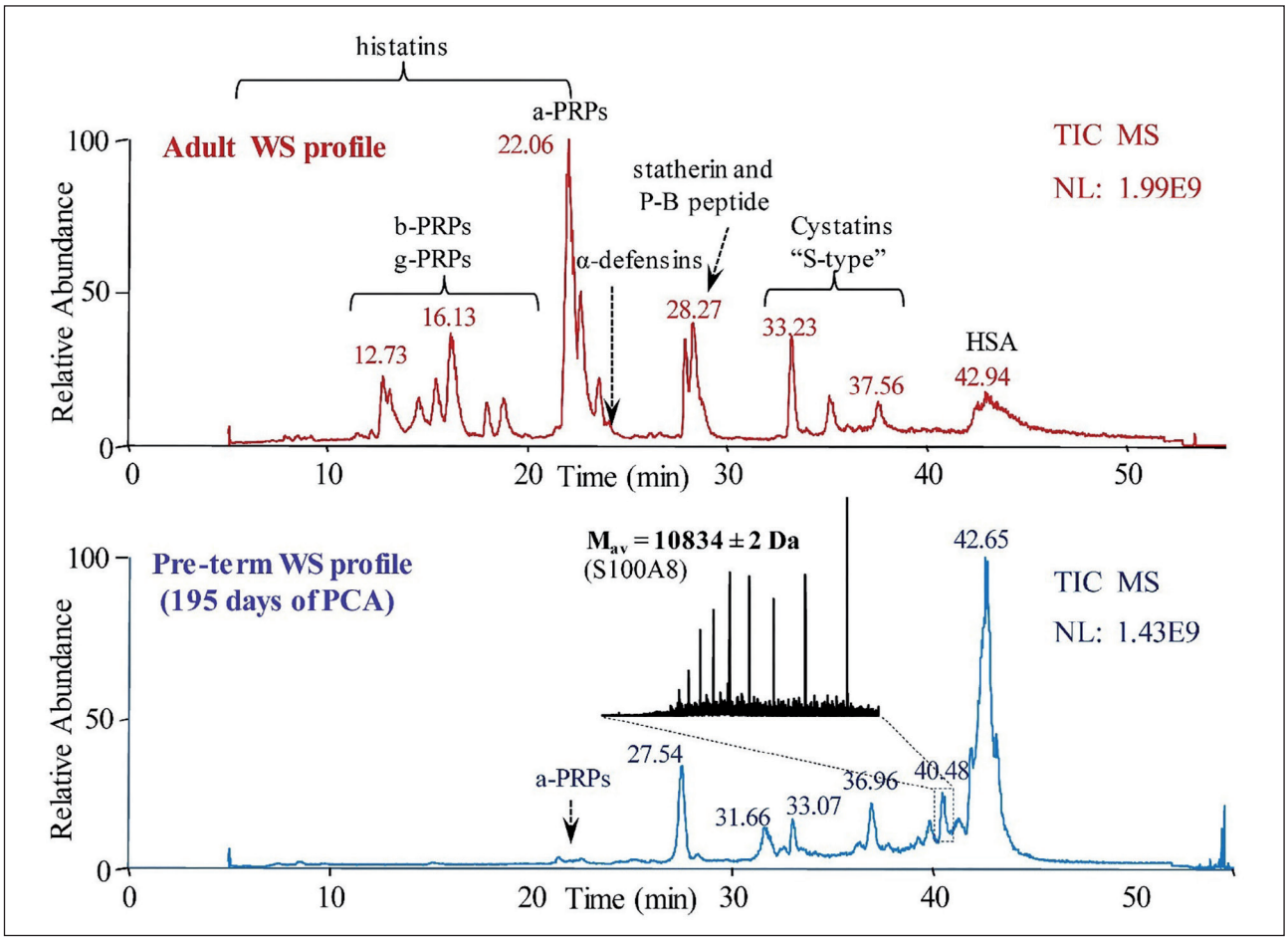

Figure 3. Comparison of low-resolution HPLC-ESI-MS TIC profiles of the acidic soluble fraction of human adult saliva (top panel) and human preterm newborn (195 days PCA) saliva (bottom panel) (modified from: Castagnola et al., 2011 [3]).
In the bottom indent the ESI spectrum of S100A8 protein (M_{av} theor. 10834 Da) is reported to evidence the high dynamic sensitivity of the procedure.
aPRPs: acidic proline-rich proteins; bPRPs: basic proline-rich proteins; ESI: electrospray ionization; gPRPs: glycosylated basic proline-rich proteins; HPLC: high-performance liquid chromatography; HSA: human serum albumin; M_{av}: average molecular mass; MS: mass spectrometry; NL: normalization level; PCA: post-conceptional age; RT: retention time; TIC: total ion current; WS: whole saliva.

Acidic proline-rich proteins

However, despite our initial instrumental limitations, we were able to carry out a year-long follow-up investigation of salivary aPRPs in preterm and at-term newborns [24]. We established that (i) aPRPs are detected (although at low levels) in saliva of preterm newborns starting from 180 days of PCA and that the expression of aPRPs encoded by the *PRH2* locus occurs before those encoded by *PRH1* locus, as evidenced by the possibility to detect in saliva of newborns the PRP-1 and PRP-2 type proteoforms (encoded by *PRH2*) some months before the Db proteoform (encoded by *PRH1*). The evaluation of the relative abundances of the different aPRPs isoforms and of their derivatives as a function of PCA showed that (ii) the proteolytic enzymes generating truncated forms are active since 180 days of PCA; (iii) the kinase involved in aPRP phosphorylation is almost inactive in preterm newborns, but its activity increases with PCA, synchronizing with that of at-term newborns and reaching the adult levels at about 500-600 days of PCA, in concomitance with the beginning of deciduous dentition [25]. This finding allowed us to detect a significant reduction of the levels of phosphorylation in salivary aPRPs, statherin and histatin 1 in a cohort of 16 (out of 27) children with autism spectrum disorders, when compared with an age-matched group of 23 healthy children [26]. This finding suggested that at least a percentage of autistic children have the prodromes of the disease during the late stages of fetal development. The acquisition of the Orbitrap MS apparatus in our laboratories around the end of 2008 allowed us to characterize the structure of a PRP-1 variant detected in 2 Italian individuals and 1 US individual. The variation was present at Ser_{22} residue, which is commonly phosphorylated, and this has outcomes on the HPLC elution time of the variant. It was called PRP-1 (PRP-3) Roma-Boston Ser_{22}(phos)→Phe variant [27].

Basic proline-rich peptide P-J and the other basic proline-rich proteins (the salivary proteome of Neanderthals and other ancient hominids)

Using the Orbitrap apparatus, we were able to characterize the structure of a new basic proline-rich peptide with a monoisotopic *m/z* value $[M^+H^+]1^+$ of 5,941.003, observed in all the adult human saliva samples analyzed [28], and which, following the nomenclature adopted by Saito, Isemura and Sanada, was called P-J [10, 11]. It is encoded by the L allele of the *PRB2* locus. It is remarkable to underline that, in the literature, it is reported the structure of a bPRP [11], never detected in granules of human parotid [9], that, according to the nomenclature of Kauffman and Bennick [29], was called IB-7. It corresponds to the sequence of peptide P-J missing two C-terminal serine residues. Since peptide P-J was always detected in saliva, while IB-7 was rarely instead, our data suggested that the latter was an artifact deriving from the proteolytic cleavage of the two C-terminal serine residues of peptide P-J and that this cleavage infrequently occurs in saliva [28]. The surprising variation of the TIC-HPLC profile we observed in preterm oral fluid [3] led us to investigate the salivary proteome in 67 subjects aged between 3 and 44 years [28]. Subjects were divided into 5 age groups: group A, 3-5 years; group B, 6-9 years; group C, 10-12 years; group D, 13-17 years; group E, 24-44 years. Salivary bPRPs, almost undetectable in the A and B groups, reached salivary levels comparable to that of the E group around puberty. The concentration of aPRPs, histatin 3 fr.1/24, histatin 3 fr.1/25, and cystatins S1 and S2 showed a minimum in the B group. These experiments revealed that the human salivary proteome shows important variations with age beyond adolescence and warns of the conclusions reached in proteomic studies that do not use age-appropriate controls [30]. Moreover, it offers relevant clues to formulate hypotheses on the specific role of salivary peptides and proteins. For instance, the low levels of bPRPs during childhood are suggestive of a role in the perception of food taste. Effectively, the perception of the bitter taste of PROP (6-n-propylthiouracil) seems related to the salivary levels of II-2 and Ps-1 [31], together with aPRPs family, whose role in adults has been recently proposed [32]. In agreement with this hypothesis, a recent work on the Neanderthals' and Denisovians' salivary genes evidenced significant variations of the convertase's consensus sequences of the bPRPs family, which should generate different cleavages processes during their secretion with a consequent different bPRPs asset in the *Homo sapiens* saliva as reported in **Fig. 4**, probably due to different feeding habits with respect to ancient hominids [33].

