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An integrated *top-down* and *bottom-up* proteomic platform to reveal potential salivary biomarkers of the rare disorders SAPHO syndrome, Wilson's disease and Hereditary angioedema

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Abstract

Wilson's disease, SAPHO syndrome and Hereditary angioedema are three rare disorders characterized by a wide spectrum of different clinical manifestations, which involve several organs and apparatus, making the diagnosis extremely difficult. In this study, the salivary proteome and peptidome of subjects affected by these pathologies has been investigated using mass spectrometry, through an integrated *top-down* and *bottom-up* platform, and compared with groups of healthy controls, with the aim to assess whether qualitative and quantitative variations of salivary proteins and peptides could be associated to the immune derangement distinctive of each disease and in order to have suggestions on potential specific salivary biomarkers.

The analysis of the salivary proteome from patients affected by Wilson's disease allowed to characterize new oxidized proteoforms of S100A8 and S100A9 and two fragments of the polymeric immunoglobulin receptor named ASVD and AVAD. Higher levels of these proteins and peptides observed in the patients are most likely connected to the oxidative stress, the activation of the inflammatory processes, and the hepatic damage caused by the altered copper transport and its subsequent accumulation in the organism, which is at the origin of the pathology. The observed increase of the level of α-defensins 2 and 4 may give a contribution to the development of the disease by the improvement of the free copper.

The proteome of patients affected by SAPHO syndrome revealed a significant decrease of cystatins, histatins, and aPRPs, which are involved in the protection against infections, suggesting a reduced ability of these subjects to contrast bacteria colonization, in particular *P. acnes* which is a possible trigger of this disease. In particular, the lower levels of histatins and the higher frequency of S100A12 observed in patients with respect to controls, may be connected with the dysregulation of the innate immunity and the neutrophil response typical of SAPHO syndrome. Cystatin SN abundance decrease correlated with the disease duration, suggesting its reduced production during the chronic phase of the

disease, while histatins showed positive correlation with serum levels of the C reactive protein.

In saliva of Hereditary angioedema patients, the increased percentage of peptides generated by the proteolytic cleavage by metalloproteinases indicates the intense metalloproteinase activity possibly connected to the activation of inflammatory pathways. Interestingly, in consideration of the possible role of cystatin B in enhancing the production of nitric oxide, and the higher salivary levels measured in the patients, we suggest that cystatin B may give a contribution to the vasodilatation and the vasopermeability responsible for the oedema formation, which is the main feature of this pathology.

In conclusion, the results obtained in these studies clearly highlighted that the salivary proteome showed some features specific of the three pathologies. Even though these results have been obtained in a small cohort of patients, due to the difficult recruitment of subjects affected by rare disorders, and need further validation by using orthogonal techniques, they strongly suggest that saliva, with easy and non-invasive collection characteristics, could be a biofluid suitable for diagnostic applications.

Introduction

- **Salivary Proteome**

Saliva is a clear body fluid hypotonic compared to plasma composed by more than 99% of water, that contains significant amounts of proteinaceous material (including enzymes such as amylase, lysozyme, lipase, acid phosphatase, lactoperoxidase, superoxide dismutase, etc.; various peptide hormones), glycoproteins (the main constituents of the mucosal secretions), lipid (hormones such as testosterone and progesterone) and inorganic ions such as sodium, chloride, potassium, calcium, magnesium, bicarbonate, phosphate. It is secreted by major (parotid, submandibular and sublingual) and minor (labial, palatine, buccal and lingual) salivary glands. According to viscosity of their secretions, that is dependent from the content of mucins and lipids, salivary glands can be clustered in serous (parotid), mucous (minor glands), and mixed (sublingual and submandibular). Under resting conditions, the contribution of parotid, submandibular and sublingual glands to saliva accounts for about 20%, 65% and 7%, respectively, and less than 10% derives from numerous minor glands. Saliva secretion follows circadian rhythms: the minimum flow rate is observed in the early morning, and the maximum of secretion is reached in the afternoon, when the parotid gland contribution accounts for more than 50% of the total secretion. The term "saliva" specifically refers to the salivary gland secretion, while "whole saliva" or "oral fluid" are both used to indicate the complex solution deriving also from the contribution of gingival crevicular fluid, and containing non-adherent bacteria and food residues (Edgar, 1992; Humphrey & Williamson, 2001). Saliva is essential for the preservation and the maintenance of the oral health, playing a wide range of different actions. This fluid is necessary to lubricate mouth tissues, forming a barrier against irritant elements, e.g. hydrolytic enzymes produced by plaque bacteria, and substances derived from smoking. Mucins, complex glycosylated proteins are the main lubricating components for their high viscosity, great elasticity, strong adherence and they participate to the formation of the acquired enamel pellicle that protect tooth

and also support speech, mastication and swallowing (Humphrey & Williamson, 2001; Messana, 2008a; Nieuw Amerongen, 2002). Some salivary elements, i.e. statherin, histatins, cystatins and salivary proline-rich proteins, regulate calcium homeostasis and mobilization, allowing the equilibrium between demineralization and remineralization necessary for the maintenance of the tooth integrity (Humphrey & Williamson, 2001; Messana, 2008a). Of great importance is saliva antibacterial activity, in which immunologic and nonimmunologic elements are involved: IgA, secreted by plasma cells; IgG and IgM, deriving from gingival crevicular fluid; glycoproteins, statherins, agglutinins, histidine-rich proteins, proline-rich peptides, mucins and enzymes secreted by salivary glands. Lactoferrin binds ferric ions that, consequently, are not available for microorganism nutrition, while, among the enzymes, lysozyme inhibits bacterial growth with the contribution of peroxidase that produces thiocynate, toxic for microorganisms, thus protecting tissues from oxidative stress caused by oral bacteria (Edgar, 1990; Edgar, 1992; Humphrey & Williamson, 2001). Also mucins give their contribution in the control of bacterial and fungal colonization promoting benign commensal flora growth. On the other hand, glycoproteins, statherin, agglutinins, histatins and salivary proline-rich proteins are involved in a "clumping" process that reduces bacteria ability to adhere and colonize oral tissues (Mandel, 1989; Humphrey & Williamson, 2001). Finally, but not less significant, is the role of saliva in nutrition giving its contribution to chewing, swallowing, digestion and taste perception (Humphrey & Williamson, 2001; Cabras, 2012a; Melis, 2015). Salty taste perception depends on saliva hypotonic nature and on gustin which is able to bind zinc ions (Humphrey & Williamson, 2001) while lubrication of the food bolus, promoted mainly by mucins, enhances the swallowing (Humphrey & Williamson, 2001; Hatton, 1985).

The great variety of functions in which this body fluid is involved and its role in the health of the oral cavity highlights the importance of a proper salivation and saliva composition. In the last years many studies, allowed to identify several different components and to characterize various classes of proteins and peptides. Most of them are specific for the oral cavity and belong to four main

families (salivary proline-rich proteins, cystatins, histatins and statherin) while others are common to other organs and body fluids.

Proline-rich proteins (PRPs) represent the major fraction of salivary proteins, more than 60% in weight of the total salivary proteome, and they can be classified in acidic (aPRPs), basic (bPRPs) and basic glycosylated (gPRPs) (Bennick, 1982). Acidic PRPs, secreted by parotid (70%) and submandibular and sublingual glands (30%), are encoded by two loci *PRH-1* and *PRH-2* localized on chromosome 12p13. *PRH-1* codifies for PIF-s, Db-s and Pa, *PRH-2* for PRP-1 and PRP-2 proteins. The acidic character is due to several glutamic and aspartic acid residues located in the first 30 amino acids. All aPRPs show a pyroglutamic acid residue at the *N*-terminus and most of them present two phosphorylated serine residues at position 7 and 22 although low levels of mono-phosphorylated, non-phosphorylated and three-phosphorylated (also on serine 17) proteoforms can be detected in whole saliva. PRP-1, PRP-2, PIF-s and Db-s can be cleaved giving rise to a common peptide of 44 amino acids, named P-C peptide, and four truncated forms named PRP-3, PRP-4, PIF-f and Db-f. Instead Pa forms dimers through a disulfide bridge between cysteine 103 (Azen, 1987; Azen, 1988; Hay, 1994; Inzitari, 2005). Basic and glycosylated PRPs, secreted only by parotid glands, are the expression product of four loci: *PRB1*, *2*, *3* and *4* located near aPRP genes. Each locus includes at least 3 alleles: *S*, small; *M*, medium; *L*, large (Lyons, 1988a; Lyons, 1988b; Maeda, 1985a; Maeda, 1985b) and a further allele *VL*, very large, has been described for *PRB1*, *PRB2* and *PRB3* (Lyons, 1988b; Azen, 1990). These alleles encode for pre-proproteins which, after peptide-signal removal, undergo extensive and complete proteolytic cleavages before secretion, thus only fragments of the proproteins can be detected in saliva. Proteins and peptides deriving from *PRB1* proproteins are: II-2 peptide (from *S*, *M*, *L* alleles), P-E peptides and IB-6 protein (from *S* allele), Ps-1 protein (from *M* allele) and Ps-2 protein (from *L* allele). From PRB2 proproteins, IB-1, P-J, P-H, P-F peptides and IB-8a protein (from *L* allele) have been characterized while *PRB3* and *PRB4* proproteins give rise to glycosylated proteins and *PRP4* proproteins also to P-D peptide (from *S*, *M*, *L* alleles). Moreover, P-J, P-F and IB-8a can be further cleaved during granule maturation

(Lyons, 1988a; Azen, 1993; Azen, 1996; Stubbs, 1998; Chan, 2001; Messana, 2004; Messana, 2008b; Cabras, 2009; Cabras, 2012b; Castagnola, 2012a; Manconi, 2015; Messana, 2015). Acidic PRPs play a role in modulating calcium ions homeostasis (Bennick, 1981), are absorbed in the hydroxyapatite forming the acquired enamel pellicle (Moreno, 1982; Bennick, 1983a) and could be involved in the bacterial colonization (Gibbons, 1991). On the other hand, basic PRPs bind tannins preventing their absorption and toxic effect on the gastro-intestinal tract (Bennick, 2002) and are involved in the perception of the bitter taste (Cabras, 2012a; Melis 2015). Glycosylated PRPs not only play lubricating actions (Hatton, 1985) but it has been also observed *in vitro* that bacteria can use their glycans as a substrate for their own metabolism and growth (Rudney, 2010).

Salivary cystatins belong to the cystatin superfamily. They are encoded by *CST1-5* genes, located on chromosome 20p11.21, and include three different families. Also called stefins, cystatin A and B are proteins ca. 100 amino acidic residues long, without disulfide bonds, and show cytoplasmic localization. Cystatin B has been observed in different body fluids and it has been detected in human saliva as S-modified derivatives: S-glutathionylated, S-cysteinylated and S-S dimeric form (Bobek, 1992; Dickinson, 2002; Cabras, 2012c). Salivary cystatins SN, SA and S (mainly secreted by the submandibular gland) (Shomers, 1982) as well as cystatin C and cystatin D (this last one secreted by parotid gland) (Freije, 1991) belong to the family 2. They are constituted by ca. 120 amino acids and present two intrachain disulfide bonds. Cystatin S can be mono-phosphorylated on serine 3 (S1) or di-phosphorylated on serine 1 and serine 3 (S2). All of them have been found in other body fluids like urine, tears and seminal plasma while cystatin C has a wider extracellular distribution (Abrahamson, 1986; Freije, 1991; Bobek, 1992; Dickinson, 2002). The third family of cystatins includes low-molecular-weight kininogen (LMWK), high-molecular-weight kininogen (HMWK) and T-kininogens: single-chain glycoproteins with multiple disulfide bonds found in plasma and secretions (Dickinson, 2002). Cystatins are inhibitor of the cysteine proteinases, thus they protect the oral cavity from the proteolytic action of host, bacterial, viral and parasitic proteinases. Furthermore, they seem to play an antibacterial and

antiviral action not related with proteinase inhibitory activity. They also showed antifungal action and the ability to modulate the immune system (Bobek, 1992; Gu, 1995; Blankenvoorde, 1996; Abe, 1998; Hiltke, 1999; Baron, 1999; Ruzindana-Umunyana, 2001; Dickinson, 2002; Magister, 2013; Lindh, 2013). Salivary cystatins also participate to the mineralization of the tooth and to the formation of the acquired enamel pellicle (Bobek, 1992; Dickinson, 2002). Cystatin SN and marginally SA are also able to control lysosomial cathepsins implicated in the destruction of periodontal tissues (Bobek, 1992; Baron, 1999). Moreover, Cystatin SA has been implicated in the induction of cytokines by human gingival fibroblasts (Kato, 2000).

Histatins are low molecular weight peptides, deriving their name from the high number of histidine residues on their structure, secreted both by major and minor salivary glands. Two genes, *HIS1* and *HIS2*, localized on chromosome 4q13, encode respectively for histatin 1 and histatin 3 that have a similar sequence. Before secretion, histatin 3 is exposed to an extensive proteolytic cleavage, leading the formation of histatin 6 (histatin$_3$ 1/25), histatin 5 (histatin$_3$ 1/24) and other fragments. On the other hand, histatin 1 is not cleaved and is mostly found phosphorylated at serine 2; minor tyrosine-sulfated derivatives have been also described (Oppenheim, 1988; Castagnola, 2004; Cabras, 2007; Messana, 2008a; Fabian, 2012; Cabras, 2014). Histatins show antifungal activity (Ruissen, 2001; Diaz, 2005), inhibitory effect on several oral bacteria (White, 2009), stimulate wound healing (Oudhoff, 2010), and they are also involved in the formation of the enamel pellicle and in the protection of the tooth structure (Humprey, 2001; Li, 2004; Yin, 2006; Vitorino, 2007; Vitorino, 2008) Histatin-derived peptides, like histatin 5, have been demonstrated to be active against various microbes (Oppenheim, 1999).

Statherin is a small peptide of 43 amino acids codified by the *STATH* gene located on chromosome 4q13.3 (Sabatini, 1987). Secreted by parotid and submandibular glands (Schlesinger, 1977), it is di-phosphorylated on serine 2 and serine 3, but also mono- and non-phosphorylated isoforms of this protein and a cycle-statherin can been observed in low quantities (Cabras, 2006;

Messana 2008a). Statherin has been demonstrated to play a key role in the oral calcium homeostasis, having high affinity for the hydroxyapatite, in the teeth mineralization and in the formation of the enamel pellicle, especially the cyclized form (Cabras, 2006; Schlesinger, 1977). Usually included in the bPRP family, the P-B peptide is encoded by *PROL3* which is strictly close to the statherin gene, suggesting a functional relationship with this protein. However, its role has not been defined yet (Messana, 2008a; Isemura, 2000; Inzitari, 2006).

Other proteins and peptides not specific of saliva are generally detected in this body fluid, i.e. defensins, thymosins, S100 proteins, α-amylases, albumin, agglutinin, carbonic anhydrase, peptide hormones and immunoglobulins. Some of these proteins, investigated in the present study, are described below.

α-defensins, also named human neutrophil peptides, are basic peptides rich in tyrosine and cysteine residues, the latter forming three disulfide bonds. *DEFA1*, *DEFA3* and *DEFA4* genes, located in chromosome 8p23.1, encode for α-defensin 1, 3 and 4 respectively, while α-defensin 2 derives from a proteolytic cleavage of the *N*-amino-terminal residue of α-defensin 1 or 3 (Valore, 1992). Detected in saliva, these four defensins derive mainly from the gingival crevicular fluid (Pisano, 2005) and in particular from neutrophils. They have antimicrobial activity and are involved in the regulation of the cell volume, cytokine production (Chaly, 2000; Lehrer, 2012), chemotaxis and inhibition of natural-killer cells (Goebel, 2000). α-defensin 4, also called corticostatin, exhibits pro-inflammatory effects through its anti-corticotropin property, which inhibits the production of cortisol (Singh, 1988).

β-thymosins are ubiquitous polar peptides, firstly isolated from calf thymus (Klein, 1965), which are involved in the prevention of actin filament polymerization, induction of metalloproteinases, chemotaxis, angiogenesis; inhibition of inflammation and bone marrow stem cell proliferation. They have been also associated to cancer and metastasis formation (Huff, 2001; Hannappel, 2007; Hannappel, 2010). Thymosin β4, and β4 oxidized (encoded by *TMSB4X* gene clustered on chromosome Xp22.2) and β10 (encoded by *TMSB10* located on chromosome 2p11.2) have been detected in whole saliva; they mainly

derive from gingival crevicular fluid (Badamchian, 2007; Inzitari, 2009; Castagnola, 2011a).

S100s are a family of low molecular weight acidic proteins with two distinct calcium ion binding domains. 25 proteins have been identified, among them S100A proteins are encoded by genes located on chromosome 1q21. They have no intrinsic catalytic activity but, after calcium binding, structural modifications allow them to bind and modulate the action of other proteins. S100 proteins, abundant in vertebrates but completely absent in invertebrates, are constitutively expressed in neutrophils, myeloid cells, platelets, osteoclasts and chondrocytes but can be induced and overexpressed in several cell types (macrophages, monocytes, keratinocytes, fibroblasts) in acute and chronic inflammatory, and oxidative stress conditions (Edgeworth, 1991; Vogl, 1999; Eckert, 2004; Carlsson, 2005; Sedaghat, 2008; Lim, 2008; Goyette, 2011). It has been demonstrated their involvement in a wide range of intracellular and extracellular functions: regulation of calcium homeostasis, cytoskeletal rearrangement, contraction and motility, cell growth and differentiation, membrane organization, arachidonic acid transport, chemotaxis, apoptosis, promotion of wound repair, protection against microbial proliferation, control of ROS formation, inflammation and protein phosphorylation and secretion (Ravasi, 2004; Santamaria-Kisiel, 2006; Lim, 2008; Sedaghat, 2008; Thorey, 2001; Donato, 2003). Their activity can be altered and regulated through formation of homodimers and heterodimers and by numerous post translational modifications: phosphorylation, methylation, acetylation and oxidation that can change their ability to bind ions or target proteins (Lim, 2008; Andrassy, 2006; Zimmer, 2003). In particular, S100A8 and S100A9 act as scavengers of ROS, protecting tissues from the excess of oxidant (Lim, 2008; McCormick, 2005; Harrison, 1999). Among them, S100A7, S100A8, S100A9, S100A11 and S100A12 were already detected in human saliva (Castagnola, 2011a).

Today, a great number of studies on the salivary proteome have provided an increasingly comprehension of the composition of this biological fluid and the wide spectrum of functions in which salivary proteins are involved has

stimulated research to fully understand their mechanisms of action, their reciprocal interaction and the relations with other components in the oral cavity. Moreover, the easy, rapid and noninvasive collection of saliva samples pushed, in the last years, many researchers to consider the possibility of using this biofluid for diagnostic and prognostic purposes, not only for oral diseases but also for systemic pathologies. Thus, several proteomic studies have been performed to evidence potential salivary biomarkers, and the research done during this PhD had this main goal.

- ***Top-down* and *Bottom-up* integrated platforms for proteomic analysis**

Proteomics studies on the complex human salivary proteome composition are mainly performed by the association of high-throughput separation methods with the different mass spectrometry techniques. Several proteomic platforms have been developed to achieve specific goals with the best results.

Proteomic platforms can be classified in qualitative and quantitative (Nikolov, 2012) as well as in *top-down* and *bottom-up* (Bogdanov, 2005).

According to the purpose of the study, qualitative or quantitative platforms can be adopted. Qualitative analyses are carried out either to characterize the whole proteome and peptidome of a sample or to analyze specific protein classes, or post-translational modifications, or the typical set of proteins specifically expressed in cellular sub-compartments, without considering their abundance. Conversely, quantitative platforms are employed to determine the amount of each protein component within and among different samples, as levels of proteins and/or their different isoforms can change under different physiological and pathological conditions.

Top-down and *bottom-up* approaches differ in the protocol applied for the sample treatment (Fig.1). *Top-down* platforms analyze proteins and peptides in their naturally occurring form, giving particular attention to avoid, as much as possible, any sample alteration (Tipton, 2011). The *bottom-up* approach derives from the shot-gun strategies developed for the detection of DNA sequences in genomic studies and consists in the analysis of the sample digested by specific

enzymes, generally trypsin, which cleave proteins in correspondence of defined amino acidic residues. Tryptic peptides showing a primary sequence that is univocal for a specific protein (unique peptides) allow to deduce the protein presence within the original sample.

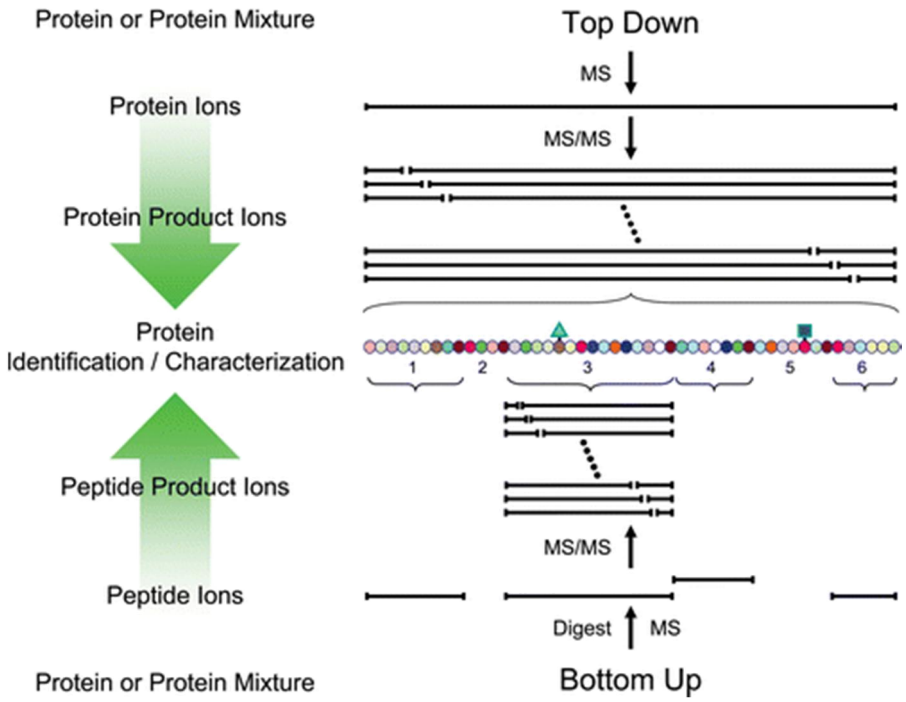

Fig.1 *Top-down* and *Bottom-up* approaches.

Both *top-down* and *bottom-up* analyses carried out by tandem mass spectrometry require previous separation steps, in order to reduce the high complexity of the mixture. Separation methods can be classified in: gel-based approaches, which can be applied for *bottom-up* analysis, such as the 2-dimensional gel electrophoresis (2-DE); or gel-free-based approaches, employed for *top-down* experiments, for example liquid chromatography.

According to the temporal order between the separation step and the digestion process, *bottom-up* strategies can be further classified in break-then-sort and sort-then break (Han, 2008). In break-then-sort approaches, the digestion is carried out on the whole set of proteins present in the sample followed by high efficient chromatographic separations coupled to tandem mass spectrometry experiments. On the other hand, in sort-then break strategies, the proteome is submitted to the separation steps in order to select and then digest only specific proteins of interest further submitted to MS/MS spectrometry analyses. The

later approach is adopted, for example, in experiments centered on the phospho-proteome characterization or on the study of the less abundant proteins by enriched fraction preparation.

Top-down mass spectrometry analyses of the human saliva acidic soluble fraction enable the simultaneous detection of all the soluble proteins and peptides in the sample (Castagnola, 2012b). High-resolution MS instruments allowed to obtain accurate structural information, as well as to identify and characterize different polymorphisms and several post-translational modifications, i.e. phosphorylation, *N*-terminal acetylation and oxidation (Messana, 2004; Inzitari, 2005; Inzitari, 2006; Messana, 2008b; Cabras, 2010; Cabras, 2012c; Castagnola, 2012b, Iavarone, 2013; Cabras, 2013). Of great interest is also the possibility to characterize the naturally occurring peptides generated in the sample by the action of –endo and exo-proteases (Amado, 2010; Thomadaki, 2011). Moreover, the *top-down* approach allowed to establish the specific origin of the proteins (glandular, ductal or oral) and even to clarify when post-translational modifications and proteolytic cleavages occur along the secretory pathway (Messana, 2008b).

Top-down proteomics allows label-free quantification of entire proteins, peptides and their different derivatives and fragments naturally present in the sample by a powerful label-free approach based on the measurement of the eXtracted Ion Current (XIC) peak area. This approach, avoiding the employment of labeled peptides, consents to perform quantification without any limitation on the number of the species under study (Castagnola, 2012b; Cabras, 2014). The relative percentages of different isoforms of the same protein in a sample can be calculated (Inzitari, 2005; Iavarone, 2013) and their diverse abundance, as well as the dissimilar patterns of protein fragmentation, can be compared in different samples and correlated to specific physiological states (Cabras, 2009; Morzel, 2012; Hardt, 2005b; Messana, 2015) or pathological conditions (Thomodaki, 2013; Cabras, 2010; Cabras, 2013).

Despite the wide spectrum of applications, the *top-down* tandem mass spectrometry platform bears some drawbacks. In fact, this technique does not allow to characterize the intact structure of higher molecular weight or

glycosylated proteins, due to the complexity of MS/MS spectra, that cannot be automatically analyzed by available software (Meyer, 2011).

Conversely, the *bottom-up* approach supported by data banks and bio-informatics tools for automatic analysis of MS/MS data allows to characterize thousands of peptides in a single experiment. However, the enzymatic fragmentation preceding the analysis reflects on the inevitable loss of qualitative and quantitative information on the naturally occurring peptidome. In addition, post-translational modification may remain undetected by this approach, and determination of the abundance of different isoforms of the same protein within the sample may be not possible (Tipton 2011, Castagnola, 2012b; Messana, 2013; Cabras, 2014).

On the basis of these considerations, the best solution to perform a deep characterization of the human salivary proteome and peptidome is the implementation of both *top-down* and *bottom-up* approaches, in order to take advantage of the two strategies and contemporaneously minimize their limitations (Cabras, 2014).

- **Wilson's disease**

Wilson's disease (WD) is an autosomal recessive genetic disorder of copper metabolism, characterized by a defective biliary excretion of copper and its failed incorporation into ceruloplasmin, a copper-protein with ferroxidase activity that normally binds 95% of blood copper (Hellman, 2002). The resulting excess of free copper in hepatocytes causes oxidative stress, apoptosis and hepatic damage. As a consequence, a massive amount of unbound copper is spilled into the blood and accumulated in other organs, such as brain, heart, kidney, and cornea, which in turn will be impaired (Ala, 2007). The pathology is connected to the activity of the transmembrane p-type ATPase called ATP7B. At low or normal copper level, the ATP7B transports copper into the trans Golgi compartment for its incorporation into ceruloplasmin, while at high level, copper is moved to be excreted with the bile (Fanni, 2005). Mutations in ATP7B block the transport of copper (Huster, 2003). In the European population, the modification H1069Q is the most common but over 350 different ATP7B

mutations have been identified, and are listed in the Wilson's disease mutation database (http://www.wilsondisease.med.ualberta.ca/database.asp) (Bugbee 2001). The majority of patients are heterozygous, and the distribution of the mutations depends on the population tested (Ala, 2007). The incidence of the disease is estimated from 1/30000 to 1/100000 with a carrier frequency of 1/90 (Wu, 2015), although the incidence is varying in different populations, i.e. in Sardinian people is about 1/3000 live births (Gialluisi, 2013). Wilson's disease is a progressive disorder with a broad spectrum of clinical manifestations: hepatic, neurological and psychiatric that may develop in variable way (Ala, 2007; Crisponi, 2012; Carta, 2012). The early diagnosis is crucial, as the disease can be lethal when untreated, and prevents neurological disability and liver cirrhosis (Gitlin, 2003). A unique diagnostic test is not available. In fact, the diagnosis, that is easy in patients with neurological symptoms but hard in individuals with liver deficiencies, is based on the evaluation of different features and on the recognition of typical symptoms, the later often non-specific, and confusing. Decreased serum ceruloplasmin, increased urinary copper excretion, elevated hepatic copper concentration and serum aminotransferases, and Kayser-Fleischer rings on cornea are considered diagnostic biomarkers of the disease (Ala, 2007; Ferenci, 2003; Roberts, 2003). Nonetheless, 5-40% of patients exhibit normal ceruloplasmin levels (Steindl, 1997), the serum/urine copper levels can be equivocal, and aminotransferase activity cannot reflect the severity of liver injury (Ala, 2007). In addition, histochemical confirmation of the liver copper content, helpful in diagnosis, requires invasive analyses, and a negative result cannot exclude the pathology (Ferenci, 2005; Sini, 2013; Liggi, 2013). *ATP7B* mutation analysis is very advantageous, but systemic genetic testing is not routinely applied in clinical practice due to the many different mutations implicated. The need of novel and unequivocal diagnostic biomarkers has stimulated the research towards proteomics investigations on animals (Lee, 2011; Simpson, 2004; Wilmarth, 2012), *in vitro* models (Roelofsen, 2004) and human serum (Park, 2009) of Wilson's disease. These studies, based on *bottom-up* proteomic platforms, highlighted altered levels of proteins involved in the oxidative stress, which is the typical feature of Wilson's disease.

- **SAPHO syndrome**

Synovitis, Acne, Pustulosis, Hyperostosis and Osteitis (SAPHO) syndrome is a rare and often unrecognized disorder characterized by cutaneous and musculoskeletal inflammations, which appear in variable combinations (Nguyen, 2012). SAPHO syndrome has been classified in the spondyloarthropathies (SpA) on the basis of clinicopathological features and similarity to psoriatic arthritis (Van Doornum, 2000). Recent evidences leads to consider this disorder within the spectrum of bone autoinflammatory diseases (Colina, 2010; Kastner, 2010). Although several studies investigated the origin of the disease and the relationship with mandibular osteomyelitis, the etiology, probably involving genetic, immunologic and infectious mechanisms, is still unknown (Mochizuki, 2012; Suei, 2003), The interaction between infectious agents (i.e. *Propionibacterium acnes*) and the immune system in a genetically predisposed subject may result in dysregulation of neutrophil responses and autoinflammation (Hurtado-Nedelec, 2008; Assman, 2011). First-line treatment is usually based on steroidal anti-inflammatory drugs (NSAIDs), systemic corticosteroids, bisphosphonates and synthetic disease-modifying anti-rheumatic drugs (DMARDs), but there is no standard therapy for SAPHO syndrome. Biological drugs, particularly anti-tumor necrosis factor alpha (TNF-α) and anti-interleukin-1 (IL-1) agents, have been successfully employed but usually reserved to resistant cases (Firinu, 2014b). Due to its rarity (estimated prevalence <1/10000), and features overlapping with other rheumatic and non-rheumatic disorders, SAPHO syndrome represents a diagnostic challenge for clinicians (Nguyen, 2012). In particular, cutaneous manifestations may overlap with psoriasis or other neutrophilic dermatoses (Braun-Falco, 2011), and bone manifestations with SpA and infectious osteitis. To date, no reliable biomarkers have been available for this disease. Despite extensive bone and skin inflammation during exacerbations, the C reactive protein (CRP) and the erythrocyte sedimentation rate (ESR) are usually normal or only slightly elevated in less than one third of the cases. Moreover, routine laboratory means do not allow to detect and evaluate the low-grade inflammatory activity that

may persist also during remission (Colina, 2009). Therefore, it would be of extreme importance the identification of new laboratory biomarkers for the diagnosis of the disease and monitoring of its activity.