It is relevant to underline that PRPs are common in the saliva of the Mammalia, but their sequence is rarely conserved, giving further support to the possible role of bPRPs in the development of the

PRB1 salivary archaic fusion-1 peptide (PRB1 SAF-1)

$R_{72}Q$: Neanderthal (13%), Chagyrskaya (8%), Vindija (6%) and Denisovan (9%)

```
  1 qNLNEDVSQE ESPSLIAGNP QGPSPQGGNK PQGPPPPPGK PQGPPPQGGN KPQGPPPPGK
 61 PQGPPPQGDK SQSPRSPPGK PQGPPPQGGN QPQGPPPPPG KPQGPPPQGG NRPQGPPPPG
121 KPQGPPPQGD KSRSP(R)
```

PRB2 salivary archaic fusion-2 peptide (PRB2 SAF-2)

$R_{93}Q$: Neanderthal (61%), Chagyrskaya (63%), Vindija (60%) and Denisovan (100%)

$R_{96}Q$: Neanderthal (100%), Chagyrskaya (100%), Vindija (100%) and Denisovan (24%)

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  1 qNLNEDVSQE ESPSLIAGNP QGAPPQGGNK PQGPPSPPGK PQGPPPQGGN QPQGPPPPPG
 61 KPQGPPPQGG NKPQGPPPPG KPQGPPPQGD KSQSPQSPPG KPQGPPPQGG NQPQGPPPPP
121 GKPQGPPPQG GNKPQGPPPP GKPQGPPPQG DNKSRSS
```

PRB2 salivary archaic cleavage-1 peptide (PRB2 SAC-1)

$Q_{59}R$: Neanderthal (87%), Chagyrskaya (77%), Vindija (67%) and Denisovan (94%)

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  1 SPPGKPQGPP PQGGNQPQGP PPPPGKPQGP PPQGGNKPQG PPPPGKPQGP PPQGDNKSRS
 61 A(R)
```

PRB2 salivary archaic cleavage-2 peptide (PRB2 SAC-2)

```
  1 SPPGKPQGPP PQGGNQPQGP PPPPGKPQGP PPQGGNKPQG PPPPGKPQGP PPQGGSKSRS
 61 S(R)
```

Figure 4. Sequences of four basic proline-rich proteins (bPRPs) hypothetical variants which should be present in archaic hominids (Neanderthal, Chagyrskaya, Vindija, Denisovan) saliva by virtue of several amino acid substitutions evidenced in their DNA sequences (modified from: Di Pietro, 2023 [33]).

In the *PRB1* salivary archaic fusion-1 peptide (*PRB1* SAF-1), the substitution $R_{72}Q$ abolishes the convertase consensus sequence responsible for the cleavage between II-2 and P-E peptides in modern *Homo sapiens*, generating a fused peptide spanning 136 (or 135) amino acids residues, which integrates the modern adult *Homo sapiens* II-2 and P-E peptides. Similarly, in the *PRB2* coded salivary archaic fusion-2 peptide (*PRB2* SAF-2), two substitutions at $R_{93}Q$ and $R_{96}Q$ abolish the convertase consensus sequence responsible for the cleavage at R_{96} between IB-1 and P-J peptides in modern *Homo sapiens* generating a fused peptide spanning 157 amino acids residues, which integrates the modern IB-1 and P-J peptide sequences of adult *Homo sapiens*. Conversely, the presence of a C nucleotide at 11,546,314 bp in archaic hominids DNAs of *PRB2* locus, instead of T in modern humans, led to the introduction of an R instead of the Q_{59} of the IB-8a Con1$^-$ isoform. This substitution generates an additional pro-protein convertase consensus sequence, $R_{59}SAR_{62}$, responsible for the cleavage of the IB-8a Con1$^-$ protein into two smaller peptides. According to the usual removal of the C-terminal arginine residue observed for all the known modern bPRPs 4,8, both peptides should be 61 aminoacidic residues long. These putative archaic hominids' *PRB2* variants were named *PRB2* salivary archaic cleavage-1 peptide (*PRB2* SAC-1 peptide) and *PRB2* salivary archaic cleavage-2 peptide (*PRB2* SAC-2 peptide). The percentages of *PRB2* SAC-2 peptide found in archaic hominids are the same as those of the *PRB2* SAC-1 peptide.

alimentation lifestyles. Moreover, several bPRPs could have a role in the formation of the protein layer termed "oral protein pellicle", elastic and resistant protein networks covering the stratified squamous epithelial layer of the oral mucosa, protecting underlying epithelial cells from various injuries. We were induced to investigate the reactivity of several purified human basic proline-rich peptides, namely P-H, P-D, P-F, P-J, and II-2, as substrates of transglutaminase-2, the enzyme responsible for the formation of the oral protein pellicle [34]. P-H and P-D peptides were very reactive and formed cyclo-derivatives, where only specific glutamine and lysine residues were involved in the cycle formation. II-2 was less reactive, and P-F and P-J almost inactive. Once the cycle was formed, other glutamine residues were hierarchically activated for further links. The heterogeneity of bPRPs is amazing, but the great structural similarity among some of them suggested the division of the bPRPs

into two main groups and a third minor hybrid group (**Fig. 5**) [10].

Group 1 includes P-E, IB-6, P-Ko, Ps-1, Ps-2, P-F, P-H, P-J, and P-D. Group 2 includes IB-1, II-2, and the gPRPs encoded by *PRB3* and *PRB4* genes, namely, GL-1, GL-2, GL-3, GPA, II-1, and Cd-II-g. The N-terminal glutamine of IB-1 and II-2 is converted to a pyro-glutamic acid moiety, and the serine 8 is phosphorylated for the presence of the SXE consensus sequence recognized by the Golgi casein kinase Fam20C [35], responsible for the phosphorylation of other salivary peptides (aPRPs, histatin 1, statherin, and cystatin S). Members of Group 2 can be divided into three subgroups: Group 2A, including IB-1 and II-2, without glycosylation sequons; Group 2B, including the glycosylated (GL) proteins encoded by the alleles of *PRB3* locus; and Group 2C including the glycosylated proteins encoded by the alleles of *PRB4* locus. Unlike the other bPRP loci, the pro-proteins expressed by the *PRB3* locus are not submitted to a proteolytic cleavage before secretion. The small Group 3 is a hybrid group, which includes the two proteoforms of IB-8a, Con1$^-$ and Con1$^+$. They derive from an SNP responsible for S_{100}→P substitution. The protein IB-8a carrying P_{100} is not glycosylated, and it is named Con1$^-$ because it does not bind concanavalin A. IB-8a Con1$^+$, carrying S_{100} and having the NKS glycosylation sequon, may be glycosylated on N_{98} [36]. In one of our studies, IB-8a was detected in 64 subjects (out of 86); 25 were homozygous for IB-8a Con1$^-$ and 22 were homozygous for IB-8a Con1$^+$, and 17 subjects were heterozygous [36]. Surprisingly, the GL-2 (or PRP-3M) glycoforms encoded by the alleles of *PRB3* locus and pertaining to Group 2B were the only gPRPs detectable in significant amounts in the newborn whole saliva, suggesting a role in the lubrication of the developing oral epithelial cells. The characterization of the GL-2 glycoforms was performed by reversed-phase (RP)-HPLC-high-resolution ESI-MS before and after N-deglycosylation of enriched fractions isolated from newborn saliva [37]. The high-resolution top-down ESI-HPLC-MS analyses of adult human saliva allowed us to detect several variants of bPRPs, namely, the P-Ko P_{36}→S (1 out of 86 samples), the P-Ko A_{41}→S (11 out of 86 samples), coded by the *PRB1* locus, the new variant P-H S_1→A (9 out of 86 samples) coded by the *PRB2* locus, and the P-D P_{32}→A variant (18 out of 86 samples) coded by the *PRB4* locus [10].