- **Hereditary angioedema**

Hereditary angioedema (HAE) is a rare autosomal dominant disease, which affect 1 out of 50000 individuals (Bafunno, 2014). Firstly described by Quincke in 1882 and fully documented by Osler in 1888 (Quincke, 1882; Osler, 1888), the disease is clinically characterized by recurrent episodes of nonpruritic acute swelling involving the face, the trunk as well as the extremities, the skin, the gastrointestinal mucosa and the upper respiratory tract, leading to airway obstruction and asphyxiation if not promptly treated (Frank, 1976; Davis, 2005; Zuraw, 2008; Bafunno, 2014).

The disease is connected to mutations that can occur in *SERPING1* gene or in *FXII* gene, which respectively encode for the C1 esterase inhibitor (C1-INH) and for the coagulation factor XII (FXII), also called Hageman factor. Alterations in *SERPING1* gene have been detected since 90's and nowadays an extremely large number of mutations are listed in a database created in 2005 by Kalmár (Kalmár, 2005; http://hae.enzim.hu/index.php). Alterations of this gene in HAE patients can determine low concentrations of serum C1-INH (Donaldson, 1963), or the production of a protein with defective inhibitor functions. These two different patterns were firstly documented by Rosen, who proposed to classify these patients as HAE type I and HAE type II, respectively (Rosen, 1965; Rosen, 1971). Another type of HAE, characterized by normal C1-INH protein and absence of *SERPING1* mutations, has been documented and initially named HAE type III (Binkley, 2000; Bork, 2000). Originally observed exclusively in women (Binkley, 2000; Bork, 2000) and correlated with high estrogen level conditions (i.e. pregnancy or use of exogenous estrogens) (Binkley, 2000; Bork 2003, Picone; 1010), this last form has been found recently also in male subjects (Bork, 2006; Bork, 2007; Martin, 2007; Vitrat-Hincky, 2010; Charignon, 2014; Moreno, 2015). Genetic studies allowed to associate HAE type III with mutations occurring in *FXII* gene (Dewald, 2006; Bork, 2011; Kiss, 2013).

The coagulation factor XII and the C1 esterase inhibitor are strictly connected. C1-INH is the main inhibitor of the classical pathway of the complement cascade and, in addition to its well-known ability to block the first component of the complement system C1 (Ratnoff, 1957; Ratnoff, 1969; Harpel, 1975), this protein is also able to inhibit some serine proteases acting in the kallikrein-kinin formation system and in the blood coagulation cascade, in particular FXII, which plays a central role in the activation of these pathways (Harpel, 1975; Schreiber, 1973a; Schreiber, 1973b; van der Graaf, 1983; de Agostini, 1984; Davis, 1986; Salvesen, 1985). Thus, in HAE type I and II, low levels and defective functionality of C1-INH protein lead to an impaired inhibition of the coagulation factor XII. Furthermore, *FXII* gene mutations have been associated with the expression of proteins characterized by a more efficient activation (Dewald, 2006; Chicon, 2006; Björqvist, 2015), and different studies demonstrated increased prekallikrein activation in HAE patients (Shapira, 1982) and the presence of higher levels of the final product of the kallikrein-kinin pathway, the bradykinin peptide, in the region of the edema during acute attacks (Nussberger, 1998; Nussberger, 1999; Cugno, 2003). These evidences suggested that this peptide plays a main role in HAE as its overproduction leads to the increased vasodilatation, endothelial permeability and vascular leakage responsible for HAE subcutaneous and submucosal swelling (Fields, 1983; Shoemaker, 1994; Dewald, 2006; Cichon, 2006).

Although different genes are responsible for HAE disease, the common involvement of C1-INH and FXII in the kallikrein-kinin system formation pathway gives rise to similar clinical manifestations.

A recent classification of different forms of angioedema proposed by Cicardi (Cicardi, 2014), which includes both hereditary and acquired typologies has been adopted in this study. HAE caused by mutations in *SERPING1* and *FXII* genes are classified as C1-INH-HAE (which embraces both type I and type II) and FXII-HAE, respectively, while a third subgroup includes subjects with familial hereditary angioedema with unknown genetic mutation (U-HAE). All these inherited forms can be distinguished from a second group in which the pathology is acquired, causing C1-INH deficiency without family history (C1-

INH-AAE) or it is triggered by treatment with inhibitors of the angiotensin-converting-enzyme (ACEI-AAE) or else is generated by non-identified causes. The latter, is further divided in idiopathic histaminergic angioedema (Ih-AAE), in which the recurrent episodes of swelling can be blocked by continue histamine administration, and idiopathic non-histaminergic angioedema (InH-AAE) which is resistant to histamine action (Cicardi, 2014).

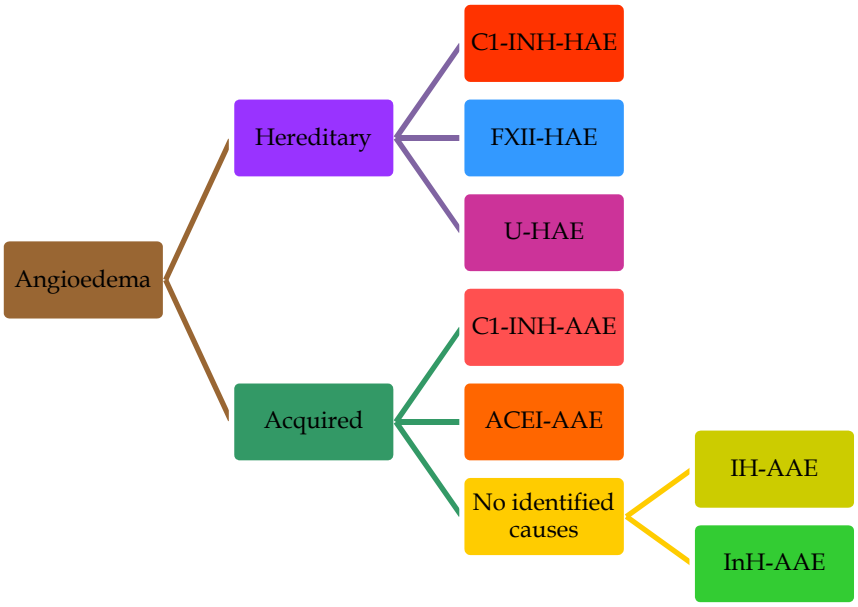

Fig.2 Classification of angioedema different forms.

Different strategies have been developed to treat HAE, focused both on the control of acute swelling attacks when they manifest, and prevention of the number and the severity of further episodes by short and long term prophylaxis. Plasma purified C1-INH or the safer recombinant C1-INH (Ruconest®, Pharming Group and Salix Pharmaceuticals) are treatment of choice to contrast acute attacks, being able to replace the deficiency of the native protein, and thus mainly used for C1-INH-HAE. Moreover, a synthetic competitive antagonist of bradykinin for Bk2R receptor binding, Icatibant (Firazyr®, Jerini AG, Sydney, Australia) has been efficiently developed, both for C1-INH-HAE and FXII-HAE. A third option is the subcutaneous administration of Ecallantide (Kalbitor®, Dyax Corp., Cambridge, MA, USA), a recombinant protein which is able to inhibit kallikrein activity and thus bradykinin formation. For long-term cures, attenuated androgens (mainly Danazol and Stanozolol which increase hepatic C1-INH production) or tranexamic acid (that

blocks plasminogen proteolytic activity) are generally used. For U-HAE patients each of the previously mentioned therapies can be chosen, although an universal therapy is not yet available. Tranexamic acid is mainly employed for long-term prophylaxis of InH-AAE patients, although the response of the patients to the treatment is different, while In-AAE symptoms can be efficiently blocked by continue histamine administration. For ACEI-AAE patients, interruption of the ACE therapy is the first choice, but also bradykinin drugs can reduce the manifestations. On the other hand, plasma C1-INH and attenuated androgens are used for C1-INH-AAE.

The similar manifestations of the pathology among these groups of patients and the unclear causes responsible for some forms of angioedema, require in-depth studies focalized to identify genetic or molecular differences which can be useful to clarify the mechanisms of the disease and to develop focused and efficient treatments.

Objectives of the study

The main goal of this study was to assess whether the immune derangement observed in the three pathologies investigated could be associated to qualitative and quantitative variations of the salivary proteins and peptides in the patients with respect to control subjects to have suggestions on potential biomarkers selective for each condition.

Materials and methods

• Materials

All chemicals and reagents were of analytical grade and were purchased from Sigma Aldrich (St. Louis, MI), Merck (Darmstadt, Germany) and Bio-Rad (Hercules, CA).

• Samples

Study subjects

The informed consent process was in agreement with the latest stipulations established by the Declaration of Helsinki. Ethics Committee approval was obtained for the study.

Wilson's disease

Wilson's disease patients were enrolled at the Hepato-Gastroenterology Service of the "Policlinico Universitario Monserrato", Cagliari. The patients were 32 (42 ± 13 years old; 18 females, 14 males), the healthy control group comprised the same number of subjects (39 ± 14 years old; 19 females, 13 males. The diagnosis was based on the combination of several criteria: clinical symptoms, Kayser-Fleisher ring, and laboratory tests. Based on clinical manifestations the patients were classified as hepatic (N = 23), hepatic/neurologic (N = 4), hepatic/psychiatric (N = 1), hepatic/neurologic/ psychiatric (N = 4). Diagnosis was confirmed by serum ceruloplasmin, total serum copper, cupruria baseline, hepatic parenchymal copper concentration following hepatic biopsy. Histological evaluation of grading and staging of liver fibrosis, and molecular biology analysis of the *ATP7B* gene mutations were also carried out.

SAPHO syndrome

10 SAPHO patients (38.0 ± 11.1 years old) with a protracted disease course, fulfilling criteria of Benhamou (Benhamou, 1988) were enrolled at the Internal Medicine and Immunology outpatients clinic of Cagliari University. 28 healthy females (33.5 ± 10.3 years old) were enrolled as controls.

Demographics, treatments and status of the disease were collected for SAPHO patients at the time of the study and they are listed in Table 1.

Table 1.

Pt #	Disease duration (Years)	Historical disease status		Current treatment	Current disease status	
		Skin	Bone		Skin	Bone (Site/s)
1	27	Hidradenitis suppurativa, psoriasis	Sternal hyperostosis, Sspondylodiscitis	Biological drug (ADA[a])	REM[b]	ACT[c] (SC[d])
					Partial remission	
2	8	Psoriasis	Sternocostoclavicular osteitis, hyperostosis	NSAIDs[e]	REM	ACT (SC)
					Partial remission	
3	16	Palmoplantar pustulosis	Sternocostoclavicular hyperostosis and osteitis, zigomatic and parietal bone, multiple foci of spondylodiscitis	Biological drug (ADA)	ACT	ACT (SC. SI[f], Spine)
					No remission	
4	7	Palmoplantar pustulosis	Sacroiliitis, sternoclavicular hyperostosis	Biological drug (ADA)	REM	ACT (SC, SI)
					Partial remission	
5	4	Palmoplantar pustulosis	Sternoclavicular osteitis	Biological drug (ADA)	REM	ACT (Jaw, SC)
					Partial remission	
6	7	Palmoplantar pustulosis	Arthritis, synovitis, sternocostal hyperostosis, femur osteitis	Biological drug (ADA)	REM	REM (None)
					Remission	
7	3	Palmoplantar pustulosis	Sacroiliitis, sternocostoclavicular osteitis	Biological drug (ADA)	REM	ACT (SC)
					Partial remission	
8	2.5	None	Sacroiliitis, sternoclavicular hyperostosis, clavicular edema	Biological drug (ADA)	-	REM (None)
					Remission	
9	23	Palmoplantar pustulosis	Sternocostoclavicular hyperostosis and osteitis, sacroiliitis, femur osteitis	NSAIDs	ACT	ACT (SC, SI)
					No remission	
10	10	Severe acne	Arthritis (elbow, knee), sternocostoclavicular hyperostosis and osteitis	Biological drug (ADA)	REM	REM (None)
					Remission	

[a]Adalimumab; [b]Remission; [c]Active; [d]Sternocostal or sternoclavicular; [e]Non steroideal anti-inflammatory drugs; [f]Sacroiliitis.

Microbiological cultures, performed on skin pustules from 7 patients, synovial fluid from 2 patients, and bone biopsy from 1 patient (#5) yielded negative results for *P. acnes* or other pathogens. All patients presented bone involvement with sternoclavicular osteitis and/or hyperostosis, 4/10 with sacroileitis and 2/10 with spondylodiscitis. Skin involvement was observed in 9/10 patients:

palmo-plantar pustulosis in 6/9, psoriasis vulgaris in 2/9, and severe acne in one patient. Patient #8 was the one without cutaneous manifestations, whereas bone manifestations were similar to those observed in the others. Mean disease duration was 10.8 years ± 8.5 (SD) and patient #8 had the lowest (2.5 years). Blood samples with lithium heparin were also collected from SAPHO patients in the same day of saliva collection. The main clinical and laboratory findings are summarized in Table 2.

Table 2. Clinical, laboratory and imaging findings of each SAPHO patient at the time of the study.

Pt #	Blood TH-17[a]	Blood TH-1[b]	TH-1/TH-17	Neutrophilia WBC[c] cells/mm^3 Neu[d] cells/mm^3	Serum CRP[e]	Serum ESR[f]
1	H 2.91	17.97	0.19	N 4900 2470	N 0.39	H 32
2	N 0.13	4.55	0.13	N 8000 4560	N 0.10	N 11
3	H 3.05	12.31	0.00	N 7200 3900	N 0.47	N 10
4	H 3.07	25.19	0.24	N 8300 3194	N 0.11	H 33
5	H 2.77	9.85	0.32	N 9400 5640	N 0.4	N 18
6	H 2.86	21.29	0.10	N 10500 6300	N 0.5	N 9
7	H 2.1	20.00	0.00	N 9700 6200	N 0.24	N 30
8	nd	nd	nd	N 7800 3300	H 1.8	H 40
9	nd	nd	nd	N 6200 3521	N 0.2	H 55
10	nd	nd	nd	N 11430 5270	H 1.9	N 19

[a]% TH-17 lymphocytes = CD4 + IL17 + Lymphocytes (normal range: 0.04-1.88%); [b]% TH-1 lymphocytes; [c]Total white cells (normal range: 4000 – 11.200/µl); [d]Neutrophils (normal range: 1800 - 7500/µl); [e]C reactive protein (normal range: 0-0.5); [f]Erythrocyte sedimentation rate (normal range: <30); H: value higher than normal; N: normal value; nd: not determined.

Blood lymphocyte subsets defined as "TH-1" (CD4 + IFNγ+), "TH17" (CD4 + IFNγ- IL-17+), "TH1/TH17" (CD4 + IFNγ+ IL-17+) were defined by analysis of intracellular cytokine production using flow-cytometry, using a protocol previously described (Firinu, 2014a). White blood cell (WBC) counts, serum CRP and ESR were determined by routine methods. WBC count (mean 8343/mm^3 ± 1980 SD) was normal in all patients, CRP (mean 0.61 mg dl^{-1} ± 0.66) was above normal range in 2 patients, and ESR (24 mm h-1 ± 16.8 SD) in 4 patients, but both were not related to disease activity status.

Hereditary and idiopathic non-histaminergic angioedema

A total of 37 patients affected by angioedema were recruited at the Unit of Internal Medicine, Allergy and Clinical Immunology of Cagliari University. On the basis of genetic analysis and familial information, patients were clustered into three subgroups: C1-INH-HAE (6 men and 8 women, 49.1 ± 17.3 years old, mutation of *SERPING1* gene), FXII-HAE (1 male and 10 females, 37.82 ± 11.31 years old, mutation of *FXII* gene), InH-AAE (5 men and 7 women, 41.4 ± 14.3 years old, no mutations of SERPING1 and FXII genes). As regards InH-AAE patients, no heredity of the pathology was documented and any positive effect was observed after histamine treatment, thus they were not classified as U-HAE or IH-AAE. 12 out of 14 patients of the C1-INH-HAE group were analyzed in the absence of symptoms (basal phase), one during an acute attack of angioedema (acute phase) and one in both conditions. 9 out of the 11 patients belonging to the FXII-HAE group were examined in basal phase, 1 in acute phase and 1 in both conditions. All the 12 InH-AAE patients were analyzed in basal phase. 31 healthy individuals (9 males and 22 females, 41.8 ± 13.0 years old) were enrolled as control group.

Salivary sample collection

Unstimulated whole saliva was collected according to a standardized protocol from patients and healthy controls using a soft plastic aspirator and transferred to a plastic tube in an ice bath. Donors did not eat or drink at least 2 h before the collection, which was established between 10.00 a.m. and 12.00 p.m. Immediately after collection, each salivary sample was diluted in 1:1 v/v ratio

with a 0.2% solution of 2,2,2-trifluoroacetic acid (TFA). Then the solution was centrifuged at 10000*g* for 10 min at 4°C (for Wilson's disease study) and at 20000*g* for 15 min at 4°C (for SAPHO syndrome and angioedema studies). The acidic supernatant was separated from the precipitate and either immediately analyzed by HPLC-ESI-MS apparatus (100 µl, corresponding to 50 µl of saliva) or stored at -80°C until low-resolution HPLC-ESI-IT-MS analysis. Also precipitates were stored at -80°C.

- ## Experimental methods

Low-resolution HPLC-ESI-IT-MS experiments

Low-resolution reversed phase (RP)-HPLC-ESI-MS analysis of the acidic soluble fraction of whole saliva was carried out by a Surveyor HPLC system connected by a T splitter to a diode-array detector, and to a LCQ Advantage mass spectrometer (ThermoFisher Scientific San Jose, CA). The mass spectrometer was equipped with an electrospray ionization source (ESI) and an ion trap (IT). The chromatographic column was a reversed phase Vydac (Hesperia, CA, USA) C8 column with 5 µm particle diameter (150 x 2.1 mm). The chromatographic separation was carried out using eluent A (0.056% TFA acidic solution) and eluent B (acetonitrile/water 80:20 with 0.05% TFA). The gradient applied for the analysis of saliva was linear from 0 to 55% of B in 40 min, and from 55% to 100% of B in 10 min, at a flow rate of 0.3 ml min^{-1}. The T splitter addressed a flow-rate of 0.2 ml min^{-1} toward the diode array detector and 0.1 ml min^{-1} towards the ESI source. During the first 5 minutes of the analysis the eluate was not directed to the mass spectrometer to avoid that the high salt concentration could damage the instrument. The photodiode array detector was set at 214 and 276 nm. Mass spectra were collected every 3 ms in the *m/z* range 300-2000 in positive ion mode. The MS spray voltage was 5.0 kV, and the capillary temperature was 260 °C. MS resolution was 6000.

Experimental mass values of each protein and peptide was obtained using the MagTran 1.0 software (Zhang, 1998) which automatically performs the deconvolution of the average ESI-MS spectra. The experimental values were

compared with the theoretical masses of the proteins registered at the Swiss-Prot Data Bank (http:/us.expasy.org/tools).

Enriched fraction preparation of S100A8 and S100A9 oxidized proteoforms from WD saliva samples and trypsin digestion

Enriched fractions of S100A8 and S100A9 oxidized proteoforms previously uncharacterized were obtained by preparative RP-HPLC (Dionex Ultimate 3000 instrument, ThermoFisher Scientific, Sunnyvale CA) of pools of Wilson's disease saliva samples. The chromatographic column was a reversed phase Vydac (Hesperia, CA) C8 column with 5 μm particle diameter (250 x 10 mm). The solutions used for preparative RP-HPLC were the same utilized for analytical low-resolution HPLC-ESI-MS experiments. The gradient was linear from 0 to 60% B in 40 min and from 60 to 100% B in 5 min, with a flow rate of 2.8 ml min^{-1}. Four fractions corresponding to peaks eluting between 39 and 44 min were collected separately and lyophilized. Each fraction was solubilized in 100 μl of ultrapure H_2O, and 1/3 of the solution was acidified with 0.2% TFA (1:1 v/v ratio) to be checked by low-resolution HPLC-ESI-MS. The remaining sample was submitted to digestion using the kit "Trypsin Singles Proteomic Grade" (Sigma-Aldrich) according to the manufacturer's instructions. Digestion was stopped after 12 h by acidification with 0.1% TFA (final concentration), and the solution stored at -80 °C until the analysis by high-resolution HPLC-ESI-MS.

High-resolution HPLC-ESI-MS/MS experiments

High-resolution HPLC-ESI-MS/MS experiments were carried out on whole saliva samples and tryptic digests of enriched fractions from Wilson's disease patients, with the aim to characterize S100A8 and S100A9 oxidized proteoforms and two fragments of the polymeric immunoglobulin receptor (pIgR). The instrument was an Ultimate 3000 Micro HPLC apparatus (ThermoFisher Scientific, Sunnyvale, CA) equipped with a FLM-3000-Flow manager module coupled to LTQ Orbitrap XL apparatus (ThermoFisher Scientific). The columns were a Zorbax 300SB-C8 column (3.5 μm particle diameter; 1.0 x 150 mm) for the *top-down* analysis, and a Zorbax 300SB-C18 column (5 μm particle diameter; 1.0 x 150 mm) for the *bottom-up*. Eluents were: (eluent A) 0.056% (v/v) aqueous

TFA, and (eluent B) 0.05% (v/v) TFA in acetonitrile-water 80/20. For both *top-down* and *bottom-up* analyses the gradient was: 0–2 min 5% B, 2-40 min from 5% to 55% B (linear), 41-43 min from 55% to 100% B, at a flow rate of 80 µL/min. MS and MS/MS spectra were collected in positive mode using the lock mass for internal mass calibration (polydimethyl cyclosiloxane, 445.1200 *m/z*) with the resolution of 60000 and 30000, respectively. The *m/z* range was from 600 to 2000 in the *top-down* experiments, from 300 to 2000 in the *bottom-up* experiments. Tuning parameters: capillary temperature was 250 °C, and the source voltage of 4.0 kV, capillary voltage and tube lens voltage were 37 V, and 150 V in the *top-down*; 48 V and 150 V in the *bottom-up* experiments. In data dependent acquisition mode the three most abundant ions were selected and fragmented by using collision-induced dissociation (CID, 35% normalized collision energy for 30 ms, isolation width of 6-10 *m/z*, activation q of 0.25. The inject volume was 20 µl.

Data were generated by Xcalibur 2.2 SP1.48 (Thermo Fisher Scientific) using default parameters of the Xtract program for the deconvolution. MS/MS data were analyzed by the Proteome Discoverer 1.2 program, based on Sequest cluster as a search engine (University of Washington, licensed to Thermo Electron Corp., San Jose, CA) against Swiss-Prot human proteome (September, 2014 released; Swiss Prot human complete.fasta; 47622 non-redundant protein sequences). For peptide matching the limits were Xcorr scores greater than 1.5 for singly charged ions, 2.0 and 2.5 for doubly and triply charged ions, respectively. Furthermore, the cleavage specificity was set to trypsin with two missed cleavages in the *bottom-up* analysis. Precursor mass search tolerance was 10 ppm and fragment mass tolerance 0.8 Da. Target FDR was 0.01 (strict), 0.05 (relaxed). The following modifications were searched: phosphorylation, acetylation, oxidation of methionine and tryptophan residues, as well as oxidative cysteine modifications, such as glutathionylation, cysteinylation, nitrosylation, sulfonic, sulfinic, and sulfenic acid. Peptide sequences and sites of covalent modifications were also validated by manual inspection of the experimental fragmentation spectra against the theoretical ones generated by

MS-Product software available at the ProteinProspector website (http://prospector.ucsf.edu/prospector/msh-ome.htm).

Fractionation of the acidic soluble fraction of salivary proteins by ultrafiltration

200 µl of the acidic soluble fraction of saliva were lyophilized, resuspended in 100 µl of 5% acetonitrile/0.1% formic acid and then centrifuged at 10000g and 20°C for 15 minutes using centrifugal filter units with a 50 kDa molecular weight cut-off (MWCO) (Vivaspin, Sartorius stedim biotech). Samples were stored at -80 °C until nano-HPLC-ESI-MS and MS/MS analyses.

Filter aided sample preparation (FASP)

Insoluble fractions obtained after whole saliva acidification and centrifugation were resuspended in 250µL of a urea/tiourea 2M 1% CHAPS buffer, sonicated for 1 minute (with an alternate cycle of 1 second of pause every 10 seconds) and then centrifuged at 10000*g* for 10 minutes at room temperature. For each sample total protein quantification was carried out using the RC-DC assay and 100 µg of protein were subjected to an endoProteoFASP (filter aided sample preparation) approach, which consists in the in-solution tryptic digestion of the sample and allows to obtain peptides for *bottom-up* LC-MS analysis. 30 µg of the tryptic digest was then loaded in a Pierce™ C18 Spin Column (ThermoFisher) for the salts elimination. The eluate was dried in a Speed Vac, re-suspended in a 5% acetonitrile/0.1% formic acid solution, sonicated for 3 minutes and finally centrifuged at 15000*g* for 3 minutes at room temperature. Samples were stored at -80 °C until nano-HPLC-ESI-MS and MS/MS analyses.

Nano-HPLC-ESI-MS and MS/MS experiments

Nano-HPLC-ESI-MS and MS/MS experiments were performed on the tryptic digests of the acidic insoluble fractions after FASP protocol and on the low-molecular weight acidic soluble fraction (MW < 50 kDa). The eluting peptides were analyzed using an LXQ linear ion trap mass spectrometer. The chromatographic separation was carried out using eluent A (5% acetonitrile/0.1% TFA) and eluent B (acetonitrile/water 90:10 with 0.1% formic acid). The gradient employed was linear from 0% to 5% of eluent B in 10

minutes, from 5% to 40% of eluent B in 35 minutes, from 40% to 60% of eluent B in 10 minutes and finally from 60% to 90% of eluent B in other 10 minutes. The flow rate was 0.3 ml min^{-1}. The first 10 minutes of the analysis were set up for the loop washing. LXQ settings were as follows: spray voltage, 1.8 kV; 1 microscan for MS scans at maximum inject time 10 ms with mass range 400–1650 *m/z*, 3 microscans for MS/MS at maximum inject time 100 ms with automatic mass range. The LXQ was operated in a data-dependent mode to execute top5, corresponding to one MS scan for precursor ions followed by four data-dependent MS/MS scans for precursor ions above a threshold ion count of 450 with normalized collision energy value of 35%. Charge state screening was enabled to reject unassigned and 4+ charge states. Mass spectrometer was set to alternate during the entire experiment 10 µs of MS analysis and 10 µs of MS/MS analysis and to repeat this sequence for the three most abundant ions identified. Each sample was analyzed in duplicate. MS/MS data by *bottom-up* analyses were processed on Mascot software, while MS/MS data obtained by *top-down* analyses were processed on BioworksBrowser v3.0 (Thermo Fisher Scientific). DTA files were generated from LC-MS/MS raw files with the following options: precursor ion tolerance 1.5 Da, group scan 1, minimum group count 1, minimum ion count 20, and filtering through charge state analysis. The generated DTA files were searched against SwissProt protein database (March 2013) for *Homo sapiens* and its reversed-sequence version. Search on data obtained by bottom-up analysis of the insoluble fractions was prformed according the following criteria: enzyme, trypsin (KR/P); full enzymatic cleavage; missed cleavage sites, 2; peptide tolerance, 2.0 Da; fragment ions tolerance, 1.0 Da; variable modifications, carbamidomethylation (+57Da), methionine oxidation (+16Da), STY phosphorylation (+80Da); modifications per peptide, 3. The search result was filtered with Xcorr versus charge state (Xcorr of 1.5, 2.0 and 2.5 for +1, +2, +3 charges, respectively) and delta CN 0.08. Only proteins identified by at least two unique peptides and found in at least two different subjects were considered confident. Search on data obtained by *top-down* analyses of the soluble fractions was carried out with the same criteria, but excluding enzymatic cleavages and

carbamidomethylation and results were filtered with Xcorr versus charge state (Xcorr of 1.5, 2.0 and 3.0 for +1, +2, +3 charges, respectively).

Bioinformatic analyses

The open source software platform Cytoscape_v3.2.1 (http://www.cytoscape.org/) and the additional plugins ClueGO (http://apps.cytoscape.org/apps/cluego) and CluePedia (http://apps.cytoscape.org/apps/cluepedia) were used in order to visualize the interaction networks among the genes encoding proteins identified with confidence, and the biological pathways in which they are involved. In order to visualize only the most significant pathways, only pathways represented by at least 3 genes and accounting for at least the 3% of the total genes were selected.

Prediction of proteases naturally acting in saliva

The peptides naturally present in the acidic soluble fractions of saliva were analyzed by the Proteasix software to perform the *in silico* prediction of the proteases present in the oral cavity responsible for their generation. A database containing the association between proteases and cleavage sites (CS) allows to have suggestions on the most probable proteases responsible for the generation of the peptide.

- ## Quantification

***Top-down* proteomics experiments**

Intact protein quantification by low resolution HPLC-ESI-MS

Proteins and peptides, previously identified and characterized in human saliva in our laboratory (Castagnola, 2004; Inzitari, 2005; Messana, 2008b; Cabras, 2010; Castagnola, 2011a; Castagnola, 2012a, Castagnola, 2013), were quantified by the XIC procedure performed on low-resolution reversed phase (RP)-HPLC-ESI-MS chromatograms. The typical low-resolution HPLC-ESI-MS total ion current (TIC) profile of the acidic soluble fraction of saliva from an healthy adult subject is shown in Fig.3. The elution ranges of the main classes of proteins and peptides, which are usually detected in saliva, are evidenced.

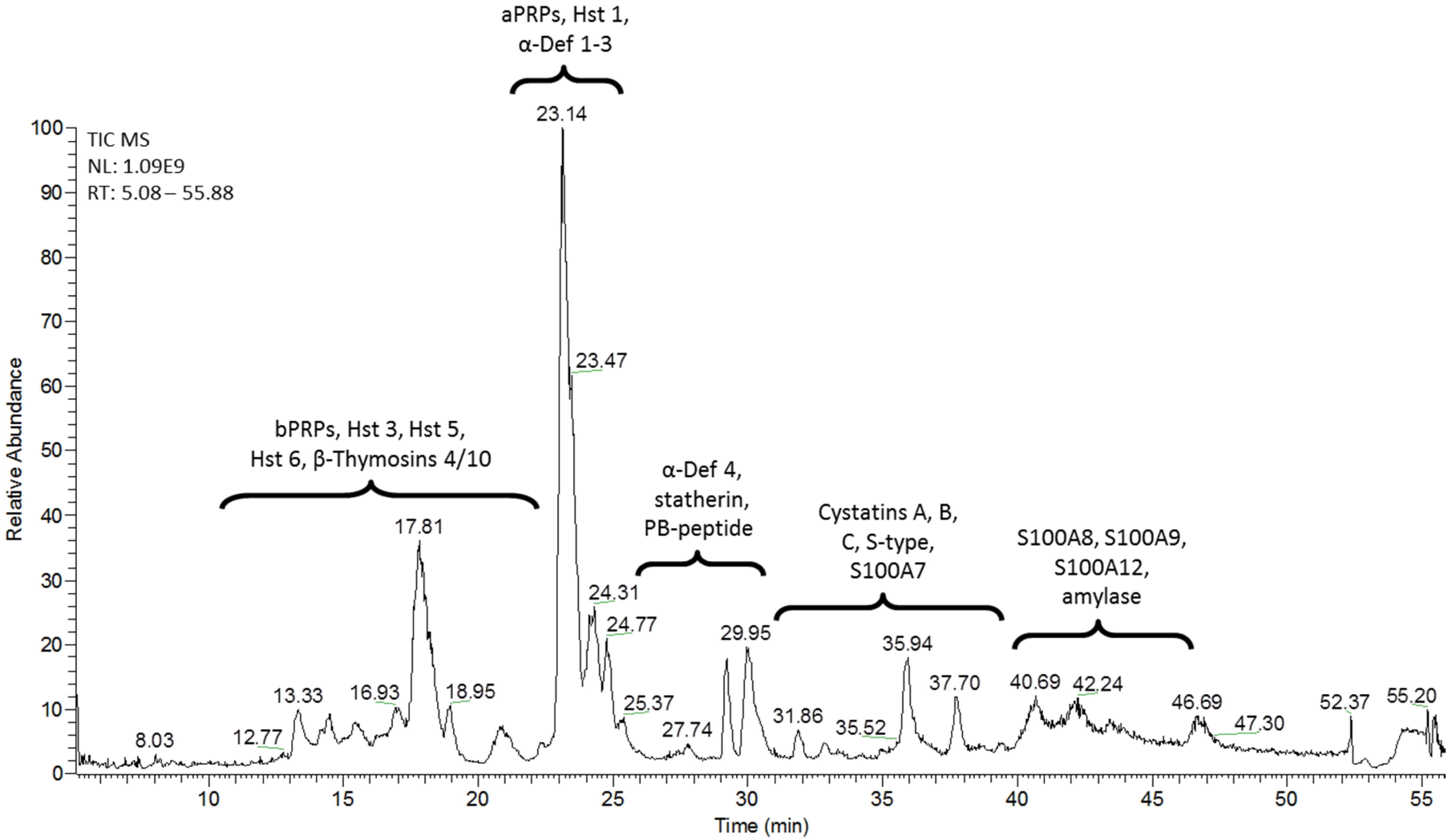
aPRPs, Hst 1,
α-Def 1-3
23.14
TIC MS
NL: 1.09E9
RT: 5.08 – 55.88
23.47
bPRPs, Hst 3, Hst 5,
Hst 6, β-Thymosins 4/10
α-Def 4,
statherin,
PB-peptide
Cystatins A, B,
C, S-type,
S100A7
S100A8, S100A9,
S100A12,
amylase
17.81
24.31
24.77
29.95
35.94
13.33
16.93
18.95
37.70
40.69
42.24
25.37
31.86
35.52
46.69
52.37
55.20
8.03
12.77
27.74
47.30
Relative Abundance
Time (min)

Fig.3

Under our experimental conditions β-thymosins 4 and 10, basic PRPs, and histatins 3, 5 and 6 elute in the initial region of the chromatogram followed by histatin 1, acidic PRPs and α-defensins 1-3. In the central region, α-defensin 4, statherin and P-B peptide can be detected and afterwards cystatins and S100A7. Finally, in the last part of the chromatogram amylase and the other S100s proteins elute. To extract the XIC peaks, selected multiply-charged ions generated by the proteins/peptides at the electrospray ionization source were searched in the chromatographic profile. The area of the XIC peaks is proportional to the protein concentration under constant analytical conditions, allowing to perform relative quantification of the same protein in different samples (Ong, 2005; Messana, 2008a). The *m/z* values selected to quantify proteins and peptides were chosen by excluding values in common with other closely eluting proteins (±0.5 *m/z*). The estimated percentage error of the XIC procedure was <8%. XIC peaks were considered when the signal to noise ratio was at least 5. Table 3 reports the Swiss-prot codes, the elution times, the experimental and theoretical average (low-resolution) and monoisotopic (high-resolution) mass values (Mav., Mmonois.), and the multiply-charged ions utilized to selectively extract the ion current peaks used to quantify proteins/peptides and their derivatives. Basic proline-rich proteins were not evaluated due to their high variability linked to the physiological status (Cabras, 2009; Cabras, 2012a).

Table 3. Proteins and peptides investigated: Swiss-prot code, elution time, experimental (exp.) and theoretical (th.) average and monoisotopic mass values (Mav., Mmonois.) and multiply charged ions used for XIC quantification are reported.

Protein (Swiss-Prot code)	**El. time (min)**	**Exp. Mav. (Th. Mav.) Da**	**Multiply-charged ions selected for the XIC procedures ($m/z_{(charge)}$)**	**Exp. Mmonois. (Th. Mmonois.) Da**
Histatin 1 (P15515)	23.3-23.8	4928.2 ± 0.5 (4928.2)	$1644.1_{(+3)}$ $1233.5_{(+4)}$	4925.22 ± 0.08 (4925.200)
Non-phosphorylated Histatin 1 (P15515)	23.4-23.8	4848.2 ± 0.5 (4848.2)	$1617.4_{(+3)}$ $1213.5_{(+4)}$	4846.25 ± 0.08 (4846.233)
Histatin 3 (P15516)	17.6-17.9	4062.2 ± 0.4 (4062.4)	$1355.1_{(+3)}$ $1016.6_{(+4)}$	4059.88 ± 0.07 (4059.979)

Histatin 5 (P15516)	14.2-14.7	3036.5 ± 0.3 (3036.3)	$1013.2_{(+3)}$ $760.1_{(+4)}$	3034.53 ± 0.06 (3034.522)
Histatin 6 (P15516)	14.0-14.4	3192.4 ± 0.3 (3192.5)	$1065.1_{(+3)}$ $799.1_{(+4)}$	3190.63 ± 0.06 (3190.623)
Statherin 2P[a] (P02808)	28.9-29.5	5380.0 ± 0.5 (5379.7)	$1794.2_{(+3)}$ $1345.9_{(+4)}$ $1076.9_{(+5)}$	5376.5 ± 0.1 (5376.450)
P-B peptide (P02814)	29.7-30.6	5792.9 ± 0.5 (5792.7)	$1932.0_{(+3)}$ $1449.2_{(+4)}$ $1159.6_{(+5)}$	5789.1 ± 0.1 (5789.036)
PRP-1 type[b] 2P (P02810)	22.9-23.6	15515 ± 2 (15514-15515)	$1293.9_{(+12)}$ $1194.4_{(+13)}$ $1035.3_{(+15)}$ $970.7_{(+16)}$ $913.6_{(+17)}$	15505.4 ± 0.2 (15505.24-15506.22)
PRP-3 type[c] 2P (P02810)	23.3-24.2	11161 ± 1 (11161-11162)	$1595.5_{(+7)}$ $1396.2_{(+8)}$ $1015.7_{(+11)}$ $931.1_{(+12)}$ $859.6_{(+13)}$	11155.1 ± 0.2 (11155.08-11156.06)
P-C peptide (P02810)	13.6-14.5	4370.9 ± 0.4 (4370.8)	$1457.9_{(+3)}$ $1093.7_{(+4)}$	4368.19 ± 0.07 (4368.183)
Cystatin S (P01036)	37.4-38.2	14186 ± 2 (14185.8)	$1774.3_{(+8)}$ $1577.2_{(+9)}$ $1419.6_{(+10)}$ $1290.6_{(+11)}$ $1183.2_{(+12)}$ $1092.2_{(+13)}$ $1014.3_{(+14)}$	14176.8 ± 0.2 (14175.81)
Cystatin S 1P (S1) (P01036)	37.7-38.4	14266 ± 2 (14265.8)	$1784.3_{(+8)}$ $1586.1_{(+9)}$ $1427.6_{(+10)}$ $1297.9_{(+11)}$ $1189.8_{(+12)}$ $1098.4_{(+13)}$ $1020.0_{(+14)}$	14255.6 ± 0.2 (14255.77)
Cystatin S 2P (S2) (P01036)	37.8-38.4	14346 ± 2 (14345.8)	$1794.3_{(+8)}$ $1595.0_{(+9)}$ $1435.6_{(+10)}$ $1305.2_{(+11)}$ $1196.5_{(+12)}$ $1104.5_{(+13)}$ $1025.7_{(+14)}$	14335.7 ± 0.2 (14335.74)
Cystatin SN (P01037)	35.8-36.4	14312 ± 2 (14313.1)	$1790.0_{(+8)}$ $1591.2_{(+9)}$ $1432.2_{(+10)}$ $1302.1_{(+11)}$ $1193.7_{(+12)}$ $1101.9_{(+13)}$ $1023.3_{(+14)}$	14303.1 ± 0.2 (14303.09)
Cystatin SA (P09228)	38.7-39.4	14347 ± 2 (14346.1)	$1794.4_{(+8)}$ $1595.1_{(+9)}$ $1435.7_{(+10)}$ $1305.3_{(+11)}$ $1196.6_{(+12)}$ $1104.6_{(+13)}$ $1025.8_{(+14)}$	14337.1 ± 0.2 (14337.01)
Cystatin A (P01040)	31.3-32.1	11006 ± 2 (11006.5)	$1835.4_{(+6)}$ $1573.4_{(+7)}$ $1376.8_{(+8)}$ $1223.9_{(+9)}$ $1101.7_{(+10)}$ $1001.6_{(+11)}$	10999.7 ± 0.2 (10999.66)
Cystatin B-SS[d]dimer (P04080)	33.6-34.4	22362 ± 3 (22361.2)	$1864.4_{(+12)}$ $1721.1_{(+13)}$ $1598.2_{(+14)}$ $1491.8_{(+15)}$ $1398.6_{(+16)}$ $1316.4_{(+17)}$ $1243.3_{(+18)}$ $1177.9_{(+19)}$ $1119.0_{(+20)}$ $1065.8_{(+21)}$ $1017.4_{(+22)}$ $973.2_{(+23)}$	nd
Cystatin B-SSG[e] (P04080)	32.5-33.1	11487 ± 2 (11486.7)	$1915.5_{(+6)}$ $1642.0_{(+7)}$ $1436.9_{(+8)}$ $1277.3_{(+9)}$ $1149.7_{(+10)}$ $1045.3_{(+11)}$	11479.6 ± 0.2 (11479.68)

Cystatin B–SSC[f] (P04080)	32.6-33.2	11301 ± 2 (11300.7)	$1884.5_{(+6)}$ $1615.4_{(+7)}$ $1413.6_{(+8)}$ $1256.6_{(+9)}$ $1131.1_{(+10)}$ $1028.3_{(+11)}$	11294.6 ± 0.2 (11294.613)
Cystatin C (P01034)	38.2-38.9	13343 ± 2 (13343.1)	$1668.9_{(+8)}$ $1483.6_{(+9)}$ $1335.3_{(+10)}$ $1214.0_{(+11)}$ $1112.9_{(+12)}$ $1027.4_{(+13)}$ $954.1_{(+14)}$	13334.5 ± 0.2 (13334.60)
Cystatin C-Mox[g] (P01034)	38.1-38.7	13360 ± 2 (13359.1)	$1670.8_{(+8)}$ $1485.2_{(+9)}$ $1336.8_{(+10)}$ $1215.4_{(+11)}$ $1114.2_{(+12)}$ $1028.6_{(+13)}$ $955.2_{(+14)}$	13354.4 ± 0.2 (13354.59)
α-defensin 1 (P59665)	24.9-25.4	3442.1 ± 0.4 (3442.1)	$1722.0_{(+2)}$ $1148.4_{(+3)}$ $861.5_{(+4)}$	3439.53 ± 0.06 (3439.519)
α-defensin 2 (P59665 and P59666)	24.9-25.4	3371.0 ± 0.4 (3371.0)	$1686.5_{(+2)}$ $1124.7_{(+3)}$ $843.8_{(+4)}$	3368.49 ± 0.06 (3368.482)
α-defensin 3 (P59666)	24.9-25.4	3486.1 ± 0.4 (3486.1)	$1744.0_{(+2)}$ $1163.0_{(+3)}$ $872.5_{(+4)}$	3483.52 ± 0.06 (3483.509)
α-defensin 4 (P12838)	27.7-28.0	3709.3 ± 0.4 (3709.5)	$1855.7_{(+2)}$ $1237.5_{(+3)}$ $928.4_{(+4)}$	3706.78 ± 0.06 (3706.767)
Thymosin β_4 (P62328)	20.7-21.0	4962.5 ± 0.4 (4963.5)	$1655.5_{(+3)}$ $1241.9_{(+4)}$ $993.8_{(+5)}$	4960.51 ± 0.08 (4960.494)
Thymosin β_4-Mox (P62328)	19.0-19.2	4979.4 ± 0.4 (4979.5)	$1660.8_{(+3)}$ $1245.9_{(+4)}$ $996.9_{(+5)}$	4976.40 ± 0.08 (4976.488)
Thymosin β_{10} (P63313)	22.0-22.4	4936.3 ± 0.4 (4936.5)	$1646.5_{(+3)}$ $1235.1_{(+4)}$ $988.3_{(+5)}$	4933.44 ± 0.08 (4933.530)
S100A7 (D27) (P31151[h])	37.4-38.0	11367 ± 2 (11367.8)	$1422.0_{(+8)}$ $1264.1_{(+9)}$ $1137.8_{(+10)}$ $1034.4_{(+11)}$	11360.3 ± 0.2 (11360.53)
S100A12 (P80511)	39.5-40.2	10444 ± 2 (10443.9)	$1306.5_{(+8)}$ $1161.4_{(+9)}$ $1045.4_{(+10)}$ $950.4_{(+11)}$	10437.7 ± 0.2 (10437.49)
S100A8 (P05109)	39.1-39.7	10833 ± 2 (10834.5)	$1355.3_{(+8)}$ $1204.8_{(+9)}$ $1084.5_{(+10)}$ $985.9_{(+11)}$	10827.8 ± 0.2 (10827.66)
S100A9(S) (P06702)	41.3-42.0	12690 ± 2 (12689.2)	$1410.9_{(+9)}$ $1269.9_{(+10)}$ $1154.6_{(+11)}$ $1058.4_{(+12)}$ $977.1_{(+13)}$	12681.4 ± 0.2 (12681.29)
S100A9(S) monophosphorylated (P06702)	41.3-42.0	12770 ± 2 (12769.2)	$1419.8_{(+9)}$ $1277.9_{(+10)}$ $1161.8_{(+11)}$ $1065.1_{(+12)}$ $983.3_{(+13)}$	12761.1 ± 0.2 (12761.26)
S100A9(S)-Mox (P06702)	41.3-42.0	12706 ± 2 (12705.2)	$1412.7_{(+9)}$ $1271.5_{(+10)}$ $1156.0_{(+11)}$ $1059.8_{(+12)}$ $978.3_{(+13)}$	12697.4 ± 0.2 (12697.29)
S100A9(S)-Mox monophosphorylated (P06702)	41.3-42.0	12786 ± 2 (12785.2)	$1421.9_{(+9)}$ $1279.5_{(+10)}$ $1163.3_{(+11)}$ $1066.4_{(+12)}$ $984.5_{(+13)}$	12777.2 ± 0.2 (12777.25)

S100A9(L)-SSG (P06702)	41.1-41.8	13459 ± 2 (13458.1)	$1346.8_{(+10)}$ $1224.5_{(+11)}$ $1122.5_{(+12)}$ $1036.3_{(+13)}$ $962.3_{(+14)}$	13449.7 ± 0.2 (13449.55)
S100A9(L)-SSG monophosphorylated (P06702)	41.1-41.8	13539 ± 2 (13538.1)	$1354.8_{(+10)}$ $1231.8_{(+11)}$ $1129.2_{(+12)}$ $1042.4_{(+13)}$ $968.0_{(+14)}$	13529.3 ± 0.2 (13529.52)
S100A9(L)-SSG/Mox	41.0-41.6	13475 ± 2 (13474.1)	$1348.4_{(+10)}$ $1225.9_{(+11)}$ $1123.8_{(+12)}$ $1037.5_{(+13)}$ $963.4_{(+14)}$	13465.6 ± 0.2 (13465.55)
S100A9(L)-SSG/Mox monophosphorylated	41.0-41.6	13555 ± 2 (13554.1)	$1356.4_{(+10)}$ $1233.2_{(+11)}$ $1130.5_{(+12)}$ $1043.6_{(+13)}$ $969.1_{(+14)}$	13545.7 ± 0.2 (13545.52)
S100A9(L)-SSC (P06702)	41.1-41.8	13273 ± 2 (13271.9)	$1328.2_{(+10)}$ $1207.6_{(+11)}$ $1107.0_{(+12)}$ $1021.9_{(+13)}$ $949.0_{(+14)}$	13263.5 ± 0.2 (13263.49)
S100A9(L)- SSC monophosphorylated (P06702)	41.1-41.8	13353 ± 2 (13351.9)	$1336.2_{(+10)}$ $1214.8_{(+11)}$ $1113.7_{(+12)}$ $1028.1_{(+13)}$ $954.7_{(+14)}$	13343.3 ± 0.2 (13343.46)

[a]Phosphorylation; [b]PRP-1 type includes the three entire isoforms PRP-1, PRP-2, and Pif-s, with a mass difference of 1 Da; [c]PRP-3 type includes the truncated isoforms PRP-3, PRP-4 and Pif-f; [d]disulfide bridge; nd: not determined; [e]glutathionylated cysteine residue; [f]cysteinylated cysteine residue; [g]methionine sulfoxide; [h]Swiss-Prot code refers to the variant E27.

Statistical analysis

GraphPad Prism was used for statistical analysis (the version 4.0 for Wilson's disease and SAPHO syndrome studies and the version 5.0 for Hereditary angioedema study). A normality test was applied to calculate ranges, medians, means, and standard deviations of the XIC peak areas of the peptides and proteins. A *t*-test was used to compare proteins/peptides XIC peak areas between two different groups. Specifically, a non-parametric *t*-test was chosen if the distribution of the XIC peak areas was not Gaussian for at least one of the two groups (Mann-Whitney *t*-test), while a parametric test was employed if the distribution was Gaussian for both groups (Unpaired *t*-test). Welch's correction was applied if the variance resulted significantly different between the groups. Statistical analysis was considered to be significant when the *p* value was <0.05 (two tailed). One-way ANOVA was used to compare three or more groups. In particular, non-parametric tests were applied if the distribution of the data was not Gaussian for at least one of the groups (Kruskal-Wallis test with Dunn's multiple comparison test) while a parametric test was employed if the distribution was Gaussian for all the groups (one-way analysis of variance with Tukey's multiple comparison test).

Bottom-up proteomics experiments

Quantification of proteins by nano-HPLC-ESI-MS experiments

Abundance of the proteins identified in the acidic insoluble fractions by Mascot software, and considered confident according to the above reported criteria, was estimated for each group on the basis of the Exponentially Modified Protein Abundance Index (emPAI). The log2 ratio between the median emPAI values for each protein in any two groups was calculated to evidence differences. Proteins with logarithm values lower than -0.7 were considered significantly increased in the group in the denominator position with respect to the group in the numerator, while proteins with logarithm values higher than +0.7 were considered significantly increased in the group in the numerator position with respect to the group in the denominator.

Results

- **Wilson's disease**

The following proteins and peptides were searched and quantified by *top-down* low-resolution HPLC-ESI-IT-MS experiments in the acidic soluble fraction of saliva from Wilson's disease patients and healthy controls: histatins, salivary cystatins, statherin, P-B peptide, aPRPs, α-defensins 1–4, cystatins A, B, C, β-thymosins 4 and 10, S100A7 (D27), S100A8, S100A9 (short (S) and long (L) isoforms), S100A12, as well as numerous their derivatives (Table 3). In addition, several masses not previously characterized were detected, and attributed to two naturally occurring fragments of pIgR and to proteoforms of S100A8 and S100A9 with a different degree of oxidation (Table 4). Characterization was performed by an integrated high-resolution HPLC-ESI-MS/MS *top-down* and *bottom-up* approach.

High-resolution *top-down* structural characterization of pIgR, and three S100A8 oxidized proteoforms

The structural characterization was performed on the acidic soluble fraction from patient whole saliva by high-resolution HPLC-ESI-MS/MS analysis.

Two peptides, eluting in the region 23.4-27.0 min of the low-resolution HPLC-ESI-MS profile of saliva, were identified as the fragment 610-648 and 623-648 of pIgR and respectively named AVAD and ASVD (Table 4). The identification was based on the high-resolution MS/MS performed on the AVAD triply-charged ion 1278.62 ± 0.02 *m/z* and on the ASVD doubly-charged ion 1246.12 ± 0.02 *m/z*, as shown in Fig.4 and Fig.5.

Table 4. Swiss-prot code, elution time, experimental (Exp.) and theoretical (Th.) average and monoisotopic mass values (Mav., Mmonois.), and multiply charged ions used for XIC quantification of ASVD and AVAD peptides, and S100A8 and S100A9 oxidized proteoforms.

Protein (Swiss-Prot code)	El. time (min)	Exp. Mav. (Th. Mav.) Da	Multiply-charged ions ($m/z_{(charge)}$)	Exp. Mmonois. (Th. Mmonois.) Da
AVAD (pIgR fr. 610-648 - P01833)	25.4-25.8	3834.4 ± 0.3 (3834.1)	$1918.0_{(+2)}$ $1279.0_{(+3)}$ $959.5_{(+4)}$	3831.85 ± 0.06 (3831.854)
ASVD (pIgR fr. 623-648 - P01833)	25.8-26.1	2490.5 ± 0.3 (2490.7)	$1246.3_{(+2)}$ $831.2_{(+3)}$	2490.23 ± 0.04 (2489.232)
S100A8-SO_2H	39.7-40.0	10866 ± 2 (10866.5)	$1359.3_{(+8)}$ $1208.4_{(+9)}$ $1087.7_{(+10)}$ $988.9_{(+11)}$	10859.6 ± 0.2 (10859.65)
S100A8-SO_3H/W54ox	40.2-40.6	10898 ± 2 (10898.6)	$1363.3_{(+8)}$ $1212.0_{(+9)}$ $1090.9_{(+10)}$ $991.8_{(+11)}$	10891.7 ± 0.2 (10891.66)
Hyper-oxidized S100A8	39.0-39.6	10915 ± 2 (10914.6)	$1365.3_{(+8)}$ $1213.7_{(+9)}$ $1092.5_{(+10)}$ $993.2_{(+11)}$	10907.6 ± 0.2 (10907.63)
S100A8-SSG[a]	38.1-38.4	11140 ± 2 (11139.8)	$1393.5_{(+8)}$ $1238.8_{(+9)}$ $1115.0_{(+10)}$ $1013.7_{(+11)}$	11133.8 ± 0.2 (11133.72)
S100A8-SNO[b]	40.6-40.9	10863 ± 2 (10863.5)	$1358.9_{(+8)}$ $1208.1_{(+9)}$ $1087.3_{(+10)}$ $988.6_{(+11)}$	10856.6 ± 0.2 (10856.65)
S100A8/A9-SS[c]dimer	41.6-41.9	23986 ± 3 (23985)	$1600.0_{(+15)}$ $1500.1_{(+16)}$ $1411.9_{(+17)}$ $1333.5_{(+18)}$ $1263.4_{(+19)}$ $1200.3_{(+20)}$ $1143.2_{(+21)}$ $1091.2_{(+22)}$ $1043.8_{(+23)}$ $1000.4_{(+24)}$ $960.4_{(+25)}$ $923.5_{(+26)}$	nd
S100A9/A9-SSdimer	41.7-42.5	26306 ± 3 (26304)	$1754.6_{(+15)}$ $1645.0_{(+16)}$ $1548.3_{(+17)}$ $1462.3_{(+18)}$ $1385.4_{(+19)}$ $1316.2_{(+20)}$ $1253.5_{(+21)}$ $1196.6_{(+22)}$ $1144.6_{(+23)}$ $1097.0_{(+24)}$ $1053.1_{(+25)}$ $1012.7_{(+26)}$	nd

[a]glutathionylated cysteine residue; [b]nitrosylated cysteine residue; [c]disulfide bridge; nd: not determined.

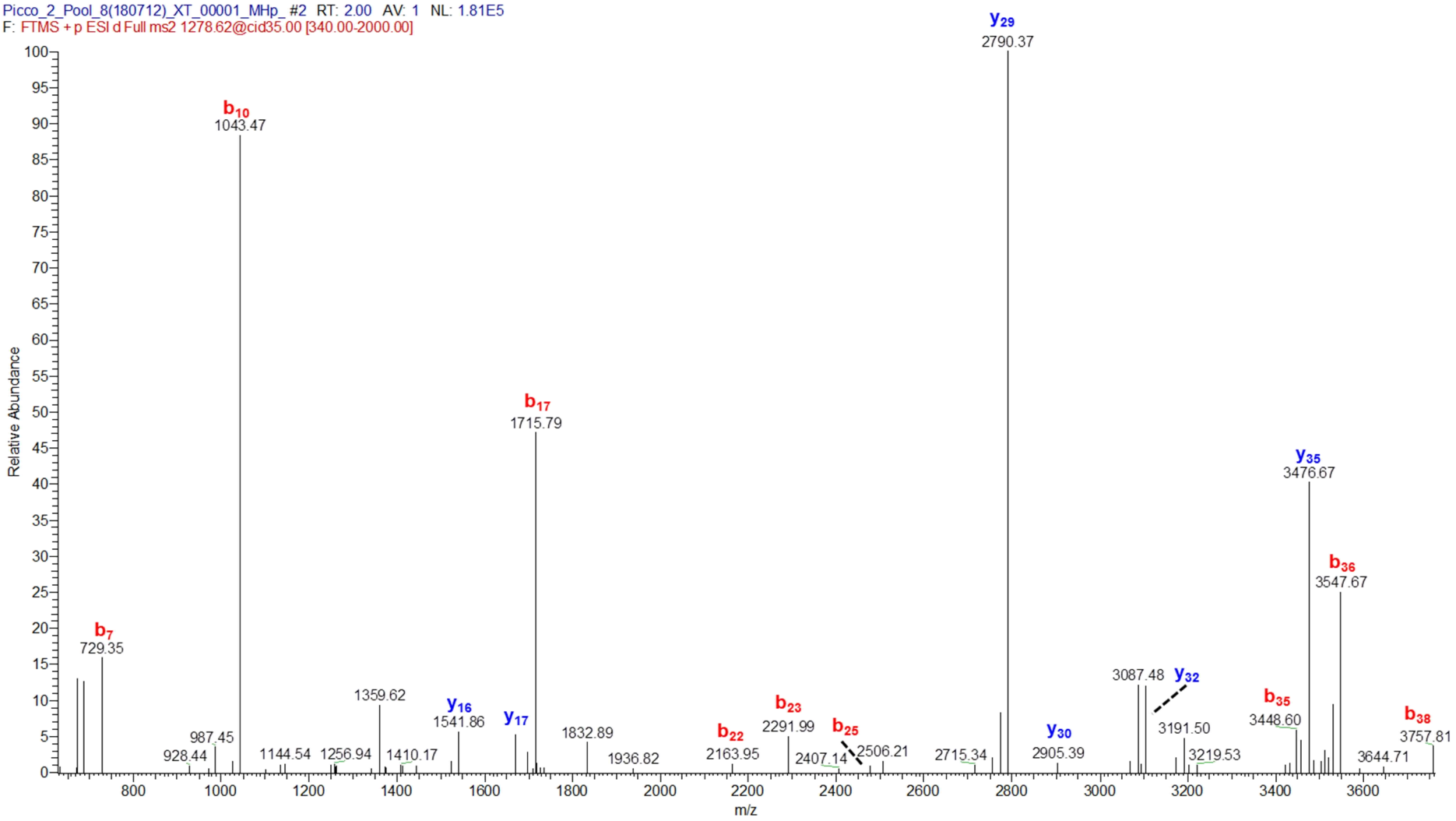

Fig.4 AVAD peptide. Annotated $[M+H]^+$ deconvoluted spectrum of high-resolution MS/MS from 1278.62 *m/z* ($[M+3H]^{3+}$).

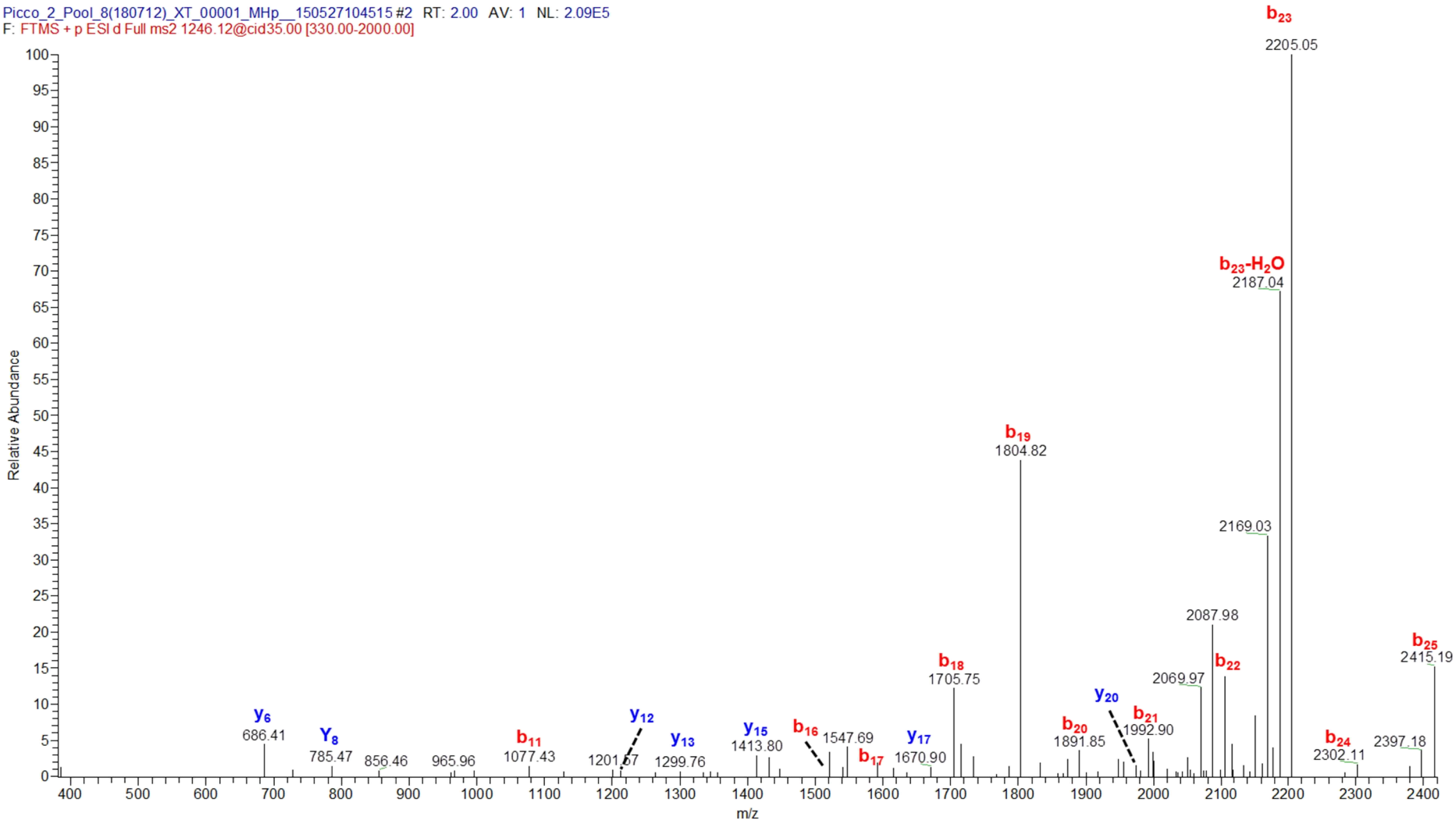

Fig.5 ASVD peptide. Annotated $[M+H]^+$ deconvoluted spectrum of high-resolution MS/MS from 1246.12 *m/z* ($[M+2H]^{2+}$).