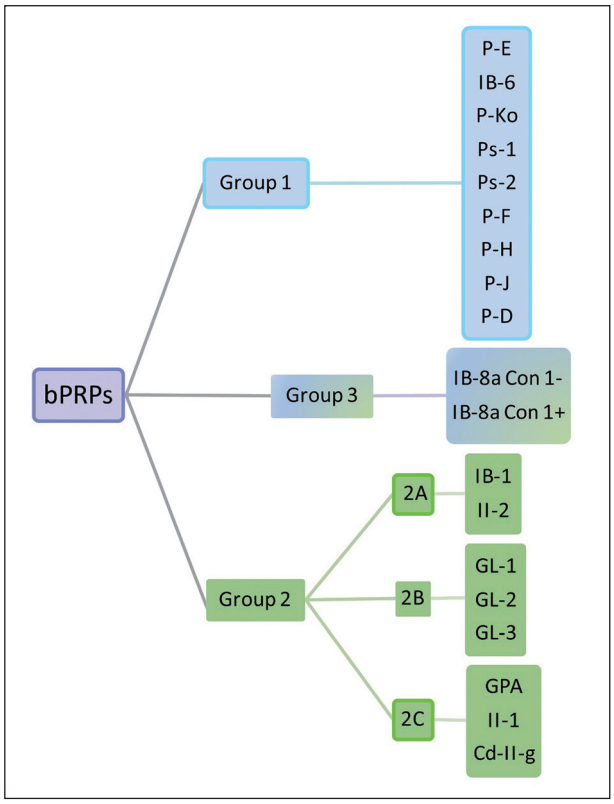

Figure 5. Schematic classification of the adult parent basic proline-rich proteins (bPRPs) based on sequence similarity (modified from: Padiglia et al., 2018 [10]).
The first group, which was named Group 1, includes P-E, IB-6, P-Ko, Ps-1, Ps-2, P-F, P-H, P-J, and P-D. The sequence of all these bPRPs starts with the same SPPGKPQGPP motif, followed by sequences somewhat similar but showing small variations among the different components. The central parts of the sequences show similar repeats. Group 2 includes IB-1, II-2, and the glycosylated basic proline-rich proteins (gPRPs), codified by *PRB3* and *PRB4* genes, namely GL-1, GL-2, GL-3, GPA, II-1, and Cd-II-g. Their sequences start with a similar motif (E/Q)XXXEDVSQEES, where XXX is LNE in IB-1, II-2, GL-1, GL-2, and GL-3 and SSS in GPA, II-1, and Cd-II-g. The central part of the sequences comprises similar repeats with differences from the repeats of the members belonging to Group 1. Based on structural differences, members of Group 2 can be divided into three subgroups: Group 2A, including IB-1 and II-2, without glycosylation sequons; Group 2B, including the glycosylated (GL) proteins codified by the alleles of *PRB3* locus; and Group 2C including the glycosylated proteins codified by the alleles of *PRB4* locus. The small Group 3 is a hybrid group, which includes the two proteoforms of IB-8a, Con1$^-$ and Con1$^+$. The initial sequences of these two proteins resemble that of Group 1, while the terminal sequence is like the repeat responsible for the glycosylation of the bPRPs of Groups 2B and 2C.

β-thymosins

By HPLC-ESI-MS on standard compounds of references, we were able to detect in preterm newborn saliva noticeable amounts of thymosin β_4 ($T\beta_4$) and thymosin β_{10} ($T\beta_{10}$), both missing the N-terminal methionine residue and N-terminally acetylated [38]. This finding stimulated a study devoted to investigating the role of these small and very polar peptides during human development.

We established that, despite their high inter-individual variability, the concentration of $T\beta_4$ at around 180 days of PCA was very high (2.0 mmol/L) and decreased with age, almost disappearing from saliva at an age corresponding to that of full-term newborns [39, 40]. In adult saliva, almost negligible amounts of $T\beta_4$ and $T\beta_{10}$ derive mainly from crevicular fluid [38]. In saliva of preterm newborns, the ratio $T\beta_4/\beta_{10}$ had a constant value of about 4 throughout the PCA range examined. The immunohistochemical analyses of major and minor salivary glands of different preterm fetuses at autopsy, starting from about 90 days of PCA, performed to investigate earlier periods of fetal development, evidenced a strong immunoreactivity of gland cells for $T\beta_4$. Cytoplasmic granules highly reactive for $T\beta_4$ were detected in all fetal salivary glands analyzed, with a maximum of expression around 140–150 days of PCA. In infants and adults' salivary glands, reactive granules in acinar cells were not observed. We just evidenced a diffuse weak cytoplasmatic immunostaining for $T\beta_4$ in ductal cells. This study outlined, for the first time, that salivary glands during fetal life express and secrete peptides such as β-thymosins probably involved in the development of the oral cavity and other organs. To sustain this hypothesis, we investigated the immunoreactivity for $T\beta_4$ in the gastrointestinal tract, finding a strong expression in the human gut and in endocrine pancreas during development [41]. The observed heterogeneity of $T\beta_4$ expression in the fetal life, ranging from a very rare detection in liver cells up to a diffuse reactivity in endocrine pancreas, was a warning to well assess the complex role of $T\beta_4$ in the fetal development. A study on the expression of $T\beta_4$ in human skin and in salivary gland tumors evidenced its very strong immunostaining for the peptide in mast cells [42]. A robust expression of $T\beta_4$ was detected in tumor-infiltrating and peritumoral mast cells in salivary gland tumors and breast ductal infiltrating carcinomas. Tumor-infiltrating mast cells also showed a strong immunoreactivity for chymase and tryptase. From these data, $T\beta_4$ could be considered a potent new marker candidate for the identification of mast cells in skin biopsies as well as in human tumors [43, 44]. The immunolocalization of $T\beta_4$ in the human fetal and adult genitourinary tract was studied in autoptic samples of kidney, bladder, uterus, ovary, testicle, and prostate [43]. The presence of the peptide was observed in cells of different origins: in surface epithelium, in gland epithelial cells and in the interstitial cells. $T\beta_4$ was mainly found in adult and fetal bladder in the transitional epithelial cells; in the endometrium, glands and stromal cells were immunoreactive for the peptide in the adult; $T\beta_4$ was mainly localized in the glands of fetal prostate while, in the adults, a weak $T\beta_4$ reactivity was restricted to the stroma. In adult and fetal kidneys, $T\beta_4$ reactivity was restricted to ducts and tubules with completely spared glomeruli; a weak positivity was observed in adult and fetal oocytes; immunoreactivity was mainly localized in the interstitial cells of fetal and adult testis. This study confirmed that $T\beta_4$ could play a relevant role during human development, even in the genitourinary tract, and confirms that immunoreactivity for this peptide may change during postnatal and adult life. A role of $T\beta_4$ in human carcinogenesis has been proposed [45]. We aimed at evaluating the correlation between $T\beta_4$ immunoreactivity and colorectal cancer, with particular attention to tumor cells undergoing epithelial-mesenchymal transition [46]. $T\beta_4$ was detected in the vast majority (59 out of 76) of colon carcinomas, showing a patchy distribution, with the well-differentiated areas significantly more reactive than the less differentiated tumor zones. We also noted a zonal pattern in many tumors, characterized by a progressive increase in immunostaining for $T\beta_4$ from the superficial toward the deepest tumor regions. The strongest expression for $T\beta_4$ was frequently detected in invading tumor cells with features of epithelial-mesenchymal transition. The increase in reactivity for $T\beta_4$ matched with a progressive decrease in E-cadherin expression in invading cancer cells. Data evidenced that $T\beta_4$ is expressed in colon cancers, with preferential immunoreactivity in deep tumor regions. The preferential expression of the peptide and the increase in intensity of the immunostaining at the invasion front suggests a possible link between the peptide and the process of epithelial-to-mesenchymal transition, suggesting its role in colorectal cancer invasion and metastasis [45]. In a study carried out on colorectal polyps and adenomas, weak cytoplasmic reactivity for $T\beta_4$ was detected in the normal colon mucosa [47]. No reactivity for $T\beta_4$ was found in hyperplastic and sessile serrated polyps/adenomas. $T\beta_4$ expression was observed in 10 (out of 15) colorectal adenocarcinomas. In adenomas with low-grade dysplasia, $T\beta_4$ immunoreactivity was mainly detected in dysplastic glands but was absent in hyperplastic glands. $T\beta_4$ immunoreactivity was characterized by a spot-like perinuclear staining, a

finding suggestive of the localization of the peptide in the trans-Golgi-network (TGN). In high-grade dysplastic polyps, immunostaining for $T\beta_4$ appeared diffuse throughout the entire cytoplasm of dysplastic cells, suggesting a translocation of the peptide from the TGN toward cytoplasmic vesicles. Spot-like perinuclear reactivity was detected in adenocarcinoma tumor cells. The shift of $T\beta_4$ immunolocalization from low-grade to high-grade dysplastic glands suggests a role for $T\beta_4$ in colorectal carcinogenesis, not restricted to the advanced phases of tumor progression, but extended to the initial steps of colorectal carcinogenesis. However, in a study on hepatocellular carcinoma, while $T\beta_4$ was completely absent in tumor cells undergoing stromal invasion, $T\beta_{10}$ showed a strong homogeneous expression, indicating a possible major role for $T\beta_{10}$ (and not for $T\beta_4$) in this tumor progression [48]. All these previous results suggested that tumor cells might utilize molecular programs previously utilized during fetal development, including $T\beta_4$ synthesis, during their expansion. In particular, the process of epithelial-to-mesenchymal transition could represent the unifying process that explains the role of $T\beta_4$ during fetal development and during the progression of cancer, with the acquisition of higher cellular motility that allows migration of tumor cells and vascular and lymphatic invasion [49]. In agreement with these hypotheses, $T\beta_4$ and $T\beta_{10}$ peptides were, for the first time, identified in the intra-cystic fluid of adamantinomatous craniopharyngioma pediatric brain tumor by low- and high-resolution MS analysis coupled with HPLC [50]. Structural analyses carried out by chemical and enzymatic cross-linking allowed us to characterize the contact sites of the complex actin-$T\beta_4$ [51]. Results showed that the complex formation of actin and $T\beta_4$ is more likely to be flexible than rigid and is localized along the subdomains 1 to 3 of actin. A study on the cyto-localization of $T\beta_4$ in HEPG2 cells showed that this peptide can translocate from different cytoplasmic domains to the nuclear membrane and back, based on different stress conditions within the cell, including cell starvation [52]. Ultrastructural studies confirmed the nuclear translocation under cellular stress, suggesting an interaction of $T\beta_4$ with nucleolar actin, probably finalized to modulate the transcription activity of RNA polymerases [53]. Stimulated by all these results, we decided to carry out an experiment in mice to investigate the potential role of $T\beta_4$ during pregnancy. Pregnant mice mothers were treated intra-peritoneally with $T\beta_4$ for two days, precisely E14 and E17 of gestation. Newborns from the $T\beta_4$-treated mothers showed a higher cranio-caudal length when compared to control newborns, suggesting an accelerated fetal growth related to the administration of the peptide during gestation. At histology, maternal mouse $T\beta_4$ treatment was associated with more advanced development of lungs, heart, kidney, cerebral cortex, and notochord [54]. These experiments showed, for the first time, that $T\beta_4$ administration during mouse gestation may act as a powerful fetal growth promoter, by accelerating the development of newborn organs and tissues. These results could be transferred, with due precautions, to the therapeutical treatment of at-risk human fetuses during late pregnancy [55].

Small proline-rich protein 3

HPLC-ESI-MS analysis of saliva from human preterm newborns revealed two proteins with average molecular mass (M_{av}) values of 17,239 ± 3 Da and 18,065 ± 3 Da co-eluting in the same chromatographic TIC peak. Matrix assisted laser desorption ionization time of flight (MALDI-TOF)-MS analysis of the proteins tryptic digest allowed the identification of two isoforms of the small PRP 3 (SPRR3, also called cornifin β, Swiss-Prot code Q9UBC9), and cDNA amplification of RNA extracts from oral mucosa, parotid and submandibular gland samples, obtained at fetal autopsy, provided two nucleotide sequences in agreement with those reported in the literature.

The two proteins differ for an octapeptide repeat (GCTKVPEP) and the substitution Leu→Val, at position Leu_{140} and Leu_{148} (**Fig. 6**). During maturation, the two proteins undergo the removal of the initiator methionine, and N-terminal acetylation [56]. cDNA amplification did not allow us to clarify if the proteins found in saliva originated from cellular shedding of the epithelium and/or secretion. The population evidenced that 9 out of 15 preterm newborns were heterozygous, 4 homozygous for the 17,239 Da isoform, and 2 homozygous for the 18,065 Da isoforms, corresponding to frequencies of about 57% and 43%, respectively, consistent with those expected for trans alleles in the Caucasian population. A study compared the levels of several salivary peptides/proteins determined by the HPLC-ESI-MS extracted ion current (XIC) peak areas in 11 edentulous subjects (age 60–76 years) with respect to 11 age-matched dentate controls [57] and evidenced statistically significant

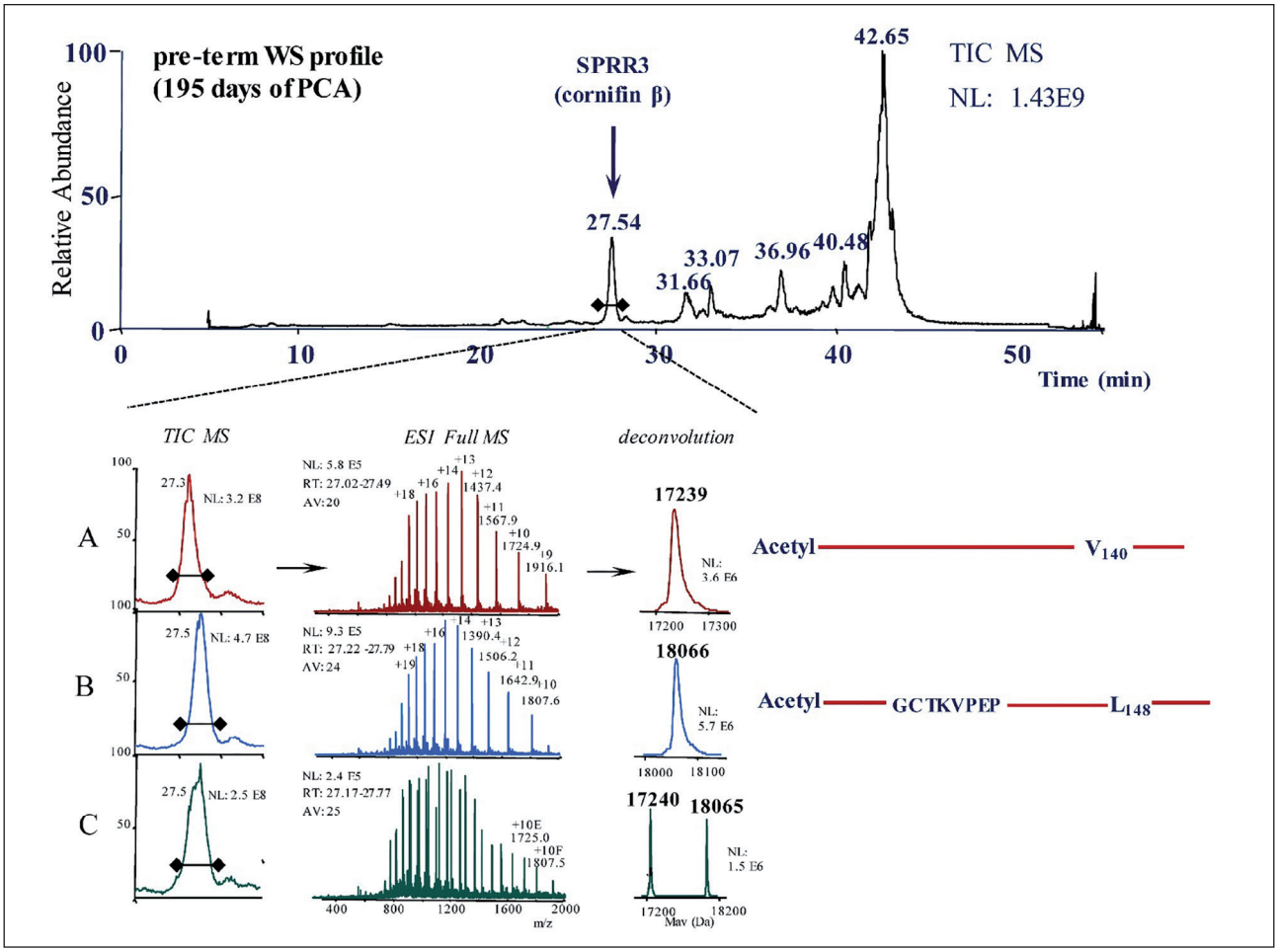

Figure 6. Typical HPLC-ESI-MS TIC profile of acidic solution from human saliva of preterm newborns (modified from: Castagnola et al., 2011 [3], and Manconi et al., 2010 [56]).