S100A8 oxidation involved methionine 1 and 78 (M1, M78), tryptophan 54 (W54), and cysteine 42 (C42). Three proteoforms of S100A8 showed C42 oxidized to sulfonic acid (S100A8-SO_3H). The first presented a further oxidation at W54 (S100A8-SO_3H/W54ox), as shown by the high-resolution MS/MS annotated spectra reported in Fig.6A-C, while Fig.7A-C shows the MS/MS spectra of the other two forms, which were isobaric derivatives of S100A8-SO_3H (Hyper-oxidized S100A8): one form was also oxidized at W54 and M78 (S100A8-SO_3H/W54ox/M78ox), the other was dioxidized at W54 (S100A8-SO_3H/W54diox). All these S100A8 proteoforms are reported in Table 4. The neutral loss of H_2SO_3 (81.97 Da) (Shetty, 2007) observed only for S100A8-SO_3H/W54ox and for S100A8-SO_3H/W54ox/M78ox was in agreement with the presence of the sulfonic acid residue, and MS/MS data of the ion 910.56 *m/z* ($[M+12H]^{+12}$) confirmed the structures of the two co-eluting and isobaric hyper-oxidized S100A8 proteoforms. *Top-down* high-resolution MS/MS annotated spectra of intact S100A8-SO_3H/W54ox and hyper-oxidized S100A8 forms are respectively shown in Fig.6A-C and Fig.7A-C.

High-resolution *bottom-up* structural characterization of S100A8, S100A9 oxidized proteoforms

Trypsinized enriched fractions of S100A8, S100A9 and their oxidized proteoforms obtained by pools of patient saliva samples were analyzed by high-resolution HPLC-ESI-MS/MS. The *bottom-up* approach allowed confirming S100A8-SO_3H and characterizing other oxidized derivatives of S100A8. MS/MS sequencing of the tryptic peptides with monoisotopic $[M+H]^+$ 1412.68 ± 0.02 and 1540.77 ± 0.03 *m/z* corresponding to S100A8-SO_3H fragments 37-47, 37-48, and 36-47 (the latter two isobaric) confirmed the sulfonic acid modification of C42 (Table 5, Fig. 8A-C).

One proteoform of S100A8 with C42 oxidized to sulfinic acid (S100A8-SO_2H, 10859.6 ± 0.2 Mmonois., Table 4) was confirmed by MS/MS of the tryptic peptide 37-47 (Table 5, Fig.9) which showed the characteristic neutral loss of H_2SO_2 (65.98 Da) (Wang, 2004). The Mmonois. 10859.6 ± 0.2 could be also in agreement with dioxidized derivatives of S100A8, i.e. at W54 and M1, or M78 and M1, or W54 and M78. This possibility could not be excluded nor confirmed

by high-resolution MS/MS due to the separation of the modification sites in the tryptic peptides (Table 5). Oxidation of M1 in S100A8 was evidenced by MS/MS data of the tryptic peptide 1-23 (Table 5, Fig.10). Furthermore, masses attributable to tryptic peptides oxidized at M78 and W54, but not confirmed by MS/MS due to the low intensity, were also detected. The tentative attributions and monoisotopic $[M+H]^+$ *m/z* values were: fragment 78-84 of S100A8-M78ox, with *m/z* 729.32 ± 0.01 (theor. 729.38) eluting at 20.7 min; fragment 50-56 of S100A8-W54ox, with *m/z* 838.35 ± 0.01 (theor. 838.41, 20.7 min); fragment 48-56 of S100A8-W54ox, with *m/z* 1110.47 ± 0.02 (theor. 1110.59, 22.0 min).

Glutathionylation of C42 in S100A8 (S100A8-SSG, Table 4) was demonstrated by MS/MS data of the 37-47 glutathionylated peptide (monoisotopic monocharged ion with *m/z* 1669.76 ± 0.03, Table 5). Fig. 11A shows the high-resolution MS/MS of the triply-charged ion with *m/z* 557.26 with the characteristic neutral loss of pyroglutamate (129.04 Da), that confirmed the presence of the glutathionyl moiety.

The monoisotopic mono-charged ions with *m/z* 1649.78 ± 0.03 and 1393.73 ± 0.02 were tentatively attributed to the following fragments of C42-nitrosylated S100A8 (S100A8-SNO, Table 4): 37-49 (or 36-48) fragment (theor. 1649.87 *m/z*) and 37-47 fragment (theor. 1393.68 *m/z*).

Cysteine 42 of S100A8 originated also a disulfide bridge with cysteine 3 of S100A9(L) (S100A8/A9-SSdimer, Table 4). The presence in saliva of Wilson's disease patients of this covalent hetero-dimer was confirmed by high-resolution MS/MS of the monoisotopic doubly-charged ion with *m/z* 877.93 ± 0.01, attributed to the tryptic peptide in which peptide fragment 2-4 of S100A9(L) was linked to fragment 37-47 of S100A8 (Fig. 11B, Table 5). The monoisotopic mono-charged ion with *m/z* 2628.26 ± 0.04 was attributed to peptide fragment 2-10 of S100A9(L) linked to peptide fragment 36-47 of S100A8, but not confirmed by MS/MS data due to its low abundance. The two peptides, with the following experimental monoisotopic $[M+H]^+$ *m/z* values: 784.49 ± 0.01 and 1528.72 ± 0.03 were tentatively attributed to Nα-Ac-TCK dimer (theor. 784.36 *m/z*), and Nα-Ac-TCK bound to Nα-Ac-TCKMSQLER (theor. 1528.72 *m/z*), respectively, generated by S100A9(L)-SSdimer (Table 4).

Both the short and the long glutathionylated S100A9 proteoforms were detected as mono-oxidized derivatives on one of the following methionine residues: M59 (or M63), M77 (or M81), M79 (or M83), and M90 (or M94) (Table 4), as confirmed by high-resolution MS/MS of the mono-oxidized tryptic peptides reported in Table 5 and shown in Fig.12 and Fig.13.

Table 5. Experimental and theoretical monoisotopic $[M+H]^+$ mass values, elution time, sequence, PTMs, and *m/z* values selected for high-resolution HPLC-ESI-MS/MS analysis of the tryptic peptides obtained by digestion of enriched fractions of S100A8 and S100A9 oxidized proteoforms**.**

Tryptic Peptides Exp. and (Th.) monois. $[M+H]^+$ (Elution time, min)	**Sequence and position**	**PTMs**	**Ion selected for MS/MS analysis *m/z* (charge)**
	S100A8		
2737.49 ± 0.04 (2737.48) (39.1)	mLTELEKALNSIIDVYHKYSLIK 1-23	M-sulfoxide	685.13 (+4)
1540.77 ± 0.03 (1540.77) (20.2)	KLLETEcPQYIR 36-47	C-sulfonic acid	770.89 (+2)
1540.77 ± 0.03 (1540.77) (18.5)	LLETEcPQYIRK 37-48	C-sulfonic acid	770.89 (+2)
1669.76 ± 0.03 (1669.76) (19.7)	LLETEcPQYIR 37-47	C-glutathionylation	557.25 (+3)
1412.68 ± 0.02 (1412.67) (20.2)	LLETEcPQYIR 37-47	C-sulfonic acid	706.84 (+2)
1396.68 ± 0.02 (1396.68) (19.9)	LLETEcPQYIR 37-47	C-sulfinic acid	698.84 (+2)
	S100A8/A9-SSdimer		
1754.86 ± 0.03 (1755.87) (19.5)	LLETEcPQYIR 37-47 (S100A8) ¦ Nα-Acet-TcK 2-4 (S100A9)	C-disulfide dimerization	877.93 (+2)
	S100A9		
1630.80 ± 0.03 (1630.80) (37.3)	QLSFEEFImLMAR 73-85	M-sulfoxide	815.90 (+2)
1630.80 ± 0.03 (1630.80) (32.7)	QLSFEEFIMLmAR 73-85	M-sulfoxide	815.90 (+2)
1758.82 ± 0.03 (1758.82) (18.3)	VIEHImEDLDTNADK 57-72	M-sulfoxide	879.91 (+2)
3354.61 ± 0.06 (3354.61) (35.8)	VIEHIMEDLDTNADKQLSFEEFI mLMAR 57-85	M-sulfoxide	839.41 (+4)
2191.96 ± 0.04 (2191.96) (12.1)	mHEGDEGPGHHHKPGLGEGTP 94-114	M-sulfoxide	731.32 (+3)

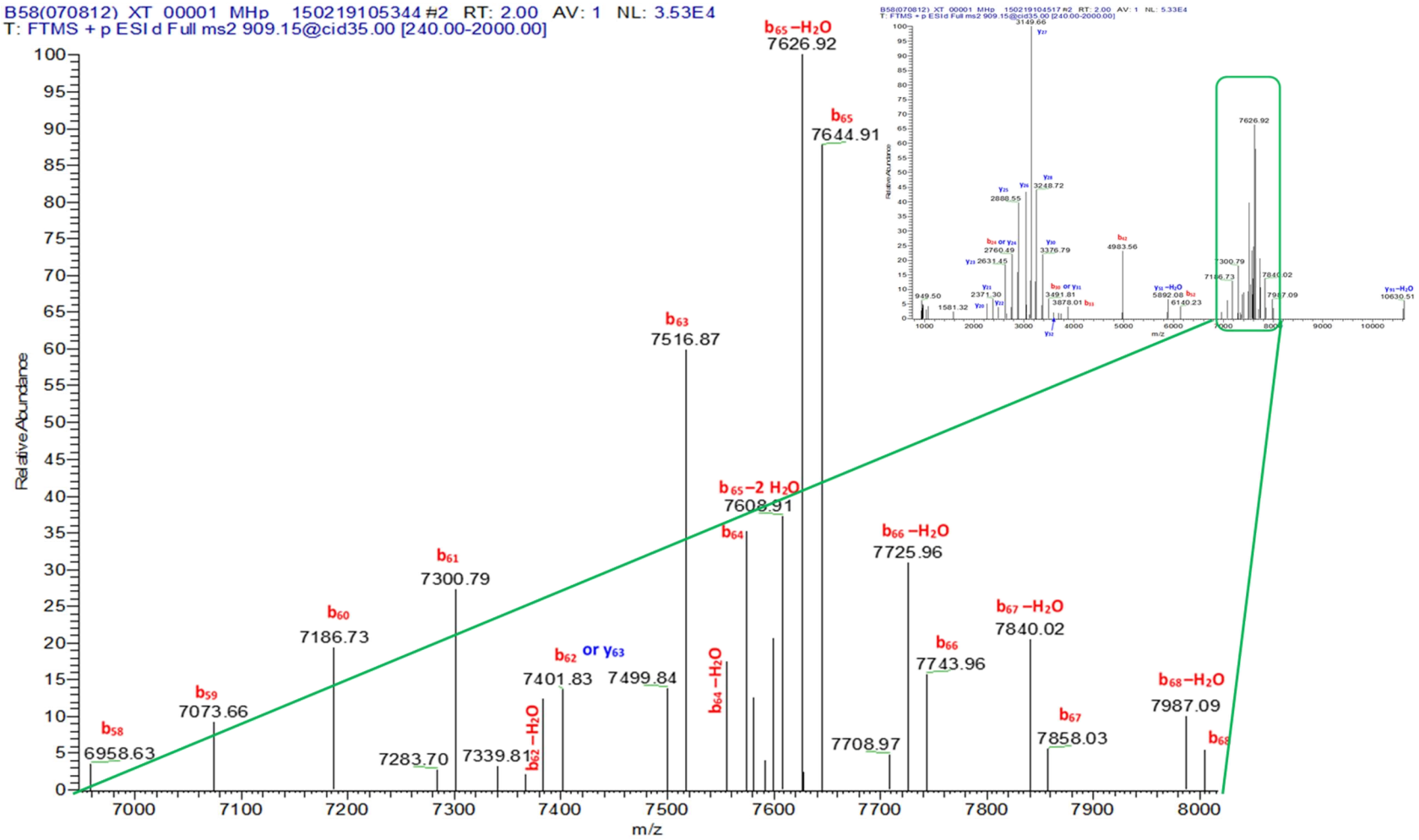

Fig.6A S100A8-SO3H/W54ox. Annotated $[M+H]^+$ and enlargement in the mass range 6949-8017 *m/z* of the deconvoluted spectrum of high-resolution MS/MS from 909.15 *m/z* ($[M+12H]^{12+}$).

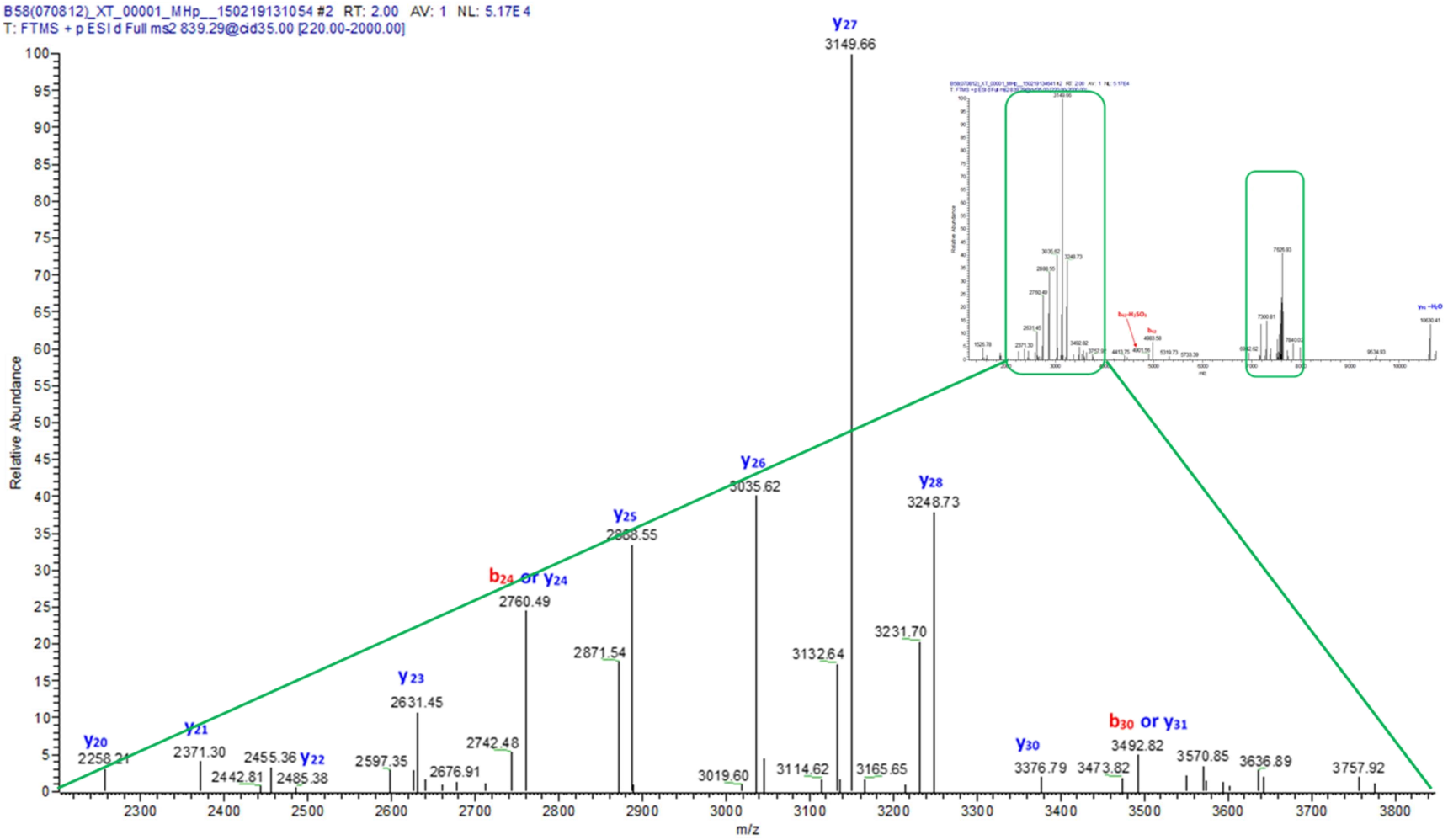

Fig.6B S100A8-SO3H/W54ox. Enlargement in the mass range 2200-3842 *m/z*. of the annotated $[M+H]^+$ deconvoluted spectrum of high-resolution MS/MS from 839.29 *m/z* ($[M+13H]^{13+}$).

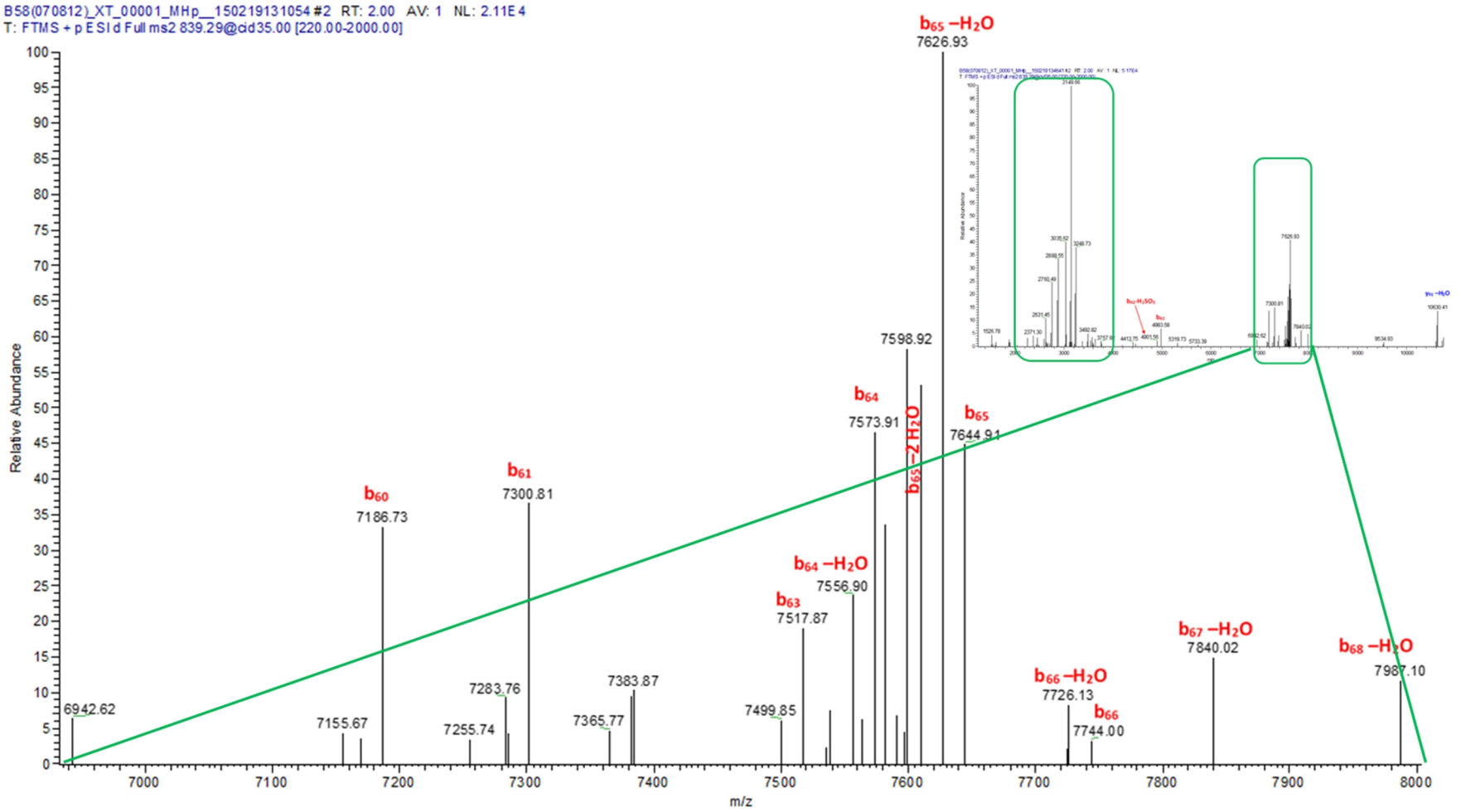

Fig.6C S100A8-$SO_3H/W_{54}ox$. Enlargement in the mass range 6938-7993 *m/z*. of the annotated $[M+H]^+$ deconvoluted spectrum of high-resolution MS/MS from 839.29 *m/z* ($[M+13H]^{13+}$).

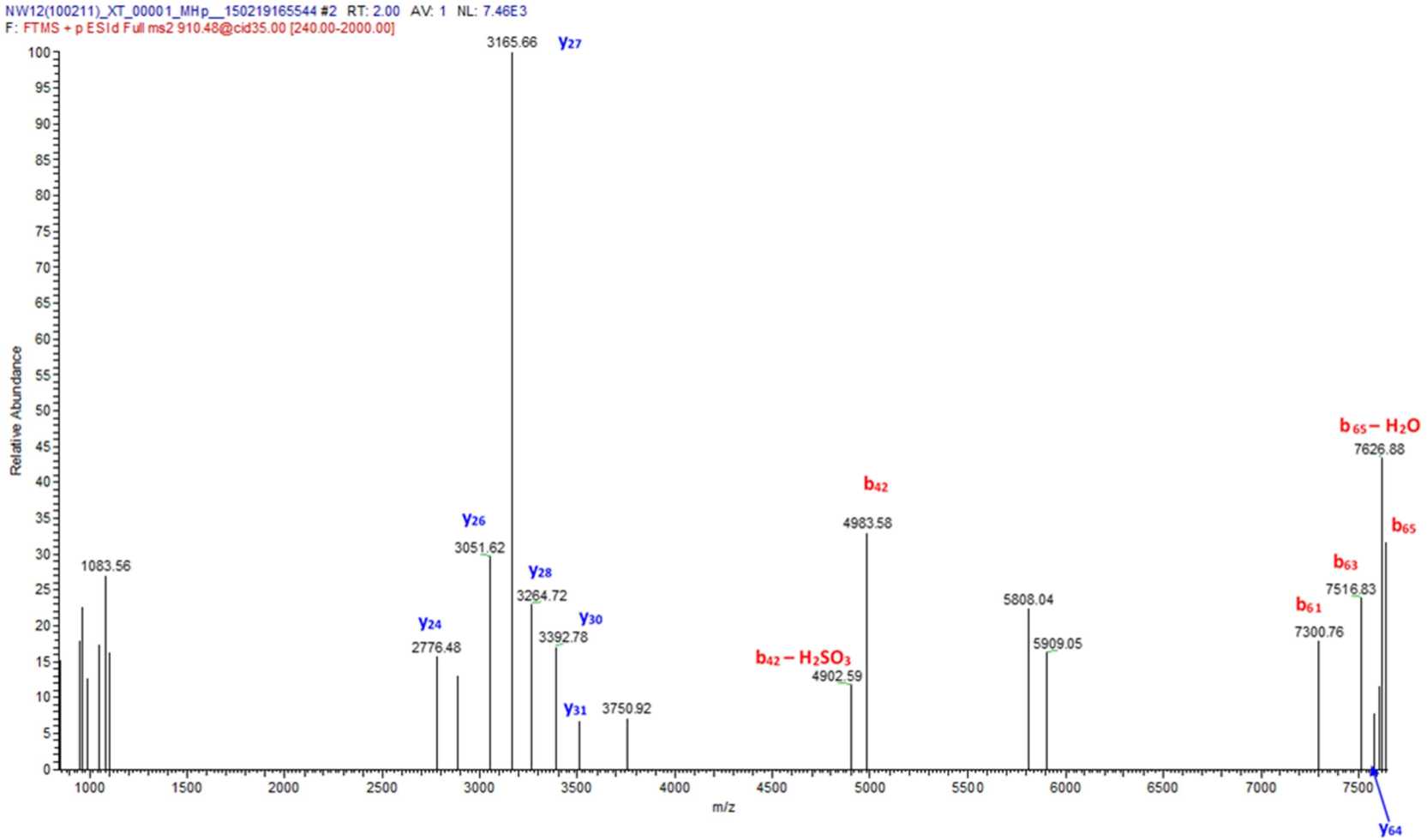

Fig.7A Hyperoxidized S100A8; S100A8-SO_3H/W_{54}ox/M_{78}ox. Annotated $[M+H]^+$ deconvoluted spectrum of high-resolution MS/MS from 910.48 *m/z* ($[M+12H]^{12+}$).

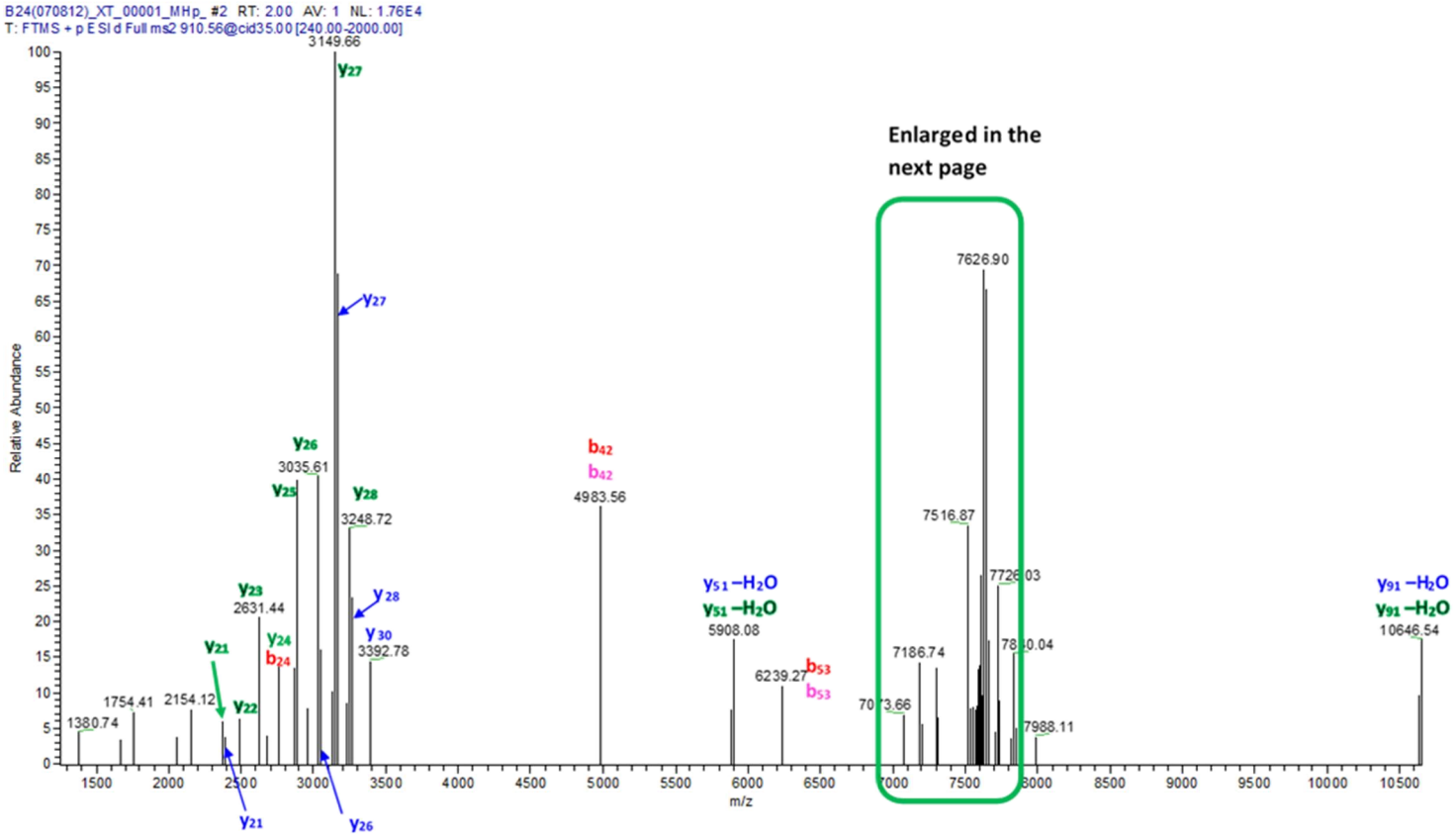

Fig.7B Hyperoxidized S100A8; S100A8-SO_3H/W_{54}ox/M_{78}ox. and S100A8-SO_3H/W_{54}diox. Annotated $[M+H]^+$ deconvoluted spectrum of high-resolution MS/MS from 910.56 *m/z* ($[M+12H]^{12+}$). The first y and b ion's series (blue and red) are in agreement with C42- SO_3H (+47.985 Da), W54-oxidized (+15.995 Da) and M78-oxidized (+15.995 Da) modifications. The second y and b ion's series (green and pink) are in agreement with C42- SO_3H (+47.985 Da) and W54-dioxidized (+31.999 Da) modifications.