The arrow in the top panel evidences the peak pertaining to two proteoforms of the small proline-rich protein 3 (SPRR3, also called cornifin β, Swiss-Prot code Q9UBC9), one with a M_{av} 17,239 ± 3 Da and the other with a M_{av} 18,065 ± 3 Da. The 18 KDa proteoform, with respect to the 17 KDa proteoform, has the insertion of an octapeptide repeat (GCTKVPEP) in the central domain (among 57-104 residues) and two substitutions L→V. Both proteoforms are acetylated on the N-terminus after removal of the initiator methionine. The three bottom panels A-C show the SPRR3 peak profiles of three salivary samples of different preterm newborns. The ESI-MS spectra of the peaks after deconvolution gave the M_{av} of SPRR3 reported in the middle. Panel A: subject homozygous for the 17 KDa proteoform. Panel B: subject homozygous for the 18 KDa proteoform. Panel C: subject heterozygous for both proteoforms.

AV: average; ESI: electrospray ionization; HPLC: high-performance liquid chromatography; M_{av}: average molecular mass; MS: mass spectrometry; NL: normalization level; PCA: post-conceptional age; RT: retention time; SPRR3: small proline-rich protein 3; TIC: total ion current; WS: whole saliva.

differences in the two groups by nonparametric Mann-Whitney test. Levels of SPRR3 were found to be significantly higher in edentulous subjects with respect to dentate controls, while the major peptides and proteins deriving from salivary glands did not show statistically significant differences. SPRR3 proteins are mainly of intracellular origin and represent the major constituents of the cornified cell envelope. They are probably a clue to inflammation of mucosal epithelia.

S100 proteins and cystatins

As reported above, the acquisition of the high-resolution Orbitrap MS around 2008, allowed us to determine the primary structure of various proteins of the S100 family and cystatins A and B detected in high amounts in the saliva of preterm newborns. The S100 family comprises members of the superfamily of Ca^{2+}-binding proteins characterized by the specific EF-hand Ca^{2+}-binding motif [58]. Our study highlighted the high levels of S100A7, S100A8, S100A9, S100A11 and S100A12 proteins in preterm newborn saliva, when compared to adult saliva [3]. Furthermore, it permitted to establish the presence of two proteoforms of S100A7 differing for Asp_{27}→Glu substitution. Unlike the structure reported in Swiss-Prot for the S100A7-D_{27} form (P31151 code), the experimental monoisotopic mass value and the fragmentation patterns agreed

with methionine N-terminal loss and N-terminal acetylation in both forms. More complex is the set of S100A9 forms: S100A9 can exist in two forms with different lengths, the "short form" (about 50%) generated by the releasing of the N-terminal MTCKM pentapeptide due to cleavage at Met_5 by the methionine aminopeptidase (MAP) and the "long form" generated by the canonical MAP removal of N-terminal methionine. The long form can undergo glutathionylation and cysteinylation at Cys_2 [58, 59]. Furthermore, since all the forms may undergo partial phosphorylation on penultimate Thr residue and oxidation of a methionine residue, the total number of possible S100A9 proteoforms is more than eight [60-62]. All the proteoforms of S100A9 are N-terminally acetylated by N-terminal acetyltransferases (NATs) [23]. Interestingly, S100A11, after removal of the N-terminal methionine residue, is N-terminally acetylated too, while S100A12 is not acetylated, and S100A8 neither misses the N-terminal methionine nor is acetylated. Cystatin B does not miss the N-terminal methionine, but it is N-terminally acetylated and was detected in the saliva of preterm newborns as glutathionylated and cysteinylated derivatives at Cys_3, further than as the unmodified form [63]. The study also evidenced the presence of the two fragments 1-53 and 54-98 of cystatin B, the first carrying the Cys_3 residue partly S-glutathionylated and S-cysteinylated in a proportion like the intact protein, suggesting that the fragmentation process is subsequent and independent from the S-modification of the protein [63]. Differently, in adult human saliva, the two fragments of cystatin B were not detectable, and the intact protein was detected only as S-modified derivatives, being about 30% of the protein present as S–S covalent dimer, about 55% as S-glutathionylated, and the remaining 15% as S-cysteinylated [64].

Reviews describing the PTMs characterized in salivary cystatins have been published [65, 66]. All the "S-type cystatins" (SA, SN, S, S1, S2) have two disulfide bridges, which, due to the sequence similarities, involve Cys_{74}-Cys_{84} and Cys_{98}-Cys_{118}. Moreover, SN, S1 and S2 cystatins are submitted to partial oxidation on Trp_{27}. A second oxidation site recognized for cystatins SN and S1 was Trp_{107}, while a precise site for the partial oxidation of SA was not characterized. Cystatin SA is sometimes submitted to partial N-terminal fragmentation, generating the Des_{1-4} SA proteoform, while the N-terminal fragmentation of cystatin SN generates the Des_{1-4} and the Des_{1-7} truncated proteoforms, for both variants with either P_{11} or L_{11} residue [65]. Cystatins A and B are both leaderless. Cystatin A can exist in human saliva both as N-terminally acetylated or not-acetylated. The heterogeneity at the N-terminus was also observed in the cystatin A Thr_{96}→M variant, found in 6 (out of 54) subjects, all heterozygous for the wild-type form [65]. Although several variants of cystatin A have been associated with severe skin diseases [67], none of the 6 subjects evidenced clinical symptoms.

As previously reported, cystatin B is detectable in adult saliva always N-terminally acetylated and only modified at the Cys_3 residue [64]. In 10 out of 54 subjects, we were able to characterize S-(carboxymethyl-Cys_3)-cystatin B, a novel PTM not found in any other human salivary protein [65]. The S-carboxymethyl-cysteine (S-CMC) is included among the advanced glycation end (AGE) products formed by the nonenzymatic reaction of the endogenously formed glyoxal and methylglyoxal, respectively, with cysteine sulfhydryl groups [68]. In contrast with the other reversible modifications, S-CMC is not reversible by transesterification reaction with cellular thiols; consequently, S-CMC derivatives are irreversible and stable products [68]. The presence of S-CMC has been described in plasma in patients with diabetes as a result of the high content of glucose and glyoxal and is considered an indicator of nephropathy [68]. In this regard, the presence of S-CMC derivatives of cystatin B and of the other salivary proteins prone to cysteine oxidation in whole saliva could be proposed as new biomarkers of oral or systemic oxidative stress.

Cystatin D, in strict analogy with "S-type cystatins" and with cystatin C, has two-disulfide bridges (Cys_{73}-Cys_{83} and Cys_{97}-Cys_{117}). It is detectable in whole saliva mainly as truncated proteoforms of the Cys_{26}→Arg variant, probably because the cysteine variant can form an insoluble complex by interacting with other partners with active cysteine residues. In human saliva, the truncated Des_{1-5} proteoform of the Arg_{26} variant was the most abundant, when compared with the Des_{1-4} and Des_{1-8} forms. The N-terminal Gln of the Des_{1-5} proteoform is converted to pyro-Glu (pGlu) [65]. The formation of pGlu can occur spontaneously or in the presence of glutamine cyclase, which can act on either N-terminal glutamine or glutamate. To date, the presence of pGlu, among the salivary proteins, has been observed in aPRP isoforms, α-amylase, and the bPRPs IB-1, IB-4, P-B, and II-2 [23]. The pGlu moiety supplies protein

resistance from degradation by amino-peptidases, and, probably for this reason, cystatin D Des_{1-5} is by far the most abundant in human saliva, even with respect to the intact proteoforms [65].