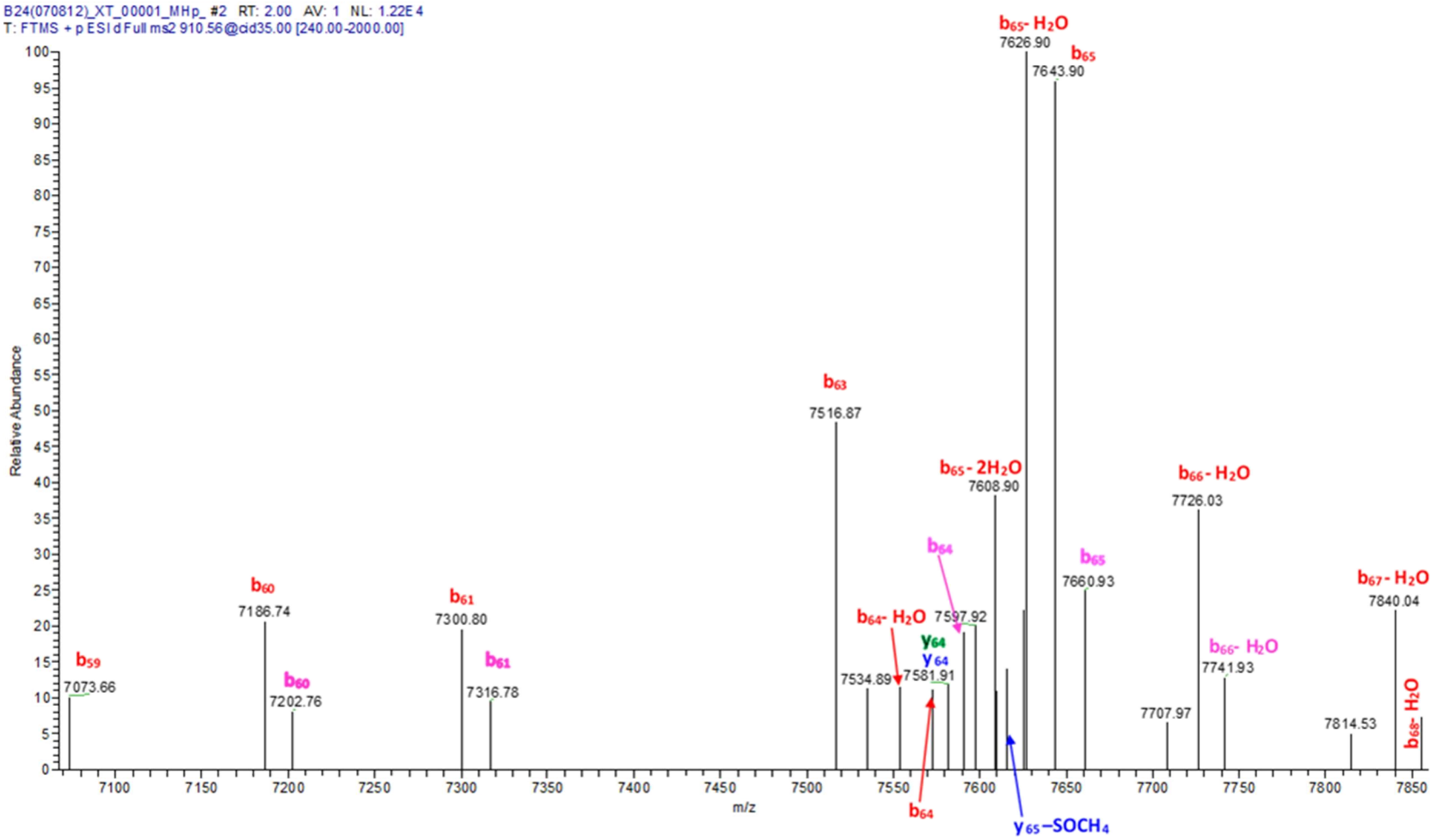

Fig.7C Hyperoxidized S100A8; S100A8-$SO_3H/W_{54}ox/M_{78}ox.$ and S100A8-$SO_3H/W_{54}diox.$ Enlargement in the range 7068-7860 *m/z* of the annotated $[M+H]^+$ deconvoluted spectrum of high-resolution MS/MS from 910.56 *m/z* ($[M+12H]^{12+}$).

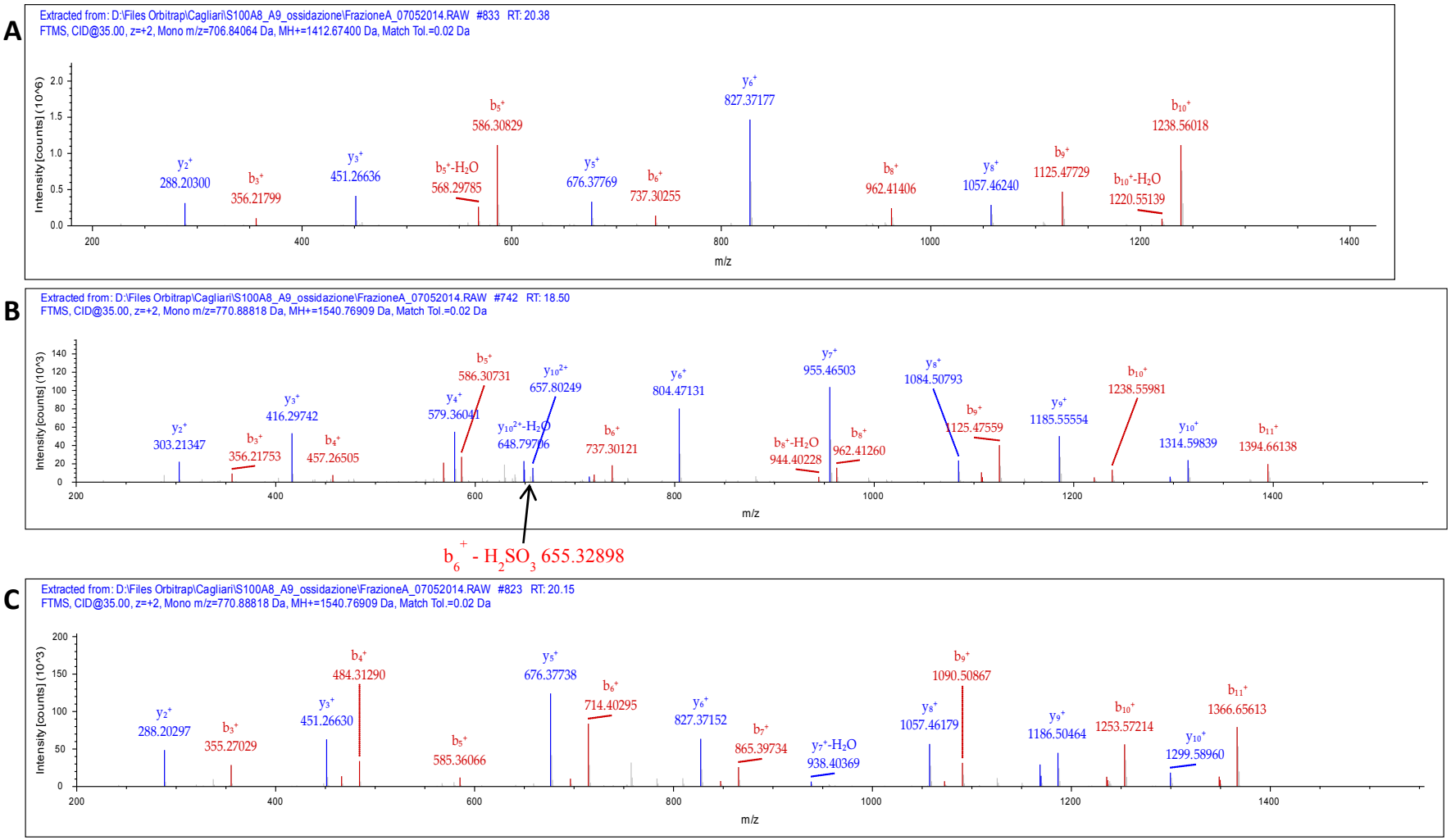

Fig.8 Sulfonic acid modification of C42 of S100A8 (C42-SO_3H): fragment 37-47, panel A; 37-48, panel B; 36-47, panel C.

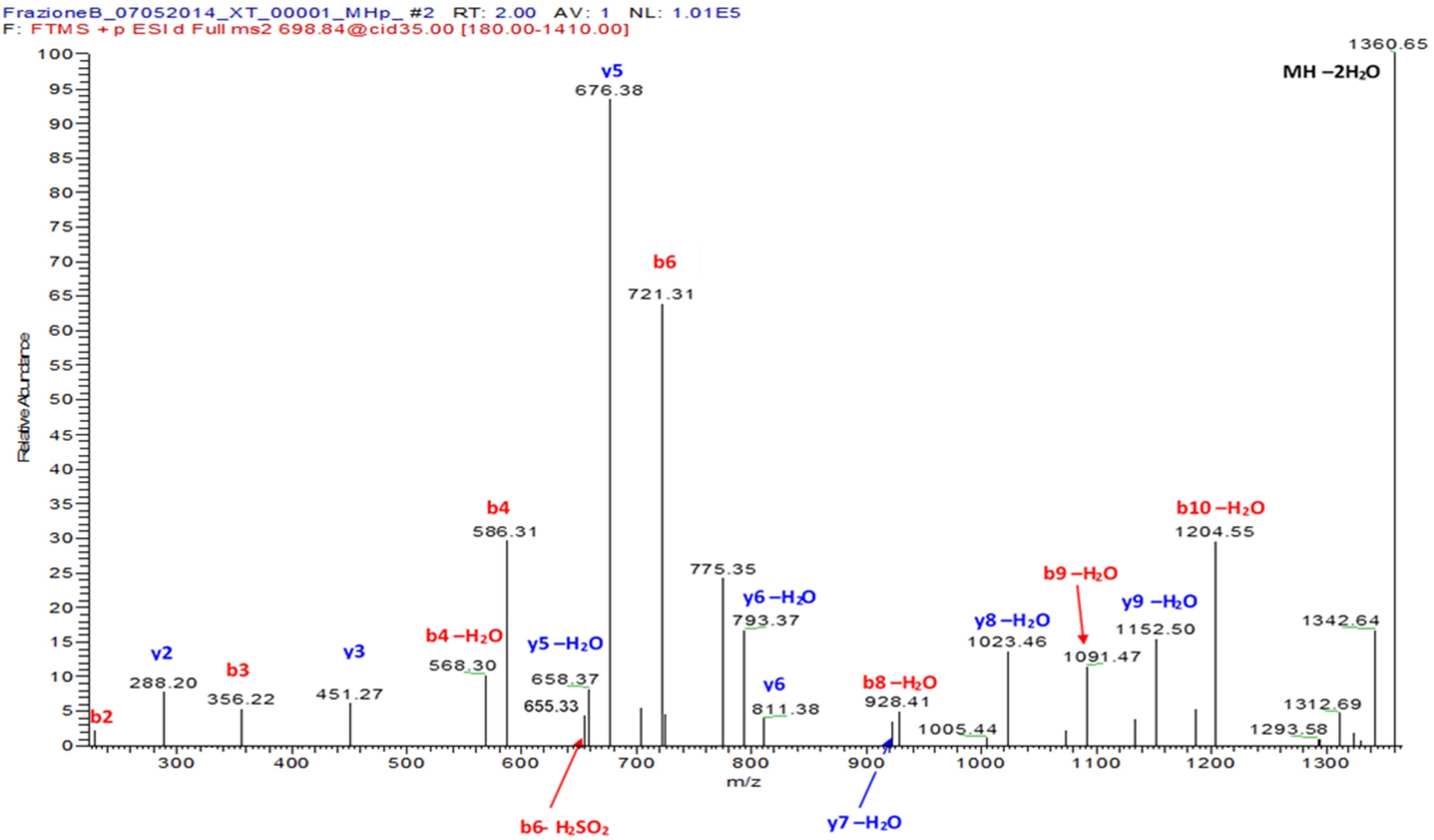

Fig.9 Sulfinic acid modification of C42 of S100A8 (C42-SO2H): fragment 37-47.

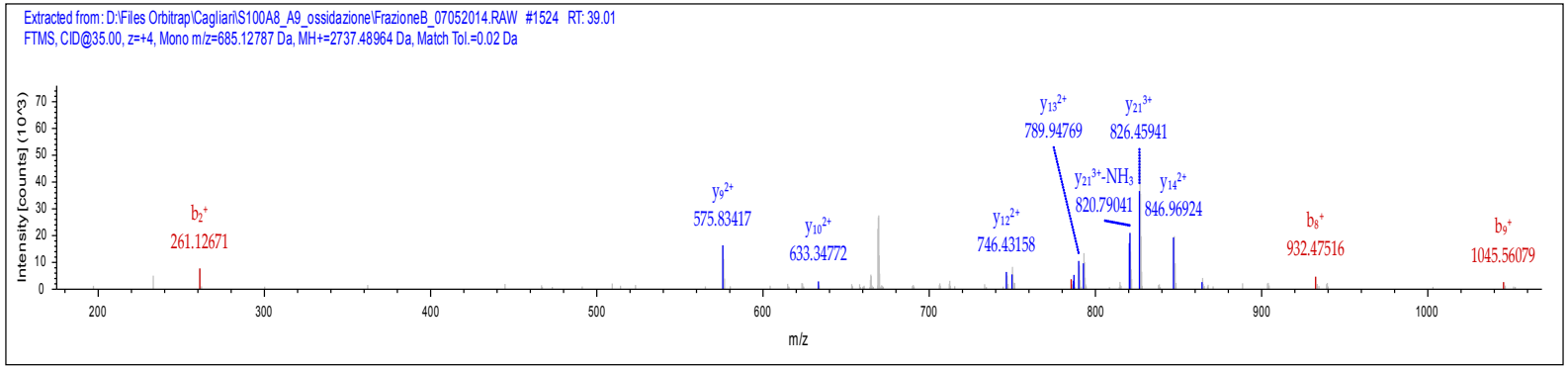

Fig.10 Oxidation on M1 of S100A8 (M1ox), fragment 1-23.

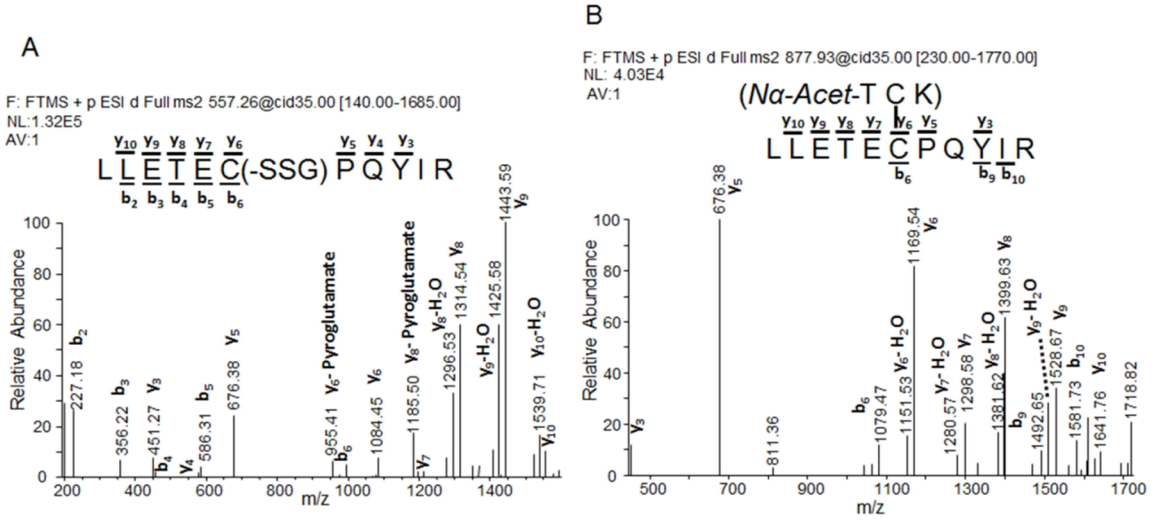

Fig.11 Glutathionylation of C42 in S100A8 (S100A8-SSG), panel A; disulfide bridge between cysteine 42 of S100A8 and cysteine 3 of S100A9(L) (S100A8/A9-SSdimer), panel B.

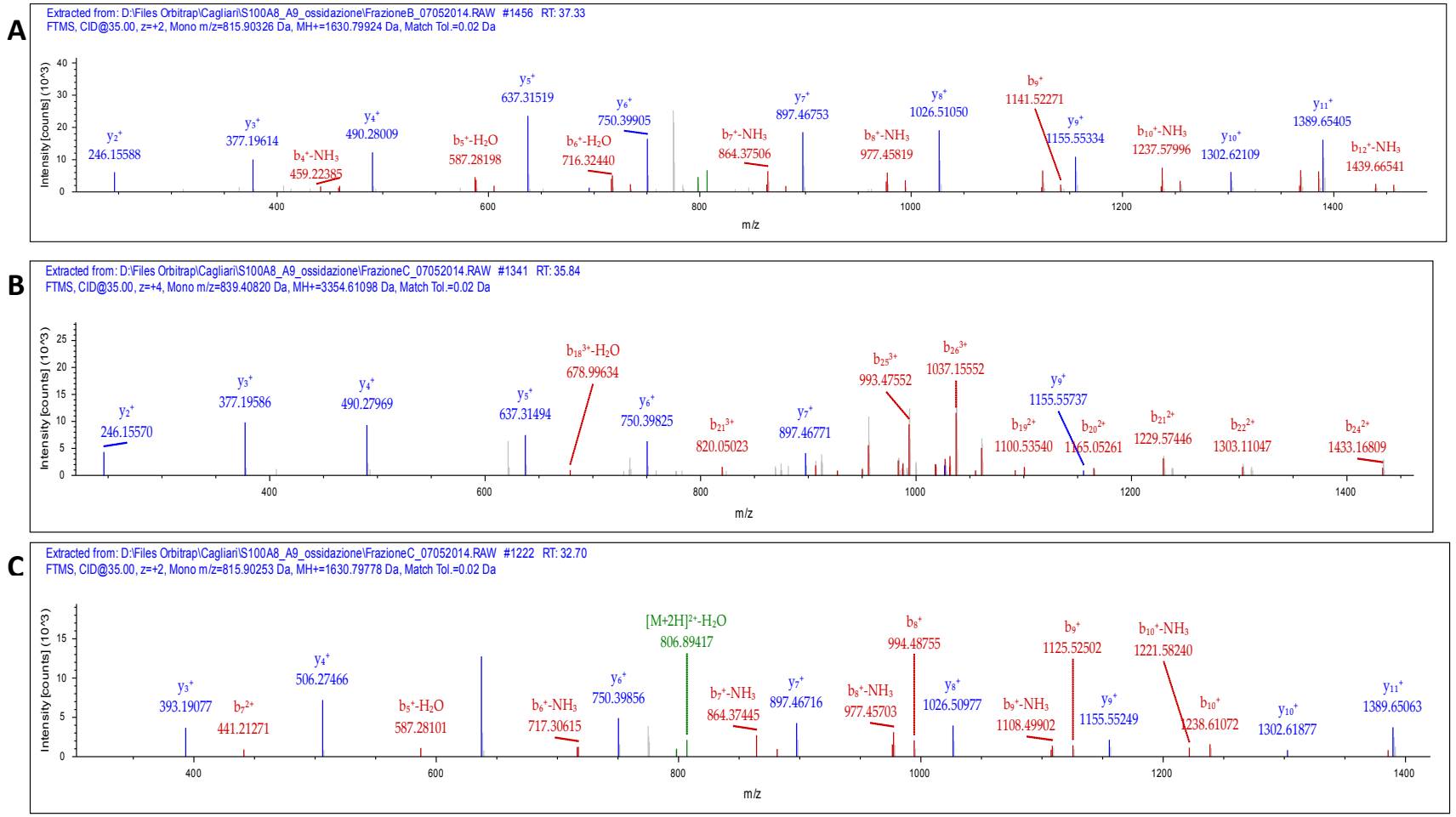

Fig.12 Oxidation on M81 of S100A9 (M81ox): fragment 73-85, panel A; fragment 57-85, panel B.

Oxidation on M83 of S100A9 (M83ox): fragment 73-85, panel C.

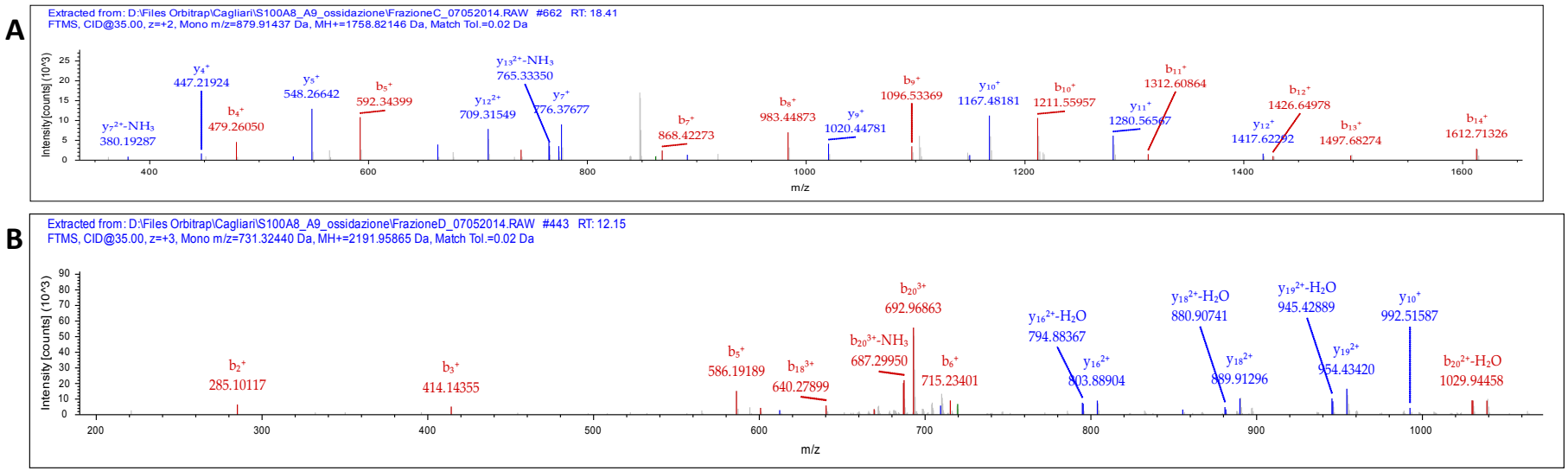

Fig.13 Oxidation on M63 of S100A9 (M63ox): fragment 57-72, panel A. Oxidation on M94 of S100A9 (M94ox): fragment 94-114, panel B.

Protein/peptide quantification and statistical analysis

T-test was used to compare the XIC peak area of each protein/peptide measured by low-resolution HPLC-ESI-IT-MS in Wilson's disease patients and healthy controls (Table 6).

Table 6. XIC peak area (mean ± standard deviation (SD) and median x10^7) and frequency of pIgR fragments, α-defensins 2-4, and several proteoforms of S100A8, and S100A9 in Wilson's disease patients and healthy controls. T-test *p* value obtained by comparing the two groups are also reported.

Peptide/protein	WD group Mean ± SD Median	Frequency	HC group Mean ± SD Median	Frequency	*p* value
ASVD	1.1 ± 0.6 1.1	30/32	0.7 ± 0.7 0.5	21/32	0.02 ↑
AVAD	2.4 ± 1.2 2.2	31/32	1.3 ± 1.1 1.4	21/32	0.003 ↑
α-Defensin 2	9.3 ± 11.0 4.3	27/32	4.7 ± 5.1 2.7	25/32	0.04 ↑
α-Defensin 4	2.5 ± 2.8 1.2	24/32	0.8 ± 1.7 0.01	11/32	0.004 ↑
S100A9(S) (1P + non-P)	27.4 ± 30.4 14.8	27/32	9.6 ± 12.1 4.5	19/32	0.004 ↑
S100A9(S)-Mox[a] (1P + non-P)	5.0 ± 8.0 1.4	16/32	2.4 ± 5.0 0.01	9/32	ns
S100A9(L)-SSG[b] (non-P)	15.9 ± 19.3 7.5	24/32	5.0 ± 7.6 0.01	15/32	0.007 ↑
S100A9(L)-SSC[c] (1P + non-P)	2.3 ± 5.1 0.01	8/32	0.3 ± 1.5 0.01	3/32	ns
S100A9/A9-SS[d]dimer	1.7 ± 4.6 0.01	5/32	0		na
S100A8/A9-SSdimer	5.1 ± 13.8 0.01	5/32	0		na
S100A9(L)ox tot	28.6 ± 31.9 18.8	29/32	8.9 ± 13.4 1.9	15/32	0.006 ↑
S100A8	3.1 ± 4.7 0.01	15/32	0.5 ± 1.6 0.01	4/32	0.01 ↑
Hyperoxidized S100A8	3.0 ± 3.7 1.6	20/32	0.7 ± 1.6 0.01	7/32	0.002 ↑
S100A8-SO_3H/W54ox	1.1 ± 3.2 0.01	11/32	0.3 ± 0.8 0.01	5/32	ns
S100A8-SSG	0.5 ± 1.5 0.01	5/32	0		na
S100A8-SNO[e]	2.7 ± 9.0 0.01	5/32	0		na
S100A8-SO_2H	0.3 ± 0.9 0.01	3/32	0		na
S100A8ox tot	10.4 ± 14.8 4.3	23/32	0.9 ± 1.8 0.01	11/32	0.0003 ↑

[a]methionine sulfoxide; [b]glutathionylated cysteine residue; [c]cysteinylated cysteine residue; [d]disulfide bridge; ns: not significant; na: not applicable; [e]nitrosylated cysteine residue; ↑: increased levels in WD group.

No statistical differences were found in the levels of proteins and peptides secreted by salivary glands, such as histatins, statherin, P-B peptide, cystatins S and C, and aPRPs, as well as in the level of cystatin A and B. Conversely, saliva of Wilson's disease patients with respect to healthy subjects showed significant higher levels of AVAD and ASVD pIgR peptides, and α-defensins 2 and 4 (Table 6, Fig.14A-B).

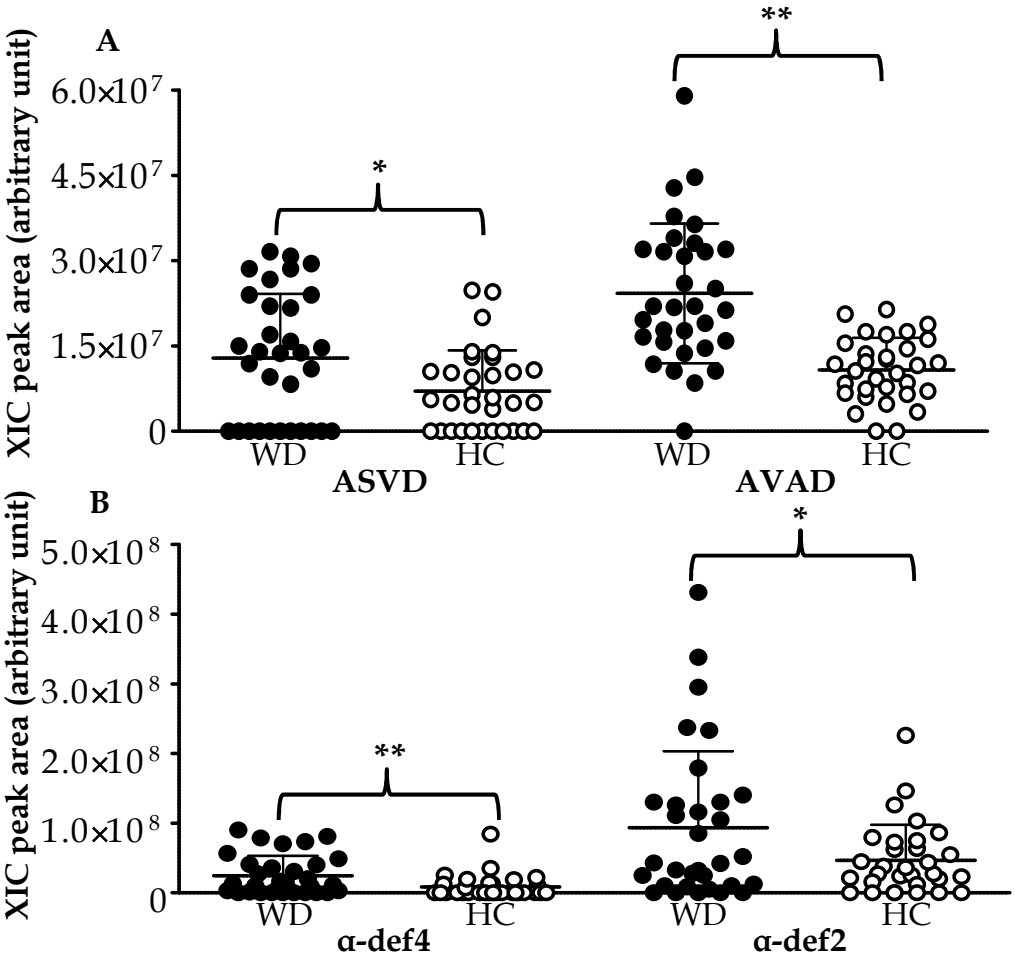

Fig.14 Distribution of the XIC peak area values of ASVD and AVAD peptides (A) and α-defensins 2 and 4 (B) measured in saliva from Wilson's disease patients and healthy controls. Asterisk indicates statistically significant differences: *($p < 0.05$), **($p \leq 0.01$).

Moreover, S100A9(S), S100A9(L)-SSG, and their phosphorylated derivatives, S100A8 and hyper-oxidized S100A8 exhibited higher levels in saliva of patients with respect to controls (Table 6, Fig.15A-B). Statistical analysis was performed

considering the totality of non-phosphorylated and phosphorylated forms of short and long S100A9 and the results are reported in Table 6.

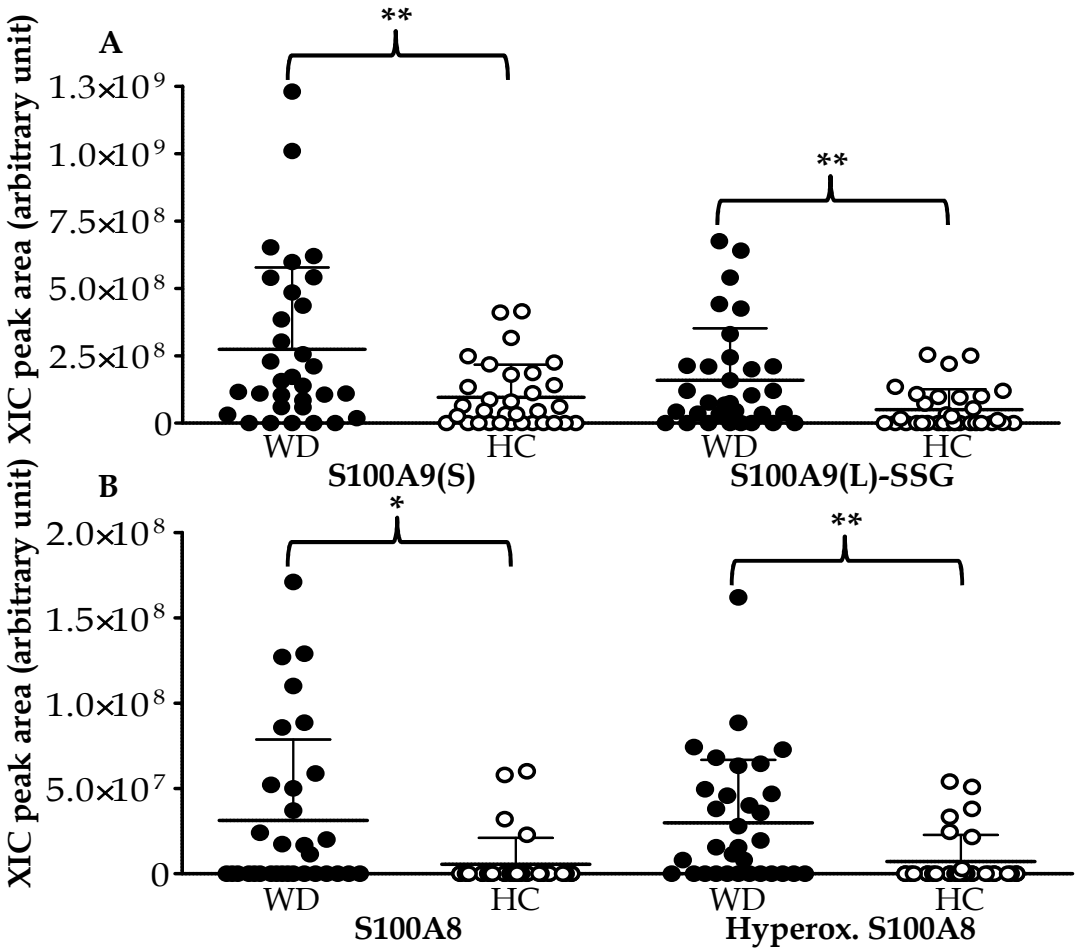

Fig.15 Distribution of the XIC peak area values of S100A9(S) and S100A9(L)-SSG (A) and S100A8 and hyper-oxidized S100A8 (S100A8-SO_3H/W54ox/W54-ox/M78-ox or -SO_3H/W54diox (B) measured in saliva from Wilson's disease patients and healthy controls. Asterisk indicates statistically significant differences: *($p < 0.05$), **($p \leq 0.01$).

Wilson's disease patients showed higher levels and frequencies of S100A8-SO_3H/W54ox, S100A9(L)-SSC, S100A9(L)-SSC phosphorylated, S100A9(S)-Mox and S100A9(S)-Mox phosphorylated with respect to controls, even though without a statistical significance (Table 6). In patients with high concentration of S100A8 and S100A9, also the following modifications were detected, even if sporadically: S100A8-SSG and S100A8-SNO in 5 patients; S100A8-SO_2H in 3 patients, S100A8/A9-SSdimer and S100A9(L)-SSdimer in 8 patients (Table 6). In

8 Wilson's disease patients S100A8 was detected only in the oxidized forms. Statistical analysis highlighted significant higher levels in patients with respect to healthy subjects of the totality of the oxidized proteoforms of S100A8 (S100A8ox tot), and the totality of the oxidized proteoforms of S100A9(L) (S100A9(L)ox tot) (Table 6, Fig.16).

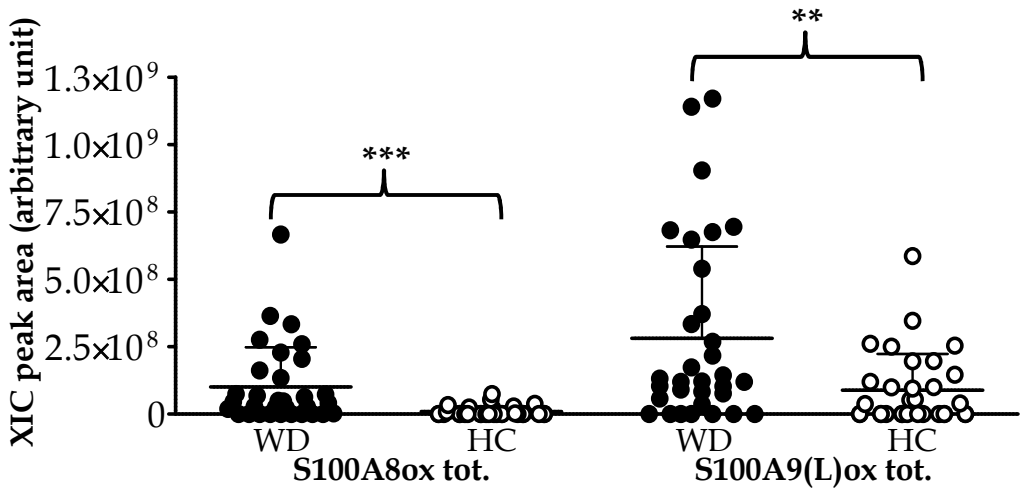

Fig.16 Distribution of the XIC peak area values of total S100A8ox and S100A9(L)ox measured in saliva from Wilson's disease patients and healthy controls. Asterisk indicates statistically significant differences: **($p \leq 0.01$), ***($p \leq 0.001$).

A calculation of the relative abundance of the different proteoforms revealed that S100A8 was mainly represented by its oxidized derivatives in saliva of both patients and controls: 75 ± 30% in Wilson's disease patients, 77 ± 42% in healthy subjects. Among the oxidized proteoforms the most abundant was the hyper-oxidized S100A8 (WD 42 ± 40%, controls 59 ± 46%), followed by S100A8-SO_3H/W54ox (WD 12 ± 23%, controls 18 ± 32%), S100A8/A9-SSdimer (WD 11 ± 27%), S100A8-SNO (WD 8 ± 22%), S100A8-SSG (WD 1 ± 3%), and S100A8-SO_2H (WD 1 ± 4%).

Both in patients and controls, S100A9(L) was detected only in the form oxidized at the cysteine residue: glutathionylated (WD 85 ± 16%, HC 98 ± 5%), cysteinylated (WD 7 ± 10%, 375 HC 2 ± 5%), and dimeric (WD 8 ± 13%). Conversely, S100A9(S) was prevalently detected in the non-oxidized forms in Wilson's disease patients (86 ± 24%), and healthy subjects (80 ± 35%).

- **SAPHO syndrome**

The following proteins and peptides were searched and quantified by *top-down* low-resolution HPLC-ESI-IT-MS experiments in the acidic soluble fraction of saliva from SAPHO patients and healthy controls: histatins, salivary cystatins, statherin, P-B peptide, aPRPs, α-defensins 1–4, cystatins A, B, C, β-thymosins 4 and 10, S100A7 (D27), S100A8, S100A9 (short (S) and long (L) isoforms), S100A12, as well as numerous their derivatives (Table 3).

Protein/peptide quantification and statistical analysis

T-test was used to compare the XIC peak area of each protein/peptide measured by low-resolution HPLC-ESI-IT-MS in SAPHO patients and healthy controls. The XIC peak areas mean ± SD (x10^7), the frequency and the *p* value obtained on proteins showing differences between the two groups are reported in Table 7.

Table 7.

Proteins	SAPHO patients		Healthy controls		*p* value
	mean ± SD	Frequency	mean ± SD	Frequency	
Salivary cystatins					
Cystatin S1	60.4 ± 58.7	10/10	122.2 ± 118.6	26/28	**0.04**
Cystatin SN	115.2 ± 132.4	10/10	199.1 ± 189.1	25/28	ns
Histatins					
Histatin 1	21.5 ± 14.1	9/10	40.8 ± 25.8	28/28	**0.007**
Histatin 3	11.1 ± 19.0	6/10	17.5 ± 16.9	18/28	ns
Histatin 6 (1/25)	10.5 ± 16.7	8/10	15.6 ± 11.5	27/28	ns
Histatin 5 (1/24)	26.7 ± 33.0	10/10	48.6 ± 30.8	28/28	ns
S100A12	3.4 ± 3.6	6/10	1.2 ± 2.6	6/28	ns
aPRPs					
PRP-1 diphos	629.8 ± 361.5	10/10	981.0 ± 511.2	28/28	ns
PRP-3 diphos	178.2 ± 77.2	10/10	364.9 ± 176.1	28/28	**<0.0001**
P-C peptide	144.1 ± 80.2	10/10	246.1 ± 95.1	28/28	**0.005**
P-B peptide	195.0 ± 115.9	10/10	304.2 ± 169.4	28/28	ns

ns: not significant

The results of the statistical analysis performed by excluding from the SAPHO group the patient #8, who unlike the others never had skin manifestations (Table 1), are reported in Table 8.

Table 8.

Proteins	SAPHO patients mean ± SD	SAPHO patients Frequency	Healthy controls mean ± SD	Healthy controls Frequency	*p* value
Salivary cystatins					
Cystatin S1	52.2 ± 55.6	9/9	122.2 ± 118.6	26/28	**0.02**
Cystatin SN	83.1 ± 90.3	9/9	199.1 ± 189.1	25/28	**0.02**
Histatins					
Histatin 1	21.1 ± 14.8	8/9	40.8 ± 25.8	28/28	**0.04**
Histatin 3	5.4 ± 6.6	5/9	17.5 ± 16.9	18/28	**0.004**
Histatin 6 (1/25)	5.4 ± 5.1	7/9	15.6 ± 11.5	27/28	**0.0009**
Histatin 5 (1/24)	17.0 ± 13.0	9/9	48.6 ± 30.8	28/28	**0.0001**
S100A12	3.8 ± 3.6	6/9	1.2 ± 2.6	6/28	**0.04**
aPRPs					
PRP-1 diphos	535.3 ± 215.9	9/9	981.0 ± 511.2	28/28	**0.0008**
PRP-3 diphos	161.2 ± 58.8	9/9	364.9 ± 176.1	28/28	**<0.0001**
P-C peptide	121.4 ± 37.5	9/9	246.1 ± 95.1	28/28	**<0.0001**
P-B peptide	167.8 ± 82.3	9/9	304.2 ± 169.4	28/28	**0.003**

Among salivary cystatins, only cystatin S1 showed a significant lower level in the SAPHO subjects with respect to healthy controls ($p = 0.04$), but exclusion of the patient #8 evidenced a significant lower concentration of also cystatin SN ($p = 0.02$) (Table 8, Fig.17).

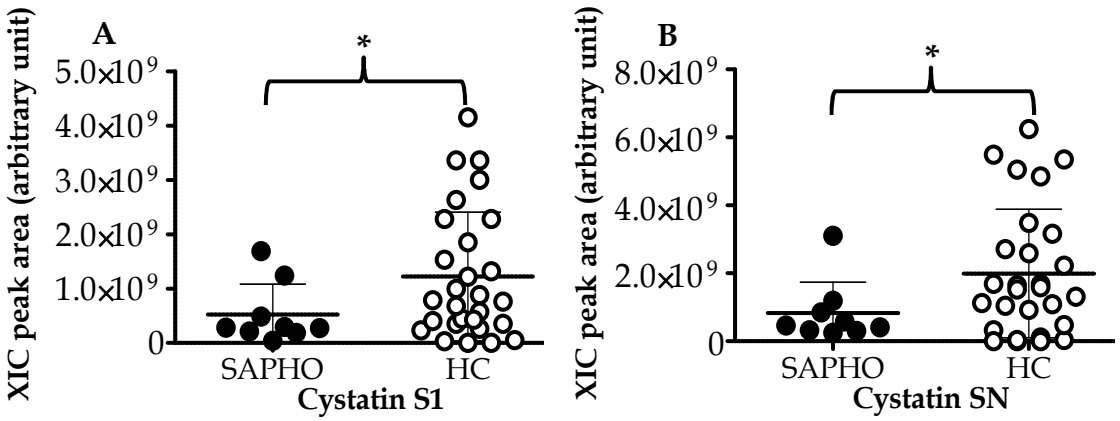

Fig.17 Distribution of the XIC peak area values of cystatins S1 (panel A) and SN (B) measured in saliva from SAPHO patients (patient #8 excluded) and healthy controls. Asterisk indicates statistically significant differences: $*p < 0.05$.

Histatin 1 concentration ($p = 0.007$) was found deeply lower in saliva of SAPHO patients as compared with controls (Fig.18A), while the level of histatins 3, 5, and 6 was similar in the two groups (Table 7). Distribution of XIC peak areas showed that concentration of the last three peptides was extremely higher in patient #8 with respect to both SAPHO and control subjects. By excluding this

subject from the patient's group, the level of histatins 3, 5 and 6 became significantly lower with respect to controls (Fig.18B-D), and the following *p* values were determined: 0.004, 0.0001, and 0.0009, for histatins 3, 5, and 6, respectively (Table 8).

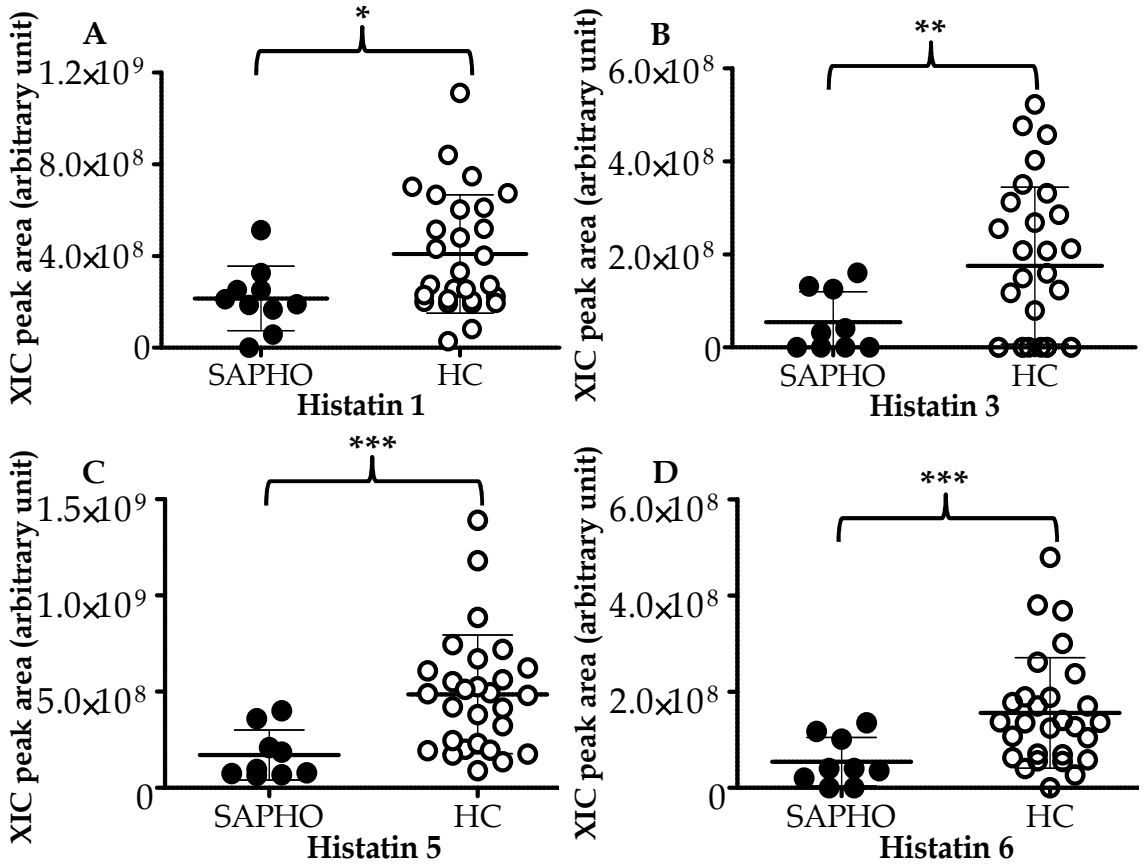

Fig.18 Distribution of XIC peak area values of histatin 1 (panel A), histatin 3 (B), histatin 5 (C) and histatin 6 (D) measured in saliva from SAPHO patients (patient #8 excluded) and healthy controls. Asterisks indicate statistically significant differences: *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

Statistical analysis evidenced significant lower levels of diphosphorylated PRP3 (p <0.0001) and the P-C peptide (p = 0.005) in saliva of SAPHO compared with the controls (Table 7). Also the levels of the P-C peptide and diphosphorylated PRP1 were higher in patient #8 than in the other patients. By excluding this subject, the level of diphosphorylated PRP1 resulted significantly different (p = 0.0008, Table 8) in the two groups, and the p value for the P-C peptide became lower than 0.0001 (Fig.19A and Table 8).

The P-B peptide was less abundant in SAPHO patients than in controls, although the statistical analysis became significant only after the exclusion of the patient #8 (p = 0.003), as shown in Fig.19B.

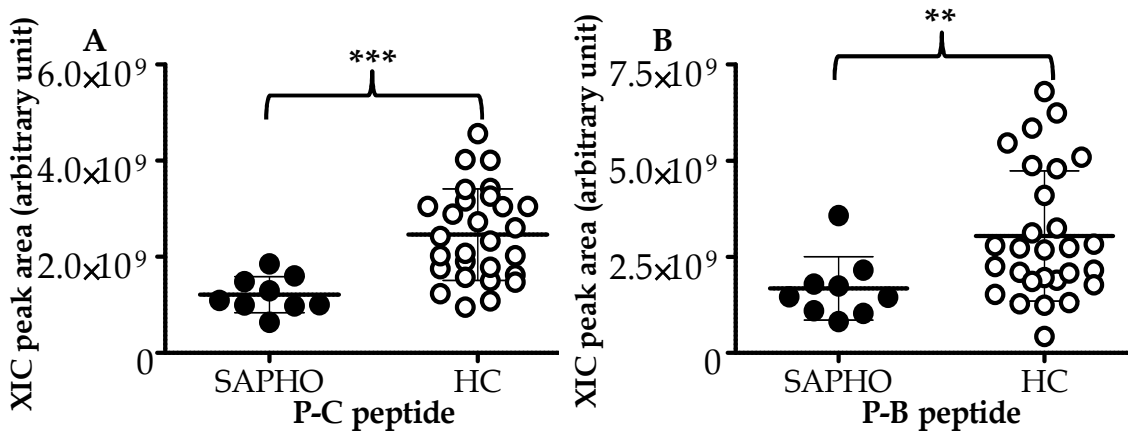

Fig.19 Distribution of XIC peak area values of the P-C peptide (panel A) and the P-B peptide (B) measured in saliva from SAPHO patiens (patient #8 excluded) and healthy controls. Asterisks indicate statistically significant differences: $^{**}p \leq 0.01$; $^{***}\ p \leq 0.001$.

S100A12 protein showed a higher frequency in the SAPHO group (6/10) with respect to the controls (6/28) (Table 7). The level of this protein became significantly higher in patients than in healthy controls by excluding the patient #8 (p = 0.04), as shown in Table 8.

Correlations between low-resolution HPLC-ESI-IT-MS data and clinical and laboratory parameters

The XIC peak areas of the salivary proteins/peptides quantified by low-resolution HPLC-ESI-IT-MS experiments and showing significant quantitative variations between the two groups were correlated with SAPHO clinical laboratory parameters. A positive correlation was observed between CRP levels and the salivary concentration of all the histatins (p = 0.005, 0.004, 0.006, 0.006, for histatins 1, 3, 5, and 6, respectively; R = 0.8 for all of them, Fig. 20A-D). Moreover, CRP showed a negative correlation with S100A12 (p = 0.02; R = -0.7, Fig.20E). The cystatin SN abundance decreased in relation to the disease duration (p = 0.03; R = -0.7, Fig.20F).

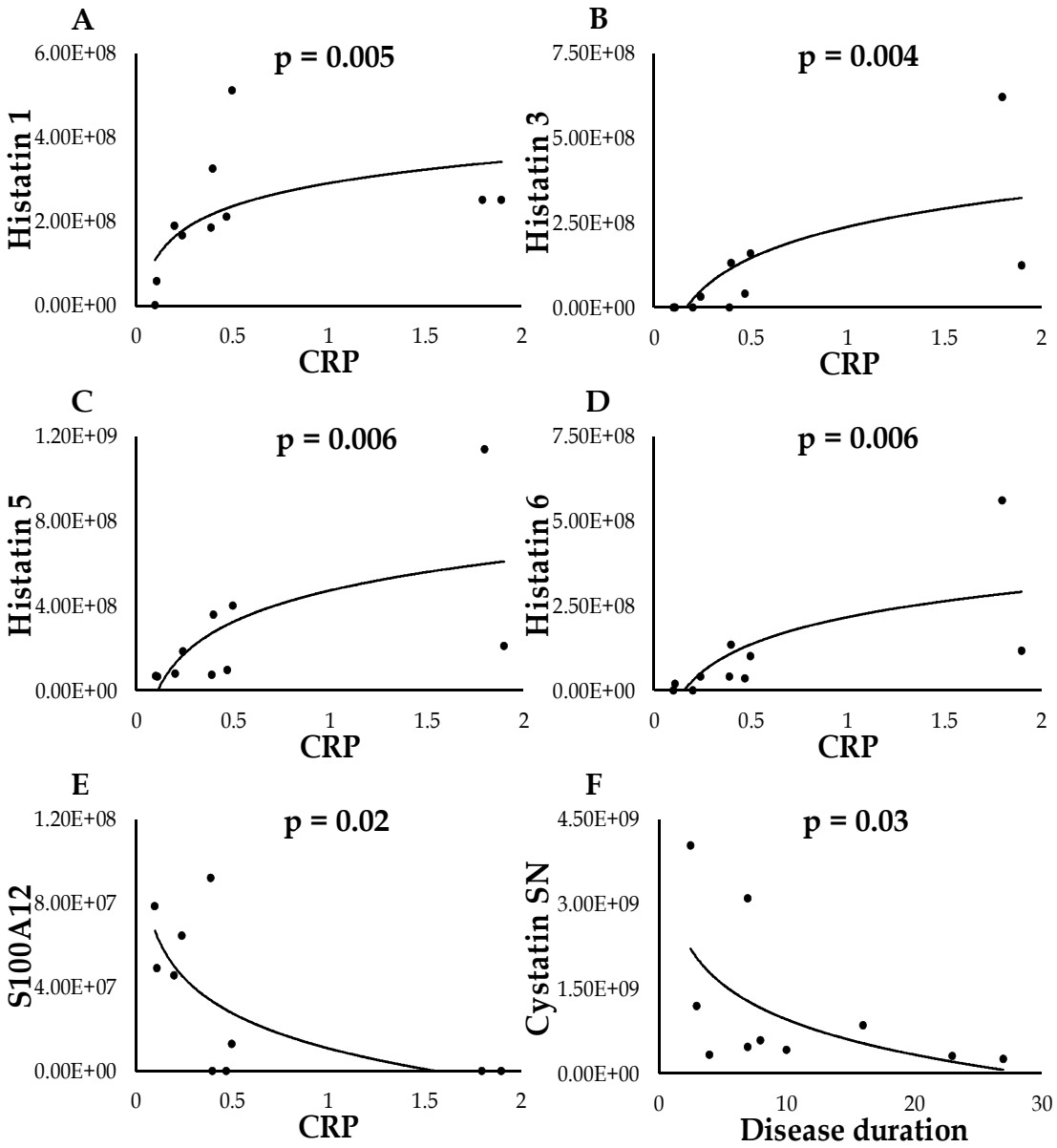

Fig.20 Correlation analysis between CRP and histatin 1 (panel A), histatin 3 (B), histatin 5 (C), histatin 6 (D), S100A12 (E), and between cystatin SN and the disease duration (F) in SAPHO patients.

By excluding patient #8, the correlations between CRP and histatins were confirmed (p = 0.005, 0.008, 0.01, 0.02; R = 0.9, 0.8, 0.8, 0.8, for histatins 1, 3, 5, and 6, respectively), as well as the correlation between CRP and S100A12 (p = 0.04; R = -0.7), while that between cystatin SN and the disease duration vanished. Histatin 3 correlated negatively with the erythrocyte sedimentation rate (p = 0.04, R = -0.7, Fig.21A), and positively with the total white cells count (p = 0.04, R = 0.7, Fig.21B). Moreover, the highest levels of histatin 3 (p = 0.01, R

= 0.8) and 5 (p = 0.01, R = 0.8) were measured in the SAPHO patients with higher blood neutrophil counts (Neu) (Fig.21C-D).

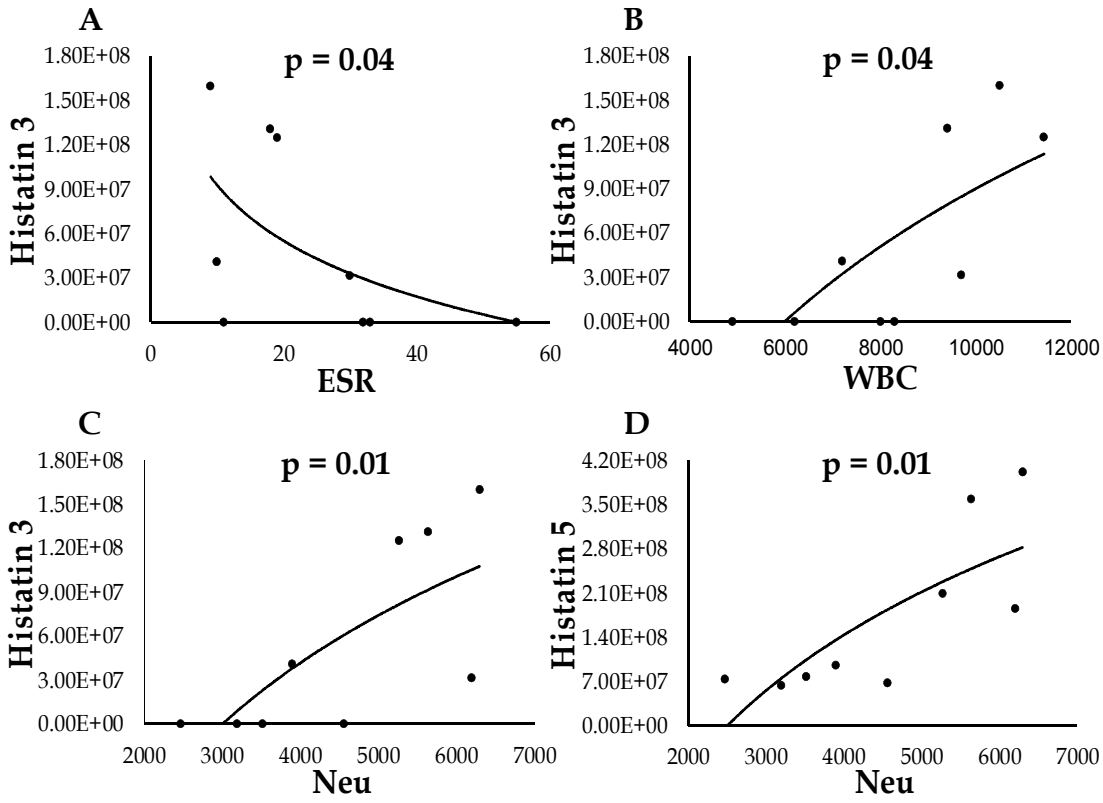

Fig.21 Correlation analysis between histatin 3 and ESR (panel A), WBC (B) and neutrophil count (C), and between histatin 5 and neutrophil count (D) in SAPHO patients (patient #8 excluded).

- **Hereditary angioedema**

The following proteins and peptides were searched and quantified by *top-down* low-resolution HPLC-ESI-IT-MS experiments in the acidic soluble fraction of saliva from angioedema patients and healthy controls: histatins, salivary cystatins, statherin, P-B peptide, aPRPs, α-defensins 1–4, cystatin B, β-thymosins 4 and 10, S100A7 (D27), S100A8, S100A9 (short (S) and long (L) isoforms), S100A12, as well as several derivatives (see Table 3).

Protein/peptide quantification and statistical analysis

One-way ANOVA and Post-hoc Dunn's multiple comparison test were used to compare the XIC peak area of each protein/peptide among the three angioedema groups and the control group and the results are shown in Table 9.

Statistically significant differences were observed for S-glutathionylated cystatin B (cystatin B-SSG) (p = 0.0007) and S-cysteinylated cystatin B (cystatin B-SSC) (p = 0.003), and Post-hoc comparisons showed that both C1-INH-HAE and InH-AAE groups had significant higher levels of cystatin B-SSG with respect to the control group, while InH-AAE had higher levels of cystatin B-SSC compared to both healthy subjects and FXII-HAE patients (Fig.22A-B).

Also lower levels of statherin Des-Thr_{42}-Phe_{43} were observed in FXII-HAE patients with respect to C1-INH-HAE and control groups (p = 0.006).

Moreover, histatin 1 and non-phosphorylated histatin 1 were significantly different among the groups (p = 0.04 and p = 0.01, respectively). In particular, in FXII-HAE patients higher levels of the two peptides were observed with respect to the healthy controls and increased levels of non-phosphorylated histatin 1 were observed also as compared to C1-INH-HAE group.

Finally, InH-AAE showed levels of S100A9(S)-Mox phosphorylated higher with respect to FXII-HAE group (p = 0.02).

Table 9. XIC peak area means (± SD), and frequencies (Freq.) of proteins and peptides showing statistically different levels among C1-INH-HAE, FXII-HAE, Inh-AAE and control groups by one-way ANOVA.

Protein name	C1-INH-HAE		FXII-HAE		InH-AAE		Controls		ANOVA	
	Mean ± SD (x10^7)	Freq.	Mean ± SD (x10^7)	Freq.	Mean ± SD (x10^7)	Freq.	Mean ± SD (x10^7)	Freq.	*p* value	Post-hoc Dunn's multiple comparison test
Cystatin B-SSG[a]	24.0 ± 16.0	13/13	14.6 ± 14.3	10/10	24.5 ± 13.5	12/12	10.4 ± 9.9	31/31	**0.0007**	C1-INH-HAE > Controls ** InH-AAE > Controls **
Cystatin B-SSC[b]	5.3 ± 3.9	13/13	2.7 ± 2.1	10/10	7.3 ± 4.2	12/12	3.7 ± 4.4	31/31	**0.003**	InH-AAE > FXII-HAE * InH-AAE > Controls **
Histatin 1	38.7 ± 39.5	11/13	52.1 ± 20.6	10/10	45.2 ± 30.2	12/12	29.5 ± 26.5	30/31	**0.04**	FXII-HAE > Controls *
Non-phosphorylated Histatin 1	4.5 ± 7.3	8/13	9.3 ± 4.5	10/10	5.0 ± 4.4	9/12	4.2 ± 5.1	24/31	**0.01**	FXII-HAE > C1-INH-HAE * FXII-HAE > Controls *
Statherin Des-Thr$_{42}$-Phe$_{43}$	6.5 ± 5.1	13/13	1.8 ± 1.5	10/10	5.1 ± 5.4	12/12	4.8 ± 3.7	31/31	**0.006**	FXII-HAE < C1-INH-HAE ** FXII-HAE < Controls *
S100A9(S)-Mox[c] **phosphorylated**	0.9 ± 1.7	4/13	0.0 ± 0.0	0/10	2.3 ± 3.5	6/12	0.6 ± 1.9	5/31	**0.02**	InH-AAE > FXII-HAE *

[a]glutathionylated cysteine residue; [b]cysteinylated cysteine residue; [c]methionine sulfoxide.