Post-translational modifications of salivary proteins during fetal development

To investigate the activity of several enzymes and the expression of several salivary proteins during late fetal growth, whole saliva was collected from 17 preterm newborns with a PCA at birth of 178–217 days. In these subjects' sample collection was performed serially starting immediately after birth and within about 1 year of follow-up, gathering a total of 111 specimens. Furthermore, whole saliva was collected from 182 subjects aged between 0 and 17 years and from 23 adults aged between 27 and 57 years. The naturally occurring intact salivary proteome of the 316 salivary samples was analyzed by low- and high-resolution HPLC-ESI-MS platforms. Proteins peculiar to the adults appeared in saliva with different time courses during human development. aPRPs encoded by *PRH2* locus and the gPRP GL2 encoded by *PRB3M* locus appeared following 180 days of PCA, followed at 7 months (± 2 weeks) by histatin 1, statherin, and P-B peptide. The other histatins and aPRPs encoded by *PRH1* locus appeared in whole saliva of babies from 1 to 3 weeks after the normal term of delivery, S-type cystatins appeared at 1 year (± 3 months), and bPRPs appeared at 4 years (± 1 year) of age [69]. All convertase(s), acting on aPRPs and histatin 3, and carboxypeptidase(s) acting on aPRPs, P-C peptide, histatin 6 and statherin were many folds more active in preterm newborns than in the other groups, suggesting that this higher activity is necessary for the molecular modeling involved in the exceptional fetal growth during pregnancy; this encourages further studies devoted to the characterization of the specific substrates [70]. Instead, the activity of the Fam20C kinase, involved in the phosphorylation of aPRPs, histatin 1, statherin and cystatin S types, started around 180 days of PCA, slowly increased, reaching values comparable to adults at about 2 years (± 6 months) of age [69]. Instead, MAPK14, involved in the phosphorylation of S100A9, was fully active since birth also in preterm newborns.

As to the glutathionylation and cysteinylation of cystatin B and S100A9 long concerns, these two proteins were mainly present in unmodified form in extremely preterm newborns; the percentage of the S-thiolated derivatives of both proteins increased according to the PCA; the greatest variation occurred up to about 280 days of PCA. Interestingly, differences in the levels of the S-thiolated derivatives only depended on the PCA and not on whether the infant was born preterm or at term. Inadequate levels of cysteine and glutathione might be responsible for the low level of S-thiolated derivatives measured in preterm newborns [71].

Concluding remarks and future investigations

This review aimed to underline how the top-down proteomic investigation of saliva of human preterm newborns integrated different competencies present in our large team, resulting in an important and fascinating incitement to investigate the possible role of some proteins in human development and to share common scientific concerns. The encouraging results of the top-down proteomic pipeline led us to additionally analyze the acidic insoluble fraction of preterm newborn saliva. The proteomic characterization of acid-insoluble proteins must necessarily and conversely exploit bottom-up strategies, i.e., a 2D electrophoretic map of the resuspended acidic insoluble salivary pellet followed by a tryptic digestion of the spots [72]. By this strategy, we identified three proteins over-expressed in at-term newborns with respect to preterm newborns and adults, i.e., bactericidal permeability-increasing protein (BPI) fold-containing family A member 1, annexin A1, and keratin type 1 cytoskeletal, and we are trying to understand their role.

Since many masses detected by MS in preterm newborn saliva are still uncharacterized, and, due to the increasing sensitivity of new MS apparatus, other masses will be surely revealed, new information useful for understanding human development will be available through the study of this bodily fluid and speed up our future interest on this topic. Although saliva is an oral fluid that can be collected more easily in humans than in other mammals, further information could be obtained by comparative studies too [73].

Declaration of interest

The Authors declare no conflicts of interest. This research received no external funding.

References

1. Tabak LA. A revolution in biomedical assessment: the development of salivary diagnostics. J Dent Educ. 2001;65:1335-9.
2. Malamud D. Saliva as a diagnostic fluid. Dent Clin North Am. 2011;55(1):159-78.
3. Castagnola M, Inzitari R, Fanali C, Iavarone F, Vitali A, Desiderio C, Vento G, Tirone C, Romagnoli C, Cabras T, Manconi B, Sanna MT, Boi R, Pisano E, Olianas A, Pellegrini M, Nemolato S, Heizmann CW, Faa G, Messana I. The surprising composition of the salivary proteome of preterm human newborn. Mol Cell Proteomics. 2011;10:M110.003467.
4. Messana I, Cabras T, Iavarone F, Vincenzoni F, Urbani A, Castagnola M. Unraveling the different proteomic platforms. J Sep Sci. 2013;36:128-39.
5. Habeck T, Lermyte F. Seeing the complete picture: proteins in top-down mass spectrometry. Essays Biochem. 2023;67(2):283-300.
6. 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;31:1948-63.
7. Oppenheim FG, Salih E, Siqueira WL, Zhang W, Helmerhorst EJ. Salivary proteome and its genetic polymorphisms. Ann NY Acad Sci. 2007;1098:22-50.
8. Manconi B, Castagnola M, Cabras T, Olianas A, Vitali A, Desiderio C, Sanna MT, Messana I. The intriguing heterogeneity of human salivary proline-rich proteins. J Proteomics. 2016;134:47-56.
9. Messana I, Cabras T, Pisano E, Sanna MT, Olianas A, Manconi B, Pellegrini M, Paludetti G, Scarano E, Fiorita A, Agostino S, Contucci AM, Calò L, Picciotti PM, Manni A, Bennick A, Vitali A, Fanali C, Inzitari R, Castagnola M. Trafficking and post-secretory events responsible for the formation of secreted human salivary peptides: a proteomics approach. Mol Cell Proteomics. 2008;7:911-26.
10. Padiglia A, Orrù R, Boroumand M, Olianas A, Manconi B, Sanna MT, Desiderio C, Iavarone F, Liori B, Messana I, Castagnola M, Cabras T. Extensive Characterization of the Human Salivary Basic Proline-Rich Protein Family by Top-Down Mass Spectrometry. J Proteome Res. 2018;17(9): 3292-307.
11. Messana I, Cabras T, Inzitari R, Lupi A, Zuppi C, Olmi C, Fadda MB, Cordaro M, Giardina B, Castagnola M. Characterization of the human salivary basic proline-rich protein complex by a proteomic approach. J Proteome Res. 2004;3:792-800.
12. Santos JL, Saus E, Smalley SV, Cataldo LR, Alberti G, Parada J, Gratacòs M, Estivill X. Copy number polymorphism of the salivary amylase gene: implications in human nutrition research. J Nutrigenet Nutrigenomics. 2012;5(3): 117-31.
13. 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;263:7472-77.
14. Castagnola M, Inzitari R, Rossetti DV, Olmi C, Cabras T, Piras V, Nicolussi P, Sanna MT, Pellegrini M, Giardina B, Messana I. A cascade of 24 histatins (histatin 3 fragments) in human saliva: suggestions for a pre-secretory sequential cleavage pathway. J Biol Chem. 2004;279:41436-43.
15. 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 Comm. 1989;160:495-502.
16. Cabras T, Fanali C, Monteiro JA, Amado F, Inzitari R, Desiderio C, Scarano E, Giardina B, Castagnola M, Messana I. Tyrosine polysulfation of human salivary histatin 1. A post-translational modification specific of the submandibular gland. J Proteome Res. 2007;6(7):2472-80.
17. Heller D, Helmerhorst EJ, Oppenheim FG. Saliva and Serum Protein Exchange at the Tooth Enamel Surface. J Dent Res. 2017;96(4):437-43.
18. Inzitari R, Cabras T, Rossetti DV, Fanali C, Vitali A, Pellegrini M, Paludetti G, Manni A, Giardina B, Messana I, Castagnola M. Detection in human saliva of different statherin and P-B fragments and derivatives. Proteomics. 2006;6:6370-9.
19. Isemura S, Saitoh E, Sanada K, Minakata K. Identification of full-sized forms of salivary (S-type) cystatins (cystatin SN, cystatin SA, cystatin S, and two phosphorylated forms of cystatin S) in human whole saliva and determination of phosphorylation sites of cystatin S. J Biochem. 1991;110(4):648-54.
20. Lupi A, Messana I, Denotti G, Schininà ME, Gambarini G, Fadda MB, Vitali A, Cabras T, Piras V, Patamia M, Cordaro M, Giardina B, Castagnola M. Identification of the human salivary cystatin complex by the coupling of high-performance liquid chromatography and ion-trap mass spectrometry. Proteomics. 2003;3:461-7.
21. Bandhakavi S, Stone MD, Onsongo G, Van Riper SK, Griffin TJ. A dynamic range compression and three-dimensional peptide fractionation analysis platform expands proteome coverage and the diagnostic potential of whole saliva. J Proteome Res. 2009;8:5590-600.
22. Iavarone F, Desiderio C, Vitali A, Messana I, Martelli C, Castagnola M, Cabras T. Cryptides: latent peptides everywhere. Crit Rev Biochem Mol Biol. 2018;53(3):246-63.
23. Messana I, Manconi B, Cabras T, Boroumand M, Sanna MT, Iavarone F, Olianas A, Desiderio C, Rossetti DV, Vincenzoni F, Contini C, Guadalupi G, Fiorita A, Faa G, Castagnola M. The Post-Translational Modifications of Human Salivary Peptides and Proteins Evidenced by Top-Down Platforms. Int J Mol Sci. 2023;24(16):12776.
24. Inzitari R, Vento G, Capoluongo E, Boccacci S, Fanali C, Cabras T, Romagnoli C, Giardina B, Messana I, Castagnola M. Proteomic analysis of salivary acidic proline-rich proteins in human pre-term and at-term newborns. J Proteome Res. 2007;6:1371-7.

25. Manconi B, Cabras T, Pisano E, Sanna MT, Olianas A, Fanos V, Faa G, Nemolato S, Iavarone F, Castagnola M, Messana I. Modifications of the acidic soluble salivary proteome in human children from birth to the age of 48 months investigated by a top-down HPLC-ESI-MS platform. J Proteomics. 2013;91: 536-43.
26. Castagnola M, Messana I, Inzitari R, Fanali C, Cabras T, Morelli A, Pecoraro AM, Neri G, Torrioli MG, Gurrieri F. Hypo-phosphorylation of salivary peptidome as a clue to the molecular pathogenesis of autism spectrum disorders. J Proteome Res. 2008;7:5327-32.
27. Iavarone F, D'Alessandro A, Tian N, Cabras T, Messana I, Helmerhorst EJ, Oppenheim FG, Castagnola M. High-resolution high-performance liquid chromatography with electrospray ionization mass spectrometry and tandem mass spectrometry characterization of a new isoform of human salivary acidic proline-rich proteins named Roma-Boston Ser22 (Phos) → Phe variant. J Sep Sci. 2014;37(14): 1896-902.
28. Cabras T, Pisano E, Boi R, Olianas A, Manconi B, Inzitari R, Fanali C, Giardina B, Castagnola M, Messana I. Age-dependent modifications of the human salivary secretory complex. J Proteome Res. 2009;8:4126-34.
29. 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;30:3351-56.
30. Castagnola M, Cabras T, Iavarone F, Fanali C, Nemolato S, Peluso G, Bosello SL, Faa G, Ferraccioli G, Messana I. The human salivary proteome: a critical overview of the results obtained by different proteomic platforms. Expert Rev Proteomics. 2012;9(1):33-46.
31. 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(2):e30962.
32. Yousaf NY, Melis M, Mastinu M, Contini C, Cabras T, Tomassini Barbarossa I, Tepper BJ. Time Course of Salivary Protein Responses to Cranberry-Derived Polyphenol Exposure as a Function of PROP Taster Status. Nutrients. 2020;12:2878.
33. Di Pietro L, Boroumand M, Lattanzi W, Manconi B, Salvati M, Cabras T, Olianas A, Flore L, Serrao S, Calò CM, Francalacci P, Parolini O, Castagnola M. A Catalog of Coding Sequence Variations in Salivary Proteins' Genes Occurring during Recent Human Evolution. Int J Mol Sci. 2023;24:15010.
34. Boroumand M, Olianas A, Manconi B, Serrao S, Iavarone F, Desiderio C, Pieroni L, Faa G, Messana I, Castagnola M, Cabras T. Mapping of transglutaminase 2 sites of human salivary small basic proline-rich proteins by HPLC high resolution ESI-MS/MS. J Proteome Res. 2020;19(1):300-13.
35. Tagliabracci, VS, Pinna LA, Dixon JE. Secreted protein kinases. Trends Biochem Sci. 2013;38:121-30.
36. Cabras T, Boi R, Pisano E, Iavarone F, Fanali C, Nemolato S, Faa G, Castagnola M, Messana I. 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(9):1079-86.
37. Manconi B, Cabras T, Sanna M, Piras V, Liori B, Pisano E, Iavarone F, Vincenzoni F, Cordaro M, Faa G, Castagnola M, Messana I. N- and O-linked glycosylation site profiling of the human basic salivary proline-rich protein 3M. J Sep Sci. 2016;39(10):1987-97.
38. Inzitari R, Cabras T, Pisano E, Fanali C, Manconi B, Scarano E, Fiorita A, Paludetti G, Manni A, Nemolato S, Faa G, Castagnola M, Messana I. HPLC-ESI-MS analysis of oral human fluids reveals that gingival crevicular fluid is the main source of thymosins beta4 and beta10. J Sep Sci. 2009;32:57-63.
39. Nemolato S, Messana I, Cabras T, Manconi B, Inzitari R, Fanali C, Vento G, Tirone C, Romagnoli C, Riva A, Fanni D, Di Felice E, Faa G, Castagnola M. Thymosin β4 and β10 levels in pre-term newborn oral cavity and fetal salivary glands evidence a switch of secretion during fetal development. PLoS One. 2009;4:e5109.
40. Fanni D, Gerosa C, Nemolato S, Locci A, Marinelli V, Cabras T, Messana I, Fanos V, Castagnola M, Faa G. Thymosin beta 10 expressions in developing human salivary glands. Early Hum Dev. 2011;87(12):779-83.
41. Nemolato S, Cabras T, Cau F, Fanari MU, Fanni D, Manconi B, Messana I, Castagnola M, Faa G. Different thymosin beta 4 immuno-reactivity in fetal and adult gastrointestinal tract. PLoS One. 2010;5:e9111.
42. Nemolato S, Cabras T, Fanari MU, Cau F, Fraschini M, Manconi B, Messana I, Castagnola M, Faa G. Thymosin beta 4 expression in normal skin, colon mucosa and in tumor infiltrating mast cells. Eur J Histochem. 2010;54:e3.
43. Nemolato S, Cabras T, Fanari MU, Cau F, Fraschini M, Manconi B, Messana I, Castagnola M, Faa G. Immunoreactivity of thymosin beta 4 in human foetal and adult genitourinary tract. Eur J Histochem. 2010;54:e43.
44. Olianas A, Serrao S, Piras V, Manconi B, Contini C, Iavarone F, Pichiri G, Coni P, Zorcolo L, Orrù G, Messana I, Faa G, Castagnola M, Fanni D, Cabras T. Thymosin β4 and β10 are highly expressed at the deep infiltrative margins of colorectal cancer – A mass spectrometry analysis. Eur Rev Med Pharmacol Sci. 2021;25:7285-96.
45. Wang WS, Chen PM, Hsiao HL, Ju SY, Su Y. Overexpression of the thymosin beta-4 gene is associated with malignant progression of SW480 colon cancer cells. Oncogene. 2003;22:3297-306.
46. Nemolato S, Restivo A, Cabras T, Coni P, Zorcolo L, Orrù G, Fanari M, Cau F, Gerosa C, Fanni D, Messana I, Castagnola M, Casula G, Faa G. Thymosin beta 4 in colorectal cancer is localized predominantly at the invasion front in tumor cells undergoing epithelial-mesenchymal transition. Cancer Biol Ther. 2012:13(4):191-7.