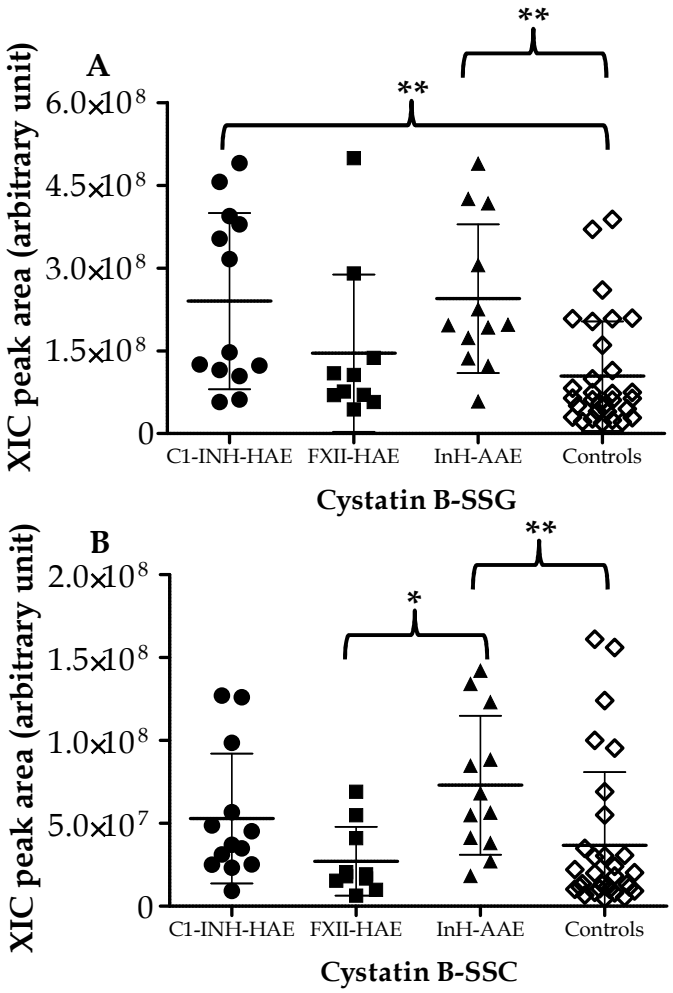

Fig.22 Distribution of XIC peak area values of cystatin B-SSG (panel A) and cystatin B-SSC (B) measured in saliva from C1-INH-HAE (circles), FXII-HAE (squares), InH-AAE (triangles) patients and healthy subjects (diamonds). Asterisks indicate statistically significant differences between pairs of groups (one way ANOVA with post-hoc Dunn's multiple comparison test): *$p < 0.05$; **$p \leq 0.01$.

Characterization of the acidic insoluble salivary proteome and bioinformatic analyses

Six available insoluble fractions obtained by treatment of whole saliva with TFA (3 from patients belonging to C1-INH-HAE group, 2 from patients belonging to FXII-HAE group and 1 from a Inh-AAE patient) were analyzed in duplicate by nano-HPLC-ESI-MS and MS/MS after tryptic digestion according with the endoProteoFASP protocol (*bottom-up* approach). A total of 191 distinct proteins were identified: 61 were common to all the three angioedema groups; 54 were shared between C1-INH-HAE and FXII-HAE groups; 31 between C1-INH-HAE

and Inh-AAE groups; and 30 between FXII-HAE and InH-AAE groups. Conversely, 7 proteins were specific of C1-INH-HAE group and 8 for FXII-HAE group (Fig.23).

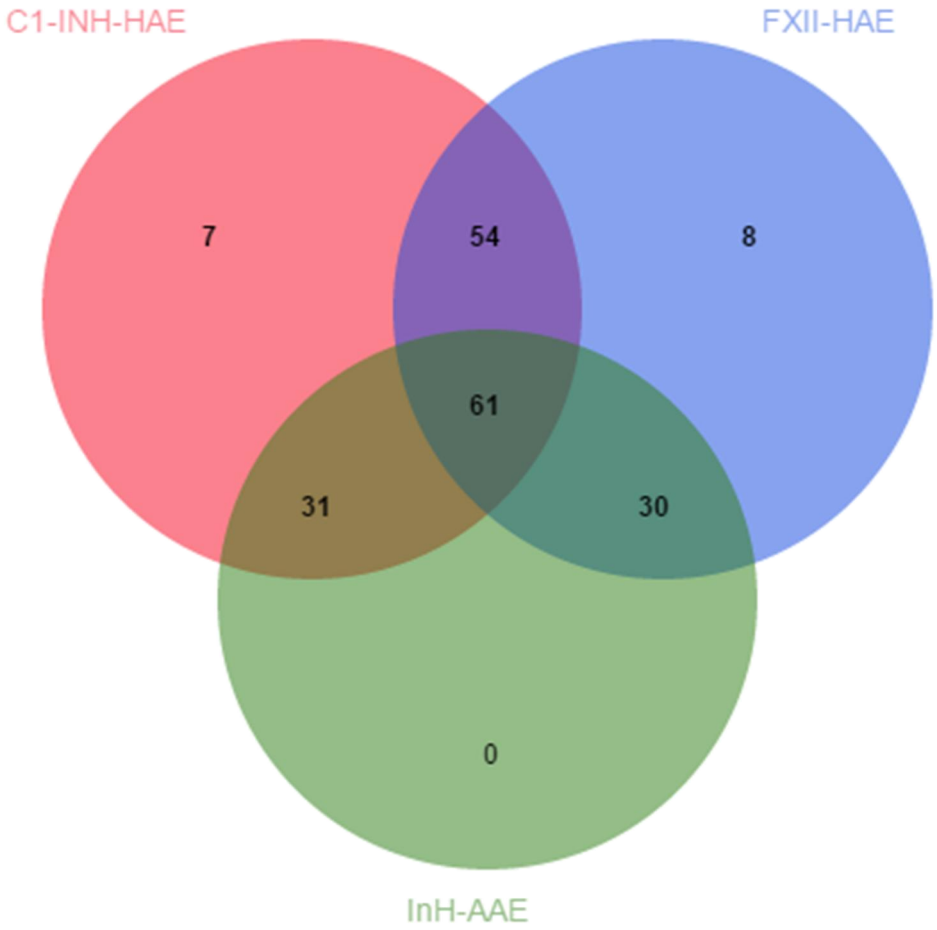

Fig.23 Venn diagram representing the distribution of identified proteins per group (C1-INH-HAE, in red; FXII-HAE, in blue; InH-AAE, in green) evidencing overlapped and unique proteins.

The integrated analysis of all proteins identified in the three patient groups performed by Cytoscape_v3.2.1 with the additional plugin ClueGO+CluePedia, generated the protein-protein interaction network reported in Fig.24, consisting of 171 proteins, distributed in 18 clusters, connected by 714 protein-protein interactions.

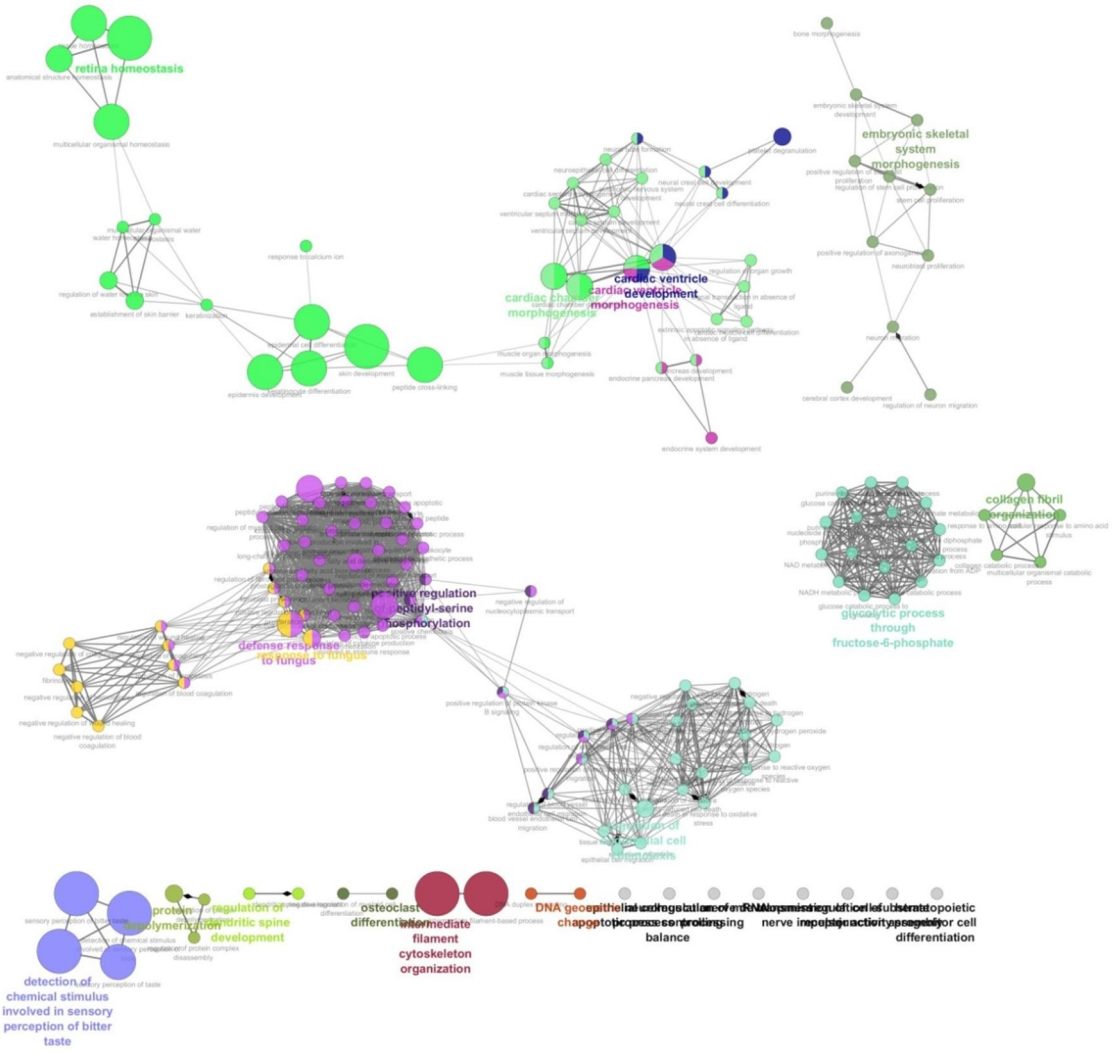

Fig.24

The most significant biological processes related to the identified proteins, shown in Fig. 25 with different colors according to their significance, were: detection of chemical stimulus involved in sensory perception of bitter taste; multicellular organismal and tissue homeostasis (retina and anatomical structure homeostasis and development); skin development (epidermal cell and keratinocyte differentiation); intermediate filament cytoskeleton organization; defense response to fungus; regulation of lipopolysaccharide-mediated signaling pathway; establishment of skin barrier; regulation of water loss via skin; protein nitrosylation; fibroblast proliferation, collagen fibril organization.

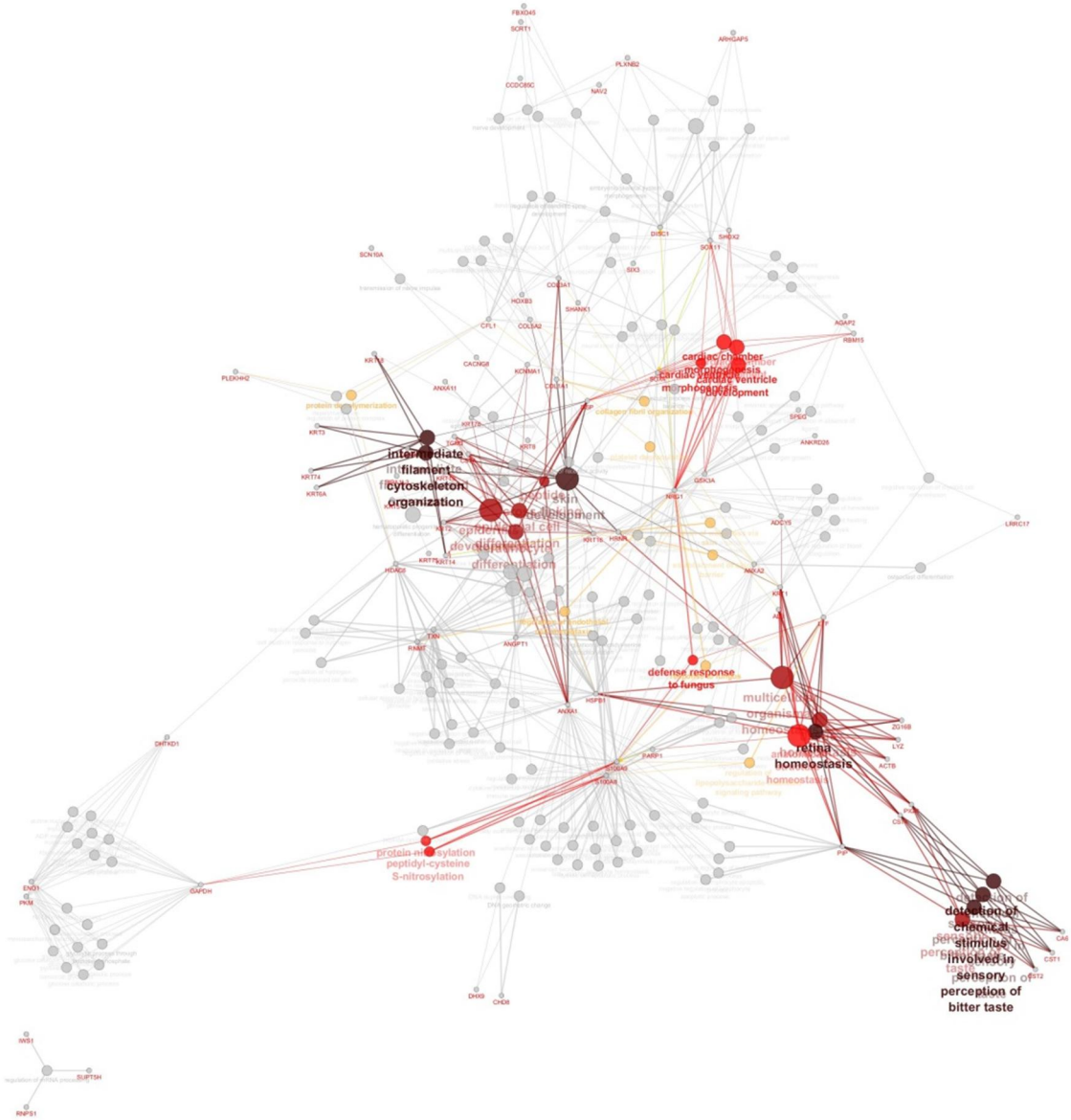

Fig.25 Protein interaction network generated with Cytoscape showing the most significant biological processes related to the identified proteins. The size of the rings indicates the number of proteins associate to each pathway, while different colors indicate the significance for each biological process represented (red: $p < 0.05$; red-purple: $p < 0.005$; brown: $p < 0.0005$).

Fig.26 shows the identified proteins evidencing the distribution among the three angioedema groups. Each color identifies a specific angioedema group: red, C1-INH-HAE; blue, FXII-HAE; green, InH-AAE.

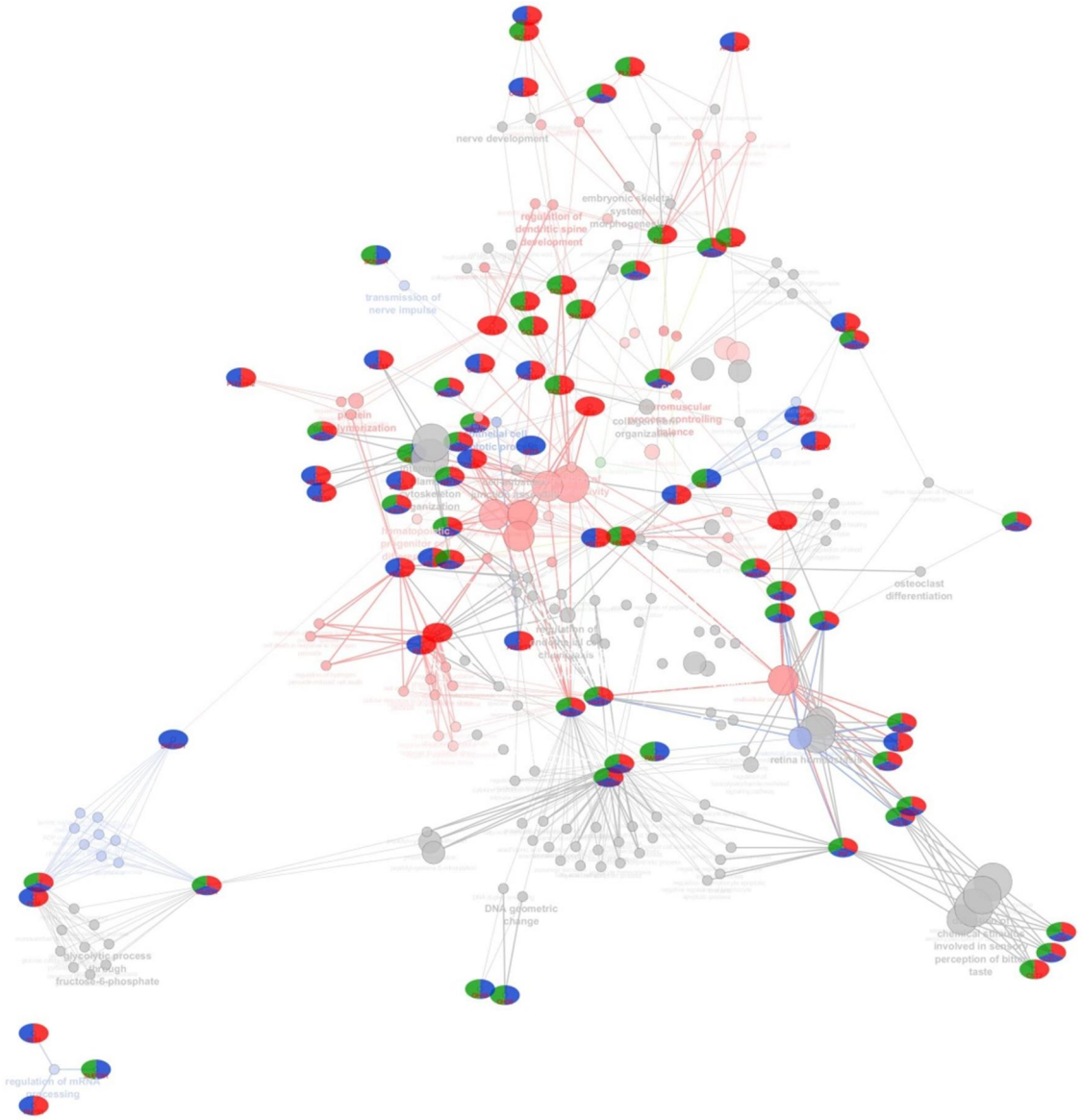

Fig.26

Quantification of proteins characterized by nano-HPLC-ESI-MS experiments and statistical analysis

Proteins which displayed significantly different abundance between pairs of the hereditary angioedema groups (log2 ratio between the median emPAI values of a specific protein in two groups lower than -0.7 or higher than +0.7) are shown in Fig.27. From the figure it is evident that 28 proteins showed a significant different level in C1-INH-HAE group with respect to InH-AAE groups, being 10 more abundant in the C1-INH-HAE group, and 18 in the Inh-AAE group (Fig. 27A). Conversely, 11 proteins were significantly more abundant and 22 less abundant in FXII-HAE patients with respect to InH-AAE (Fig.27B). C1-

INH-HAE group showed 18 proteins less abundant and 18 more abundant with respect to FXII-HAE group (Fig.27C).

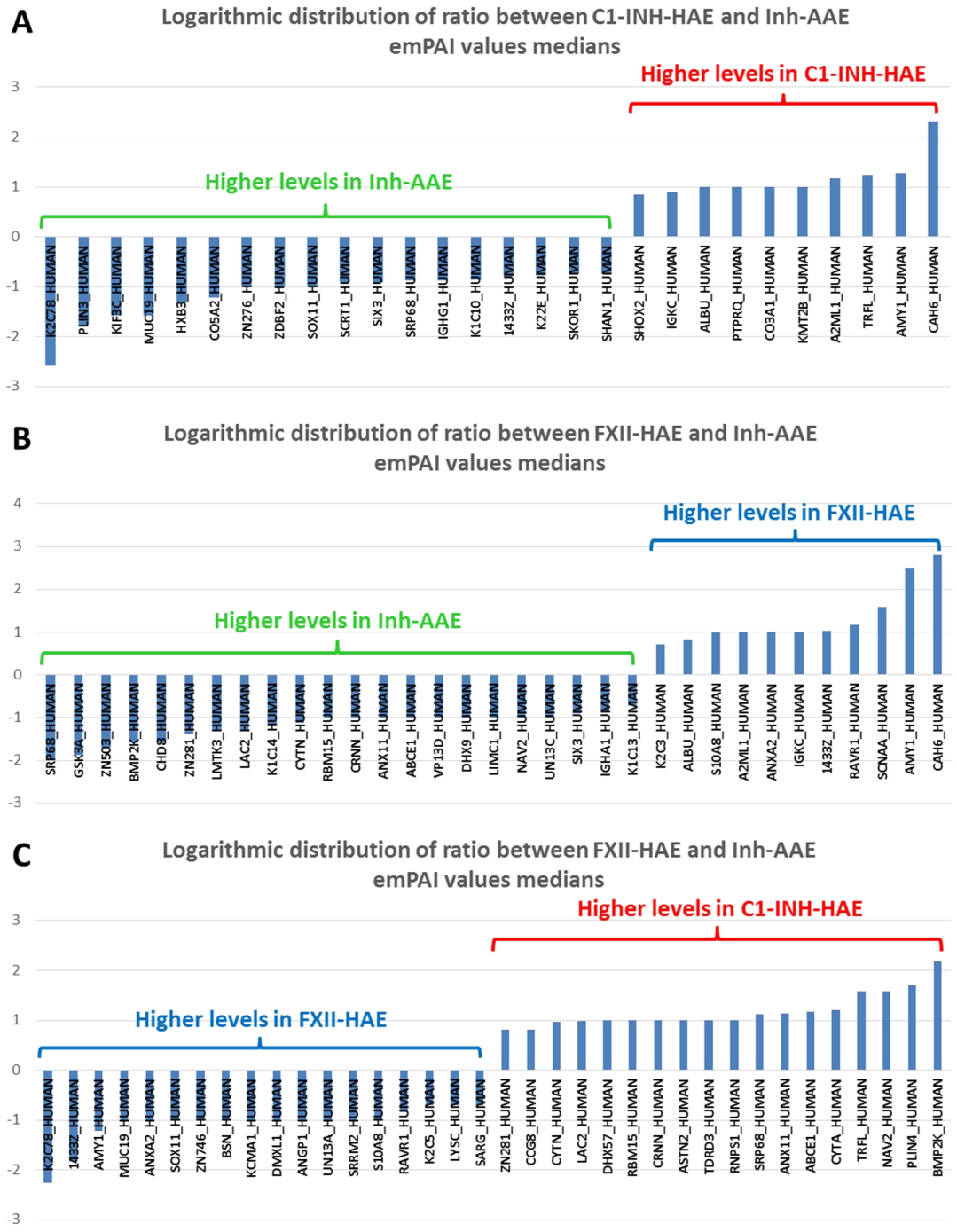

Fig.27 Base two logarithmic distribution of the emPAI values ratio calculated between pairs of angioedema groups. Comparison between C1-INH-HAE and Inh-AAE (panel A); FXII-HAE and Inh-AAE (panel B) and C1-INH-HAE and FXII-HAE (panel C).

In C1-INH-HAE group albumin (ALBU), amylase (AMY1) and carbonic anhydrase 6 (CAH6) were increased with respect to InH-AAE group (Fig.27A), while higher levels of cystatin A (CYTA) and salivary cystatin SN (CYTN) were observed with respect to FXII-HAE (Fig.27C). Moreover, lactotransferrin (TRFL) showed higher abundance with respect to both FXII-HAE (Fig.27C) and InH-AAE (Fig.27A) groups. FXII-HAE group displayed increased levels of albumin and amylase than InH-AAE group (Fig.27B). This last protein resulted more abundant with respect to C1-INH-HAE group too (Fig.27C). Differences between these two groups were observed also for lysozyme (LYSC) and angiopoietin-1 (ANGP1), which showed higher levels in FXII-HAE group (Fig.27C). In FXII-HAE, S100A8 protein (S10A8), was increased with respect to the other two angioedema groups (Fig.27B-C). Similarly to C1-INH-HAE group, also in InH-AAE higher levels of salivary cystatin SN with respect to FXII-HAE group were observed (Fig.27B), while the signal recognition particle 68 kDa (SRP68) was increased with respect to both C1-INH-HAE (Fig.27A) and FXII-HAE (Fig.27B) groups.

Prediction of the proteases acting in saliva by the characterization of the salivary peptidome in the different groups

The acidic soluble fraction of saliva from a subgroup of 10 patients (3 C1-INH-HAE in basal phase, 1 in acute state and 1 in both cases; 2 FXII-HAE in basal condition, 1 in acute phase and 1 in both situations, and 1 Inh-AAE in basal phase) and 12 healthy controls were selected among the whole number of samples under study. The low-molecular-weight fraction (MW < 50 kDa) of each sample was analyzed in duplicate by *top-down* nano-HPLC-ESI-MS and MS/MS experiments. A total of 360 peptides, of which 246 different, were identified with confidence into the three angioedema groups and the control group, and their distribution can be observed in Fig.28. Only two peptides were common to all groups, belonging to proteins involved in the interaction with cytoskeletal structures, while no specific peptides were shared among the three angioedema groups with respect to controls. Thus, most of the identified peptides were exclusive for each group: 69 for C1-INH-HAE, 56 for FXII-HAE, 7 for InH-AAE and 89 for controls.

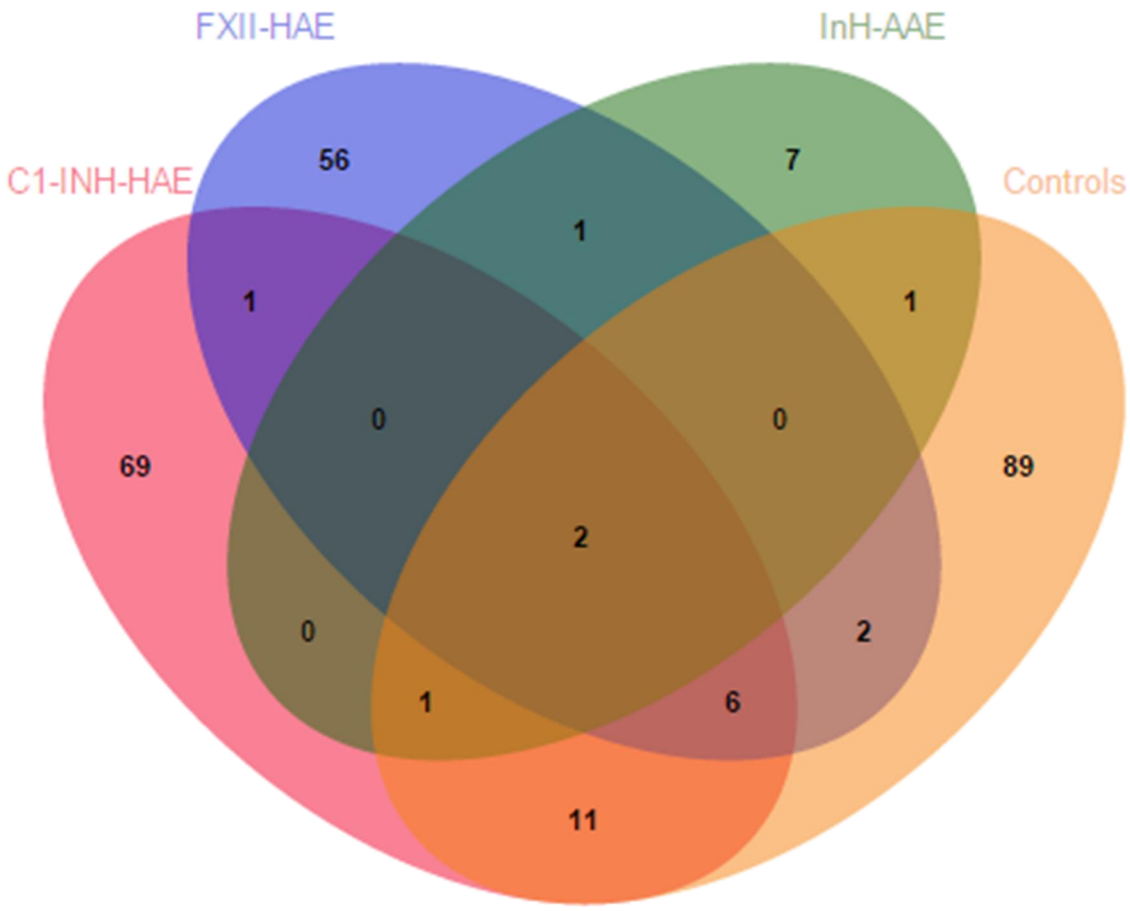

Fig.28 Venn diagram representing the distribution of identified different peptides per group (C1-INH-HAE, red; FXII-HAE, blue; InH-AAE, green; and controls, orange).

The list of the characterized peptides in each group was used to perform the *in silico* prediction of the proteases responsible for the naturally occurring peptidome, and thus to evidence possible differences among the three angioedema patient groups and the healthy controls. Fig.29-31 shows the percentage of peptides generated by the activity of different proteolytic enzymes predicted by Proteasix. From Fig.29 it is evident that different cathepsins (CTSB, CTSL1 and CTSS), several matrix metalloproteinases (MMPs) and the signal peptidase complex catalytic subunit (SEC11C) showed higher proteolytic activity in all the angioedema groups with respect to the healthy controls. On the other hand, the activity of meprin A (MEP1A and MEP1B) resulted higher in controls. However, it should be outlined that the robustness of these data is limited due to the small number of samples analysed, i.e. only one in the case of InH-AAE group. Moreover, the Proteasix software was

employed to evidence possible different proteolytic activity in saliva collected from C1-INH-HAE (Fig.30) and FXII-HAE (Fig.31) patients during the basal phase of the disease and the acute attack. The results highlighted increased proteolytic activities for different proteases in salivary samples collected during an acute attack as compared to both the basal phase and the control samples. Among them, matrix metalloproteinases (mainly MMP9) showed the most evident differences in both C1-INH-HAE (Fig.30) and FXII-HAE (Fig.31) groups. The disintegrin and metalloproteinase domain-containing protein 17 (ADAM17) showed higher activity in acute samples of both angioedema groups with respect to basal samples and controls (Fig.30 and Fig.31). Conversely, inferior activity of MEP1A and kallikreins (KLK6 and KLK4 in C1-INH-HAE and FXII-HAE groups, respectively) was observed in patients, especially in their acute phase samples, as shown in Fig.30 and Fig.31). In FXII-HAE group, cathepsins D (CATD) and granzyme B (GZMB) displayed a decreased proteolytic action (Fig.31).