47. Nemolato S, Cabras T, Restivo A, Zorcolo L, Di Felice E, Fanni D, Gerosa C, Messana I, Castagnola M, Faa G, Casula G. Thymosin ß4 expression in colorectal polyps and adenomas. Clinics. 2013;68(9):1220-4.
48. Theunissen W, Fanni D, Nemolato S, Di Felice E, Cabras T, Gerosa C, Van Eyken P, Messana I, Castagnola M, Faa G. Thymosin beta 4 and thymosin beta 10 expression in hepatocellular carcinoma. Eur J Histochem. 2014;58(1):2242.
49. Faa G, Nemolato S, Cabras T, Fanni D, Gerosa C, Fanari M, Locci A, Fanos V, Messana I, Castagnola M. Thymosin β4 expression reveals intriguing similarities between fetal and cancer cells. Ann NY Acad Sci. 2012;1269(1):53-60.
50. Desiderio C, Martelli C, Rossetti DV, Di Rocco C, D'Angelo L, Caldarelli M, Tamburrini G, Iavarone F, Castagnola M, Messana I, Cabras T, Faa G. Identification of thymosin β4 and β10 in paediatric craniopharingioma cystic fluid. Childs Nerv Syst. 2013;29(6):951-60.
51. Knop J, App C, Horn AH, Iavarone F, Castagnola M, Hannappel E. High-Resolution HPLC-ESI-MS characterization of the contact sites of the actin-thymosin β4 complex by chemical and enzymatic cross-linking. Biochemistry. 2013;52(33): 5553-62.
52. Pichiri G, Coni P, Nemolato S, Cabras T, Fanari MU, Sanna A, Di Felice E, Messana I, Castagnola M, Faa G. Cellular trafficking of thymosin beta-4 in HEPG2 cells following serum starvation. PLoS One. 2013;8(8):e67999.
53. Piludu M, Piras M, Pichiri G, Coni P, Orrù G, Cabras T, Messana I, Faa G, Castagnola M. Thymosin beta 4 may translocate from the cytoplasm into the nucleus in HepG2 cells following serum starvation. An ultrastructural study. PLoS One. 2015;10(3):e0119642.
54. Faa G, Piras M, Mancuso L, Coni P, Pichiri G, Orrù G, Fanni D, Gerosa C, Cao G, Taibi R, Pavone P, Castagnola M. Thymosin beta-4 prenatal administration improves fetal development and halts side effects due to preterm delivery. Eur Rev Med Pharmacol Sci. 2021;25(1):431-7.
55. Castagnola M, Cao G, Kalcev G, Orrù G, Fanos V, Faa G, Carta MG. Antenatal thymosin β4: a new tool for accelerating fetal development in preterms? Thymosin beta-4: a breakthrough in the "Physiological" regenerative medicine in preterm newborns. Biointerface Res Appl Chem. 2021;11(6): 13860-5.
56. Manconi B, Cabras T, Pisano E, Nemolato S, Inzitari R, Iavarone F, Fanali C, Sanna MT, Tirone C, Vento G, Romagnoli C, Faa G, Castagnola M, Messana I. Characterization of two isoforms of SPRR3 from preterm human newborn saliva, autoptic fetal oral mucosa, parotid and submandibular gland samples. Biochem Biophys Res Comm. 2010;398:477-81.
57. Manconi B, Liori B, Cabras T, Iavarone F, Manni A, Messana I, Castagnola M, Olianas A. Top-down HPLC-ESI-MS proteomic analysis of saliva of edentulous subjects evidenced high levels of cystatin A, cystatin B and SPRR3. Arch Oral Biol. 2017;77:68-74.
58. Heizmann CW. S100 proteins: Diagnostic and prognostic biomarkers in laboratory medicine. Biochim Biophys Acta Mol Cell Res. 2019;1866(7):1197-206.
59. Lim SY, Raftery MJ, Goyette J, Geczy CL. S-Glutathionylation regulates inflammatory activities of S100A9. J Biol Chem. 2010;285:14377-88.
60. Castagnola M, Cabras T, Iavarone F, Vincenzoni F, Vitali A, Pisano E, Nemolato S, Scarano E, Fiorita A, Vento G, Tirone C, Romagnoli C, Cordaro M, Paludetti G, Faa G, Messana I. Top-down platforms for deciphering the human salivary proteome. J Matern Fetal Neonatal Med. 2012;25(Suppl 5):27-43.
61. Cabras T, Iavarone F, Manconi B, Olianas A, Sanna MT, Castagnola M, Messana I. Top-down analytical platforms for the characterization of the human salivary proteome. Bioanalysis. 2014;6(4):563-81.
62. Castagnola M, Cabras T, Iavarone F, Fanali C, Messana I. Detection of Ca(2+)-binding S100 proteins in human saliva by HPLC-ESI-MS. Methods Mol Biol. 2013;963:357-71.
63. Iavarone F, Cabras T, Pisano E, Sanna MT, Nemolato S, Vento G, Tirone C, Romagnoli C, Cordaro M, Fanos V, Faa G, Messana I, Castagnola M. Top-down HPLC-ESI-MS detection of S-glutathionylated and S-cysteinylated derivatives of cystatin B and its 1-53 and 54-98 fragments in whole saliva of human pre-term newborns. J Proteome Res. 2013;12(2): 917-26.
64. Cabras T, Manconi B, Iavarone F, Fanali C, Nemolato S, Fiorita A, Scarano E, Passali GC, Manni A, Cordaro M, Paludetti G, Faa G, Messana I, Castagnola M. RP-HPLC-ESI-MS evidenced that salivary cystatin B is detectable in adult human saliva mostly as S-modified derivatives: S-glutathionyl, S-cysteinyl and S-S 2-mer. J Proteomics. 2012;75:908-13.
65. Manconi B, Liori B, Cabras T, Vincenzoni F, Iavarone F, Castagnola M, Messana I, Olianas A. Salivary cystatins: exploring new post-translational modifications and polymorphisms by top-down mass spectrometry. J Proteome Res. 2017;16(11):4196-207.
66. Turk V, Brzin J, Kotnik M, Lenarcic B, Popović T, Ritonja A, Trstenjak M, Begić-Odobasić L, Machleidt W. Human cysteine proteinases and their protein inhibitors stefins, cystatins and kininogens. Biomed Biochim Acta. 1986;45(11-12):1375-84.
67. Blaydon DC, Nitoiu D, Eckl KM, Cabral RM, Bland P, Hausser I, van Heel DA, Rajpopat S, Fischer J, Oji V, Zvulunov A, Traupe H, Hennies HC, Kelsell DP. Mutations in CSTA, encoding Cystatin A, underlie exfoliative ichthyosis and reveal a role for this protease inhibitor in cell-cell adhesion. Am J Hum Genet. 2011;89(4):564-71.
68. Zeng J, Davies MJ. Evidence for the formation of adducts and S-(carboxymethyl)cysteine on reaction of alpha-dicarbonyl compounds with thiol groups on amino acids, peptides, and proteins. Chem Res Toxicol. 2005;18(8):1232-41.
69. Messana I, Cabras T, Iavarone F, Manconi B, Huang L, Martelli C, Olianas A, Sanna MT, Pisano E, Sanna M, Arba M, D'Alessandro A, Desiderio C, Vitali A, Pirolli D, Tirone C,

Lio A, Vento G, Romagnoli C, Cordaro M, Manni A, Gallenzi P, Fiorita A, Scarano E, Calò L, Passali GC, Picciotti PM, Paludetti G, Fanos V, Faa G, Castagnola M. Chrono-proteomics of human saliva: variations of the salivary proteome during human development. J Proteome Res. 2015;14(4):1666-77.

70. Boroumand M, Iavarone F, Manconi B, Pieroni L, Greco V, Vento G, Tirone C, Desiderio C, Fiorita A, Faa G, Messana I, Cabras T, Olianas A, Castagnola M. HPLC-ESI-MS top-down analysis of salivary peptides of preterm newborns evidenced high activity of some exopeptidases and convertases during late fetal development. Talanta. 2021;222:121429.
71. Boroumand M, Manconi B, Serrao S, Iavarone F, Olianas A, Cabras T, Contini C, Pieroni L, Sanna MT, Vento G, Tirone C, Desiderio C, Fiorita A, Faa G, Messana I, Castagnola M. Investigation by top-down high-performance liquid chromatography–mass spectrometry of glutathionylation and cysteinylation of salivary S100A9 and cystatin B in preterm newborns. Sep Sci Plus. 2021;5(1):1-11.
72. Arba M, Iavarone F, Vincenzoni F, Manconi B, Vento G, Tirone C, Cabras T, Castagnola M, Messana I, Sanna MT. Proteomic characterization of the acid-insoluble fraction of whole saliva from preterm human newborns. J Proteomics. 2016;146:48-57.
73. Castagnola M, Cabras T, Vitali A, Sanna MT, Messana I. Biotechnological implications of the salivary proteome. Trends Biotechnol. 2011;29:409-18.