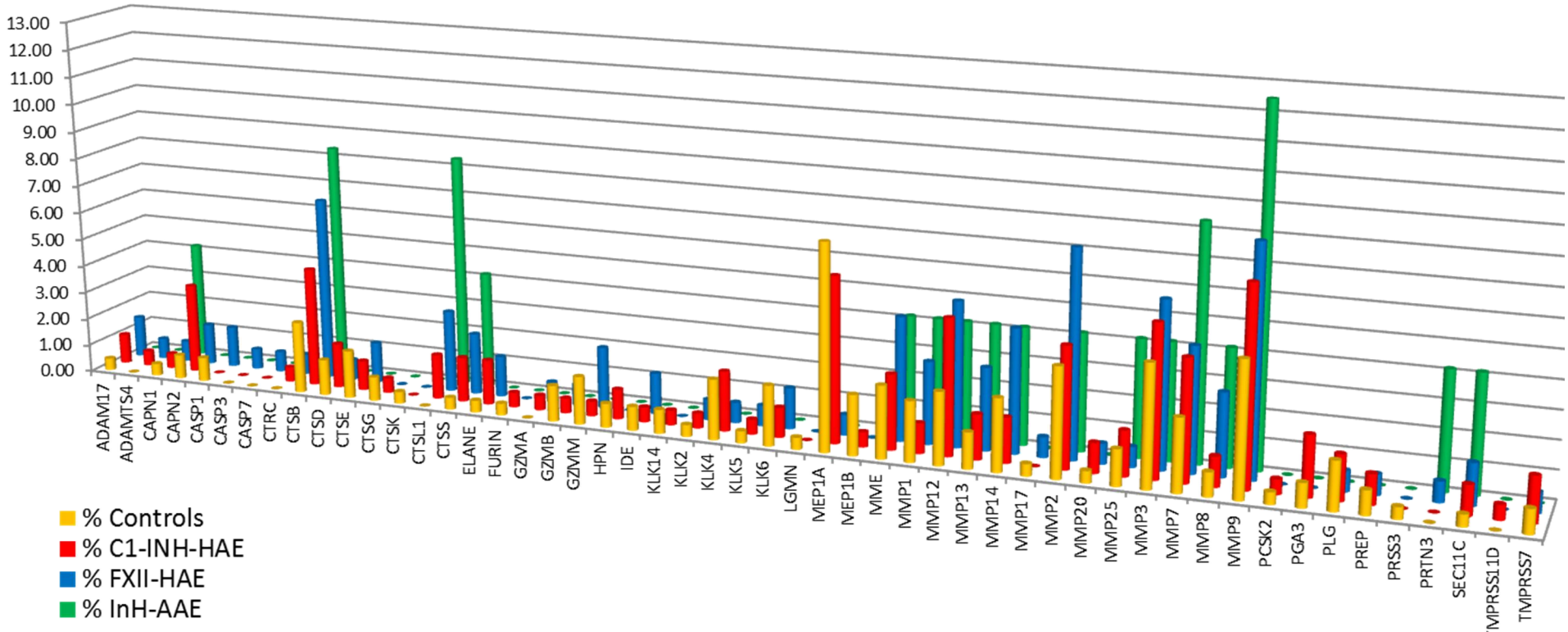

Fig.29 Percentage of peptides generated by the proteolytic activity of proteases predicted by Proteasix in the three groups of patients (C1-INH-HAE, red; FXII-HAE, light blue; Inh-AAE, green) and in the control group (yellow).

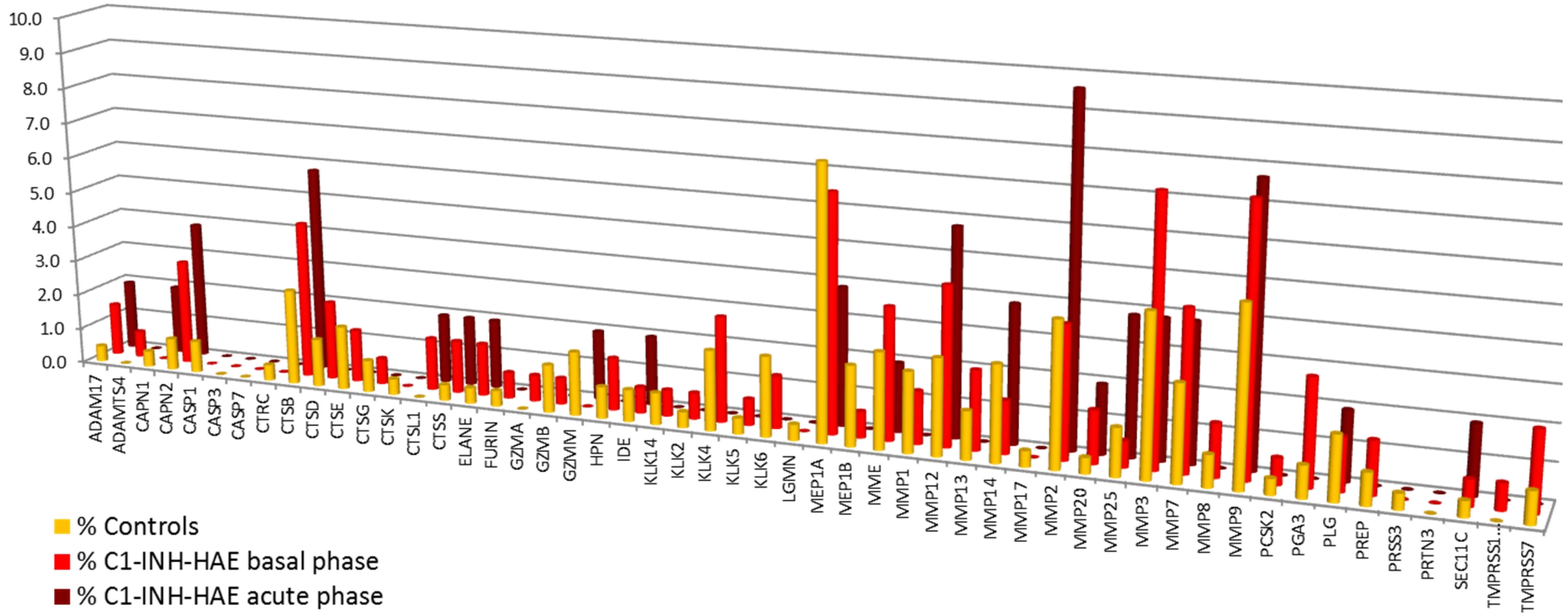

Fig.30 Percentage of peptides generated by the proteolytic activity of several proteases predicted by Proteasix in C1-INH-HAE basal phase (red), acute phase (brown) and control group (yellow).

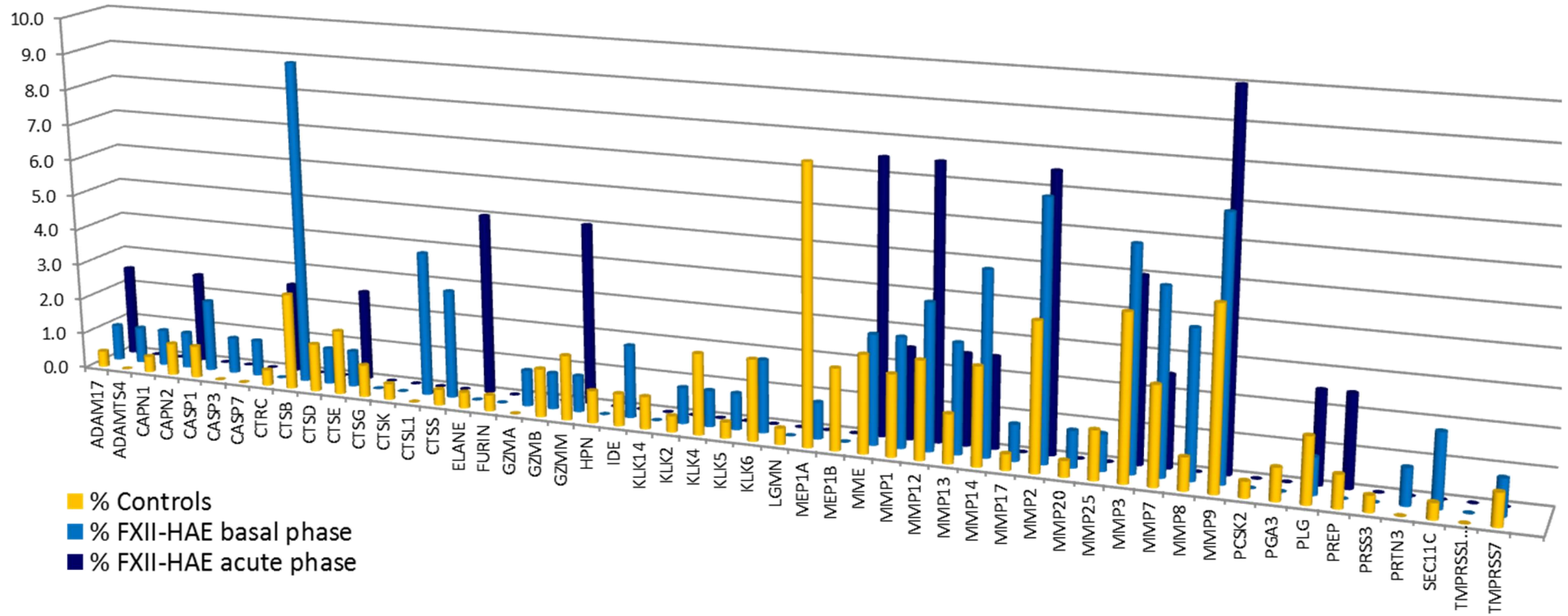

Fig.31 Percentage of peptides generated by the proteolytic activity of several proteases predicted by Proteasix in C1-INH-HAE basal phase (light blue), acute phase (blue) and control group (yellow).

Discussion

- **Wilson's disease**

One relevant result obtained by Wilson's disease saliva investigation concerns the *in vivo* characterization of several oxidized derivatives of S100A8, and S100A9. The two proteins showed different patterns of oxidation, in particular at the cysteine residue 42. In fact, while S100A8 was detected with C42 both reduced and oxidized, S100A9(L) was never detected in the reduced form. Moreover, S100A8 showed a major proneness with respect to S100A9(L) to the irreversible cysteine oxidation, and sulfonic or sulfinic acid were its main oxidized derivatives. On the other hand, S100A9 was detected principally glutathionylated and in a minor extent cysteinylated or in the dimeric form. S100A8 and S100A9 are the most abundant neutrophil proteins (about 45% of cytosolic proteins), constitutively expressed also by myeloid cells, platelets, and osteoclasts (Edgeworth, 1991; Goyette, 2011) and strongly induced in several cell types (i.e. macrophages, monocytes, keratinocytes, fibroblasts) during acute and chronic inflammatory status and in oxidative stress conditions (Goyette, 2011; Carlsson, 2005). Interestingly, it has been reported that following liver damages, upregulated S100A8 and S100A9 induce neutrophils mobilization (Moles, 2014; Wiechert, 2012). In activated granulocytes and macrophages, S100A8 and S100A9 are involved in the activation of NADPH oxidase 2 (Schenten, 2011; Doussiere, 2002), and thus contribute to the generation of reactive oxygen/nitrogen/chlorine species (ROS/RNS/RCS) with a subsequent progression and exacerbation of the inflammatory status. In addition to excess of unbound copper, different factors can be responsible for the redox unbalance in Wilson's disease. In fact, Hussain et al. observed an increased expression of the inducible nitric oxide synthase (iNOS) (Hussain, 2000), while Ogihara et al. demonstrated a decreased level of plasma antioxidants (Ogihara, 1995). Furthermore, the decreased concentration of serum peroxiredoxin 2 isoform b was demonstrated in asymptomatic, early-stage patients (Park, 2009). Thus, the oxidative stress condition occurring in Wilson's disease could be at the basis of

the high concentration of S100A8 and S100A9 proteoforms observed in our patients, and the up regulation of S100A8 should be responsible for the high levels of its oxidized forms, which however did not differ in percentage between patients and healthy controls. S100A8 in the form of sulfonic, or sulfinic acid, or oxidized at M1, M78 and W54 residues, has been already observed *in vitro*, by treating recombinant S100A8 with HOCl, as well as *in vivo* in human asthmatic sputum (Gomes, 2013), while the contemporaneous oxidation of C42 and the methionine residues has not been previously observed. The present study not only highlighted that more oxidative modifications can co-occur in S100A8, but also showed that the hyper-oxidized derivatives represented the major proteoforms of S100A8 in saliva of both patients and controls. Cysteine oxidation to sulfinic and sulfonic acid is generally considered an irreversible modification, which damages the protein. However, the enzymatic reversion of the sulfinic acid to the thiol form has been also observed (Biteau, 2003; Budanov, 2004). Reversible cysteine oxidation, such as glutathionylation, cysteinylation, and dimerization, can be involved in a variety of redox signaling/regulation events, and in the protection of critical cysteines from irreversible oxidative damages (Martínez-Ruiz, 2011; Hill, 2012; Lindahl, 2011). For instance, it has been reported that reversible cysteine oxidation of S100A8 and S100A9 can modulate their activity switching it from pro- to anti-inflammatory (Goyette, 2011; Gomes, 2013; Sroussi, 2012). In a previous study, it has been demonstrated *in vitro* that both S100A9 and S100A8 can undergo glutathionylation via GSSG or GSNO intermediates, but only S100A9-SSG was detected *in vivo* in activated neutrophils (Lim, 2010). Thus, this is the first study which detected S100A8-SSG *in vivo*. Glutathionylation of S100A9 alters its capacity to form complexes with S100A8 (calprotectin), to bind endothelial cells, and limits neutrophil migration in inflammatory lesions. It has been suggested that this modification protects the protein from its oxidation to higher oligomers (Lim, 2010). The great sensitivity to oxidation of S100A8 and S100A9 is in agreement with the detection in saliva of Wilson's disease patients of disulfide-linked homo-S100A9(L) and hetero-S100A8/A9(L) dimers. The S100A9(L)-SSdimer was previously detected in the activated neutrophils (Lim,

2010), and similar modifications of human S100A8, and murin S100A8 and A9 were observed *in vitro* in the presence of HOCl, Cu2+ and/or H_2O_2 at low concentration (Kumar, 2001; Harrison, 1999). It has been reported that S100A8 disulfide-linked dimers do not exhibit chemotactic action (Lim, 2009).

AVAD and ASVD peptides were found at high concentration in almost all patients, while in 11/32 controls they were undetected. pIgR, a type I transmembrane glycoprotein playing the main role in the adaptive immune response on mucosal surfaces (Asano, 2011; Kaetzel, 2005), transports polymeric IgA across mucosal epithelial cells. It is upregulated by pro-inflammatory cytokines, hormones and microbial factors, through a signaling pathway involving toll-like receptors 3 and 4 (Kaetzel, 2005). A proteolytic cleavage occurring in the glycosylated extracellular portion of pIgR generates the secretory component (19-603 residues), which has been detected also in human saliva (Ramachandran, 2006). The cleavage occurs by action of unknown proteases, probably released by activated neutrophils (Kaetzel, 2005), and the highly conserved sequence 602-613 (PRLFAEEKAVAD) is believed to be the cleavage signal (Asano, 2011). The AVAD peptide originates by a cleavage occurring in this region at the level of K609, and the ASVD peptide derives from AVAD by the trypsin-like cleavage at R622. The cleavage releasing the C-terminal glycine from both fragments could be made by several proteases, including cathepsins and matrix metallopeptidases. AVAD and ASVD peptides do not derive from the secretory component, and have a sequence partially overlapped to the transmembrane portion (639-661) of pIgR. Thus, they should originate by degradation of pIgR after its release from disrupted cell membranes. The increased levels of the AVAD and ASVD observed in our patients may be a consequence of an increased disruption of cell membranes, due to the high production of ROS characteristic of the pathology. However, it cannot be excluded that pIgR itself may be increased in Wilson's disease patients, even though conflicting data about pIgR levels and liver diseases have been reported. For instance, high levels of pIgR have been associated to the invasion and metastasis of the hepatocellular carcinoma (Ai, 2011), while a

down regulation of pIgR in intestinal mucosa of animal models subsequent to acute liver necrosis has been observed (Fu, 2012).

Wilson's disease saliva was characterized by the higher concentration of α-defensin 4 and α-defensin 2 with respect to controls. Unexpectedly, none variation of α-defensin 1 and 3 levels was observed, even if α-defensin 2 derives from their proteolytic cleavage (Valore, 1992). Besides antimicrobial activity, α-defensins can modulate the inflammatory responses through regulation of cytokine production (Chaly, 2000; Lehrer, 2012). The property of corticostatin to inhibit cortisol production (Singh, 1988) is intriguing, since it has been demonstrated that in humans and in other mammals glucocorticoids induce the metallothioneins, which are important intracellular copper storages (Gedamu, 1993; Miles, 2000). High levels of α-defensin 4, therefore, can indirectly down-regulate the metallothioneins by contributing to increase the free copper, a feature not negligible in Wilson's disease. From this point of view, it could be interesting to test metallothionein levels in a future study.

- **SAPHO syndrome**

SAPHO patients were characterized by low salivary levels of acidic PRPs. It is recognized that these proteins play an important role in the homeostasis of the calcium phosphate in the oral cavity, in the creation of a protective environment for the teeth, and in the modulation of the bacteria adhesion to the oral surfaces (Bennick, 1983b). Moreover, it has been shown that PRPs are cleaved by *exo*- and *endo*-proteases generating several small peptides (Messana, 2008b; Hardt, 2005a; Sun, 2009; Helmerhorst, 2009) and it is interesting to outline the detection of fragments containing the GGRPQ *C*-terminal sequence among them (Helmerhorst, 2009; Drobni, 2006). Indeed, Huang *et al.* demonstrated that the selective binding of the GPPPQGGRPQ peptide inhibits colonization and growth of *P. acnes*, suggesting that these salivary peptides can participate to the innate immune system through the inhibition of specific microorganisms (Huang, 2008). Interestingly, GPPPQGGRPQ peptide matches with the 148-157 sequence of PRP-1 and with the 26-35 sequence of the P-C peptide. Therefore, the lower concentration of PRP-1, P-C peptide, and PRP-3 (generated by PRP-1

proteolysis before secretion) in SAPHO patients may result in a reduced abundance of the active fragment GPPPQGGRPQ and an impaired resistance to colonization of *P. acnes*, which has been variably associated with SAPHO syndrome (Hayem, 2013). The isolation of *P. acnes* in SAPHO subjects has been linked to the hypothesis of this syndrome being triggered by a low-virulence pathogen in the initial phase, then perpetuated by a subsequent inflammatory process (Hayem, 2007).

Also salivary cystatins can suppress some viral infections (Ruzindana-Umunyana, 2001; Gu, 1995) and cystatin SN plays a key role in controlling the proteolytic activity of the parasite *Trypanosoma cruzi* (Baron, 1999). It exhibits antifungal activity against *Candida albicans*, and candidiasis onset in autoimmune polyendocrine syndrome type 1 has been correlated with cystatin SN deficiency (Lindh, 2013). The low abundance of cystatin S1 and SN in SAPHO patients, demonstrated in the present study, may reflect in a reduced protection against microorganism infections. SAPHO patients with a shorter disease duration showed higher levels of cystatin SN than those with a longer disease duration, suggesting that cystatin SN production might decrease over time, during the chronic course of the disease.

Another class of salivary proteins, playing a protective role in the mouth, is represented by histatins, which show inhibitory effect on several oral bacteria (White, 2009). Moreover, histatin-derived peptides have been demonstrated to be active against various microbes, such as *Propionibacterium acnes* (Oppenheim, 1999). On the basis of the present data we may also speculate that histatins could play a role in the down-regulation of pro-inflammatory mediator production in SAPHO syndrome, characterized by increased concentrations of IL-8, IL-18, and TNF and enhanced TH-17 lymphocyte response (Hurtado-Nedelec, 2008; Borgwardt, 2014). Indeed, histatin 3 has been observed to bind the heat shock cognate protein 70 (HSC70), blocking its interaction with the toll-like receptors TLR2 and TLR4, and thus suppressing the production of cytokines IL-6 and IL-8 in gingival fibroblasts (Firinu, 2014a). The same cytokine suppression was also demonstrated for histatin 5, both in gingival

fibroblasts and in dendritic cells stimulated by *P. gingivalis* (Imamura, 2014; Imatani, 2000).

The anti-inflammatory role of the histatins is also supported by the negative correlation found between the level of histatin 3 and ESR, the latter being a marker of inflammation. However, a positive correlation between salivary histatins and the serum CRP level, which was within the normal range in most of the SAPHO patients, was observed in this study. This result, is apparently in contrast with the anti-inflammatory role of histatins previously discussed, highlighting that further investigations are necessary to clarify whether and through which mechanisms histatins could be involved in the inflammatory pathways.

CRP negatively correlated also with salivary S100A12. This negative correlation is in disagreement with the results reported by Pradeep *et al.* demonstrating that S100A12 positively correlated with CRP in gingival crevicular fluid and serum from patients with chronic periodontitis and type II diabetes (Pradeep, 2014). However, the high abundance of S100A12 in SAPHO patients suggests a probable involvement of this protein in the inflammatory status typical of the disease. Antimicrobial and antifungal activities have been attributed to S100A12 (Cole, 2001), it has been associated with chronic inflammatory conditions (Foell, 2003a) such as cystic fibrosis (Lorenz, 2008), rheumatoid arthritis (Foell, 2003b), psoriasis (Foell, 2003b), and autoinflammatory diseases (Kessel, 2013). In particular, S100A12 has been found up-regulated in inflamed synovial tissue of patients affected by rheumatoid and psoriatic arthritis (Lorenz, 2008), and in saliva of HIV-1 positive patients with dysregulation of neutrophil response (Zhang, 2013). Interestingly, S100A12 has been shown to be a sensitive parameter to detect subclinical inflammation in familial Mediterranean fever (Kallinich, 2010). Its inflammatory properties are related to the chemoattractant capacity for monocytes and mast cells (Yan, 2008), to the ability to operate as a Damage Associated Molecular Pattern (DAMP) molecule (Foell, 2007), and to induce NFκB mediated expression of pro-inflammatory cytokines IL-1β, IL-18, IL-6 and TNF-α (Hofmann, 1999). The binding of S100A12 to TLR-4 and RAGE receptors on monocytes and granulocytes has been demonstrated to amplify

and perpetuate inflammation (Schmidt, 2001). The antagonization of IL-1β or TNF-α induced by these events represents an effective treatment for SAPHO syndrome (Firinu, 2014b).

- **Hereditary angioedema**

Increased levels of cystatin B, in particular of its S-glutathionylated derivative, were observed in the acidic soluble fraction of saliva from C1-INH-HAE and InH-AAE patients. This protein belongs to the cystatin family, inhibitors of cysteine proteases, mostly by interacting with the plant derived papain and the mammalian cathepsins B, L, S and H (Abrahamson, 1986; Turk, 1991). Cystatin B, that prevents the activity of cathepsin L (Ceru, 2010), shows mainly an intracellular localization (Abrahamson, 1986; Maher, 2014a) but it has been also detected in various body fluids and associated with neurodegenerative diseases, cancer and inflammation. For example, Cystatin B gene mutations have been reported to be responsible for the progressive myoclonus epilepsy of the Unverricht-Lunfborg type (EPM1), a degenerative disease of the central nervous system (Pennacchio, 1996; Lalioti, 1997; Bespalova, 1997; Joensuu, 2007), high–levels of the protein were observed in different malignant tumors (Feldman, 2009; Zhang, 2011), and in activated macrophages (Maher, 2014b; Kopitar-Jerala, 2015). In immune cells, cystatins have been reported to participate in the release of nitric oxide, phagocytosis, and expression of cytokines (Kopitar-Jerala, 2006; Maher, 2014a, Kopitar-Jerala, 2015), although its role is debatable. In fact, higher expression of the inducible nitric oxide synthase together with increased amounts of NO were observed in IFN-γ and LPS-activated cystatin B-deficient bone marrow-derived macrophages by the Kopitar-Jerala group (Kopitar-Jerala, 2015). Conversely, various studies demonstrated the ability of cystatin B and others members of cystatin families 2 and 3 (chicken and filarial cystatins, rat T-kininogen) to stimulate, in a concentration-dependent manner, the release of NO from IFN-γ activated murine macrophages (Verdot, 1996; Hartmann, 2002). Furthermore, it was demonstrated that the high and rapid NO production by the inducible NO synthase was not related to the inhibitory properties of cystatins (Verdot, 1996;

Hartmann, 2002). The role of cystatin B in NO production is interesting as this molecule not only directly acts against the pathogen infection (James, 1995), but also induces vasodilatation and inhibits platelet aggregation (Riddell, 1999). Thus, it might be hypothesized that the increased levels of salivary cystatin B in our patients could be connected to an enhanced NO release, participating, together with bradykinin, to the vasodilatation and the higher vascular permeability responsible for hereditary angioedema.

Furthermore, recent studies demonstrated that the C1-INH protein plays anti-inflammatory functions, not related to its protease activity, preventing the binding of LPS to macrophages and, consequently, preventing their activation (Liu, 2003). Therefore, the reduced activity of the C1 inhibitor in C1-INH patients may results in an impaired macrophage activation, with the production of higher levels of cystatin B and in the increased stimulation of nitric oxide formation.

The high proteolytic activity of several matrix metalloproteinases observed in all the groups of angioedema patients, which increases during an acute attack of edema, is in agreement with the strong degradation of the extracellular matrix proteins (i.e. collagen, elastin, gelatin and fibronectin), which is a typical feature of inflammation, enhancing leukocyte migration and the vascular leak responsible for edema formation. Active matrix metalloproteinases are unstable and can cleave and inactivate themselves. This autolytic process is modulated and prevented by the tissue inhibitors of matrix metalloproteinases, TIMPs (Ochieng, 2010). Curiously, different cystatin members are able to stabilize metalloproteinases, protecting them from their own inactivation, without interfere with their enzymatic activity against their natural substrates (Ray, 2003; Ochieng, 2010). Therefore, the higher levels of cystatin B measured in our patients may contribute to MMPs stability which can reflect in their increased proteolytic activity observed in saliva samples of angioedema patients.

Conversely, the higher activity of cathepsins in saliva of our angioedema patients, apparently contrasts with the increased levels of cystatin B, which belong to the family of their classical inhibitors.

Among the proteins characterized in the acidic insoluble fraction and showing significant different levels in the three angioedema groups, several proteins involved in inflammation are represented. Cystatin A, higher in C1-INH-HAE than FXII-HAE group, is involved in the skin development and cellular proliferation. It has been observed in psoriatic plaques of the psoriasis vulgaris, an inflammatory disease of the skin (Bowcock, 2001), and polymorphisms of its gene have been associated with atopic dermatitis (Cork, 2006; Vasilopoulos, 2007) and exfoliative ichthyosis (Blaydon, 2011). On the other hand, S100A8 protein, strongly expressed in activated macrophages, monocytes, keratinocytes and fibroblasts during inflammation and oxidative stress (Goyette, 2011; Carlsson, 2005), was over-expressed in FXII-HAE patients. Other proteins, which showed different levels among angioedema groups, play anti-microbial activities, i.e. cystatin SN (higher in C1-INH-HAE and InH-AAE groups), lactotransferrin (increased in C1-INH-HAE with respect to the other patients) and lysozyme (more abundant in FXII-HAE group). The bioinformatic analysis performed on proteins and peptides identified in the acidic insoluble fractions of saliva is in agreement with the significant activation of pathways involved in the organism response against microorganisms, in the development of the skin and in the extracellular matrix organization. Moreover, it is interesting to observe the high S-nitrosylation activity associated to the glyceraldehyde-3-phosphate dehydrogenase (GADPH), which could be connected with a massive production of NO.

Conclusions

Wilson's disease, SAPHO syndrome and hereditary angioedema are characterized by a wide spectrum of clinical manifestations, involving different organs and apparatus, which can be extremely dangerous and even lead to the death of the patient if not immediately recognized and treated. The absence of a confident method of diagnosis and the overlap of some clinical symptoms with other similar diseases, implicate difficulty in diagnosis, which represents a challenge for clinicians; thus, the characterization and validation of new disease-specific biomarkers urges.

Saliva investigations interestingly demonstrated that proteomic and peptidomic variations could be related not only to oral but also to systemic disorders (Grigoriev, 2003; Sayer, 2004; Vitorino, 2006; Peluso, 2007; Ohshiro, 2007; Castagnola, 2008; Ito, 2008; Hu, 2008; Wu, 2009; Rao, 2009; Cabras, 2010; Cabras, 2013; Caseiro, 2013), suggesting a possible employment of this body fluid for diagnostic and prognostic purposes (Castagnola, 2011b; Caseiro, 2013).

Indeed, the results obtained in this study devoted to the characterization of the salivary proteome of Wilson's disease, SAPHO syndrome and Hereditary angioedema patients highlighted that saliva composition is affected by the pathology and reflects the typical features of the disorders. In particular, in Wilson's disease patients the salivary proteome, characterized by high levels of S100A8, S100A9 and their oxidized isoforms, reflected the intense oxidative stress and the activation of the inflammatory processes caused by the accumulation of copper in the organism. In addition, the high abundance of corticostatin suggests new mechanisms involved in the augmented levels of free copper. Of particular interest is the increased concentration of pIgR fragments, which deserves further evaluation as possible index of hepatic damage. The salivary proteome of patients affected by SAPHO syndrome evidenced a significant reduction of cystatins, histatins, and aPRPs, which may be related to a reduced ability to contrast colonization from bacteria, such as *P. acnes*, often associated as possible exogenous trigger of the disease. Moreover, the high abundance of S100A12 in saliva of SAPHO patients encourage further studies to

confirm its association with the disease and to evaluate its possible application as salivary biomarker. The activation of the immune system is also reflected in saliva from all angioedema patients, by the increased abundance of several peptides generated by metalloproteinases. The high level of cystatin B in C1-INH-HAE patients, could be related to the macrophage activation caused by a reduced C1-INH activity, and the similar cystatin B levels observed in C1-INH-HAE and InH-AAE with respect to both FXII-HAE and control groups may suggest that the acquired angioedema involves mechanisms of the immune system more similar to the angioedema caused by *SERPING1* mutations than angioedema related to *FXII* gene mutations.

Eventhough the small number of the subjects under study, due to the rarity of the investigated pathologies, represents a limitation for the statistical power of the results, it should be outlined that the proteomic and peptidomic modifications with respect to controls observed in saliva of patients were distinctive for each disease, also reflecting the typical derangement of the disorder. Further studies on a larger cohort of patients to confirm the variations of the salivary proteome/peptidome observed and their disease-specificity as well as validation of the results by orthogonal methods will allow in the future to establish the actual applicability and the diagnostic power of a salivary test for these pathologies.

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2) Cabras T, **Sanna M**, Manconi B, Fanni D, Demelia L, Sorbello O, Iavarone F, Castagnola M, Faa G, Messana I.
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