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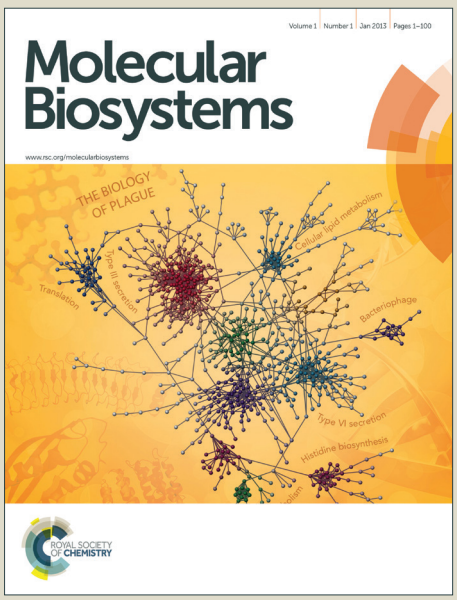

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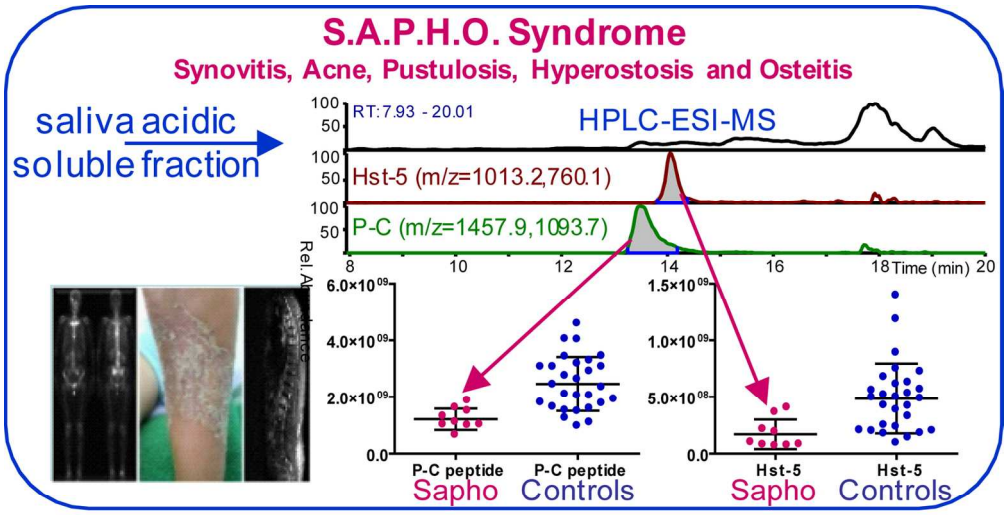

Salivary proteomic investigation in SAPHO syndrome reveals significant variations of cystatins, histatins, aPRP, S100A12, suggesting their potential role as biomarkers.

72x49mm (600 x 600 DPI)

The Salivary Proteome Profile in Patients Affected by SAPHO Syndrome characterized by a top-down RP-HPLC-ESI-MS platform.

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Abstract: SAPHO syndrome is a rare and often unrecognized disease with prominent inflammatory cutaneous and articular symptoms characterized by musculoskeletal manifestations (synovitis, hyperostosis, osteomyelitis) associated with dermatological conditions (severe acne and pustulosis). The acidic soluble fraction of whole saliva from 10 adult women affected by SAPHO syndrome and from a group of 28 healthy women was analysed by RP-HPLC-ESI-MS with the aim to discover salivary biomarkers of the disorder. The levels of the oral proteins and peptides were correlated with clinical data. The following proteins showed a significant decreased concentration in saliva of SAPHO subjects with respect to controls: cystatin S1 and SN, histatins, the major acidic PRPs, P-C and P-B peptides. The cystatin SN abundance lowered according to the disease duration and histatins showed positive correlations with the C reactive protein. Statistical analysis performed excluding one patient with a different pattern of salivary proteins/peptides highlighted a positive relationship between cystatin S1, histatins 3, histatin 5, and neutrophil count. Moreover, histatin 3 correlated positively with the total white cell count and negatively with the erythrocyte sedimentation rate. Levels and frequency of S100A12 protein showed a trend to increase in SAPHO patients. The high expression of this pro-inflammatory protein is probably related to the inflammatory response and to the altered neutrophil responses to functional stimuli that characterize SAPHO syndrome suggesting a possible application as salivary biomarker.

Introduction

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.[1] SAPHO syndrome has been classified in the spondyloarthropathies (SpA) on the basis of clinicopathological features and similarity to psoriatic arthritis.[2] Recent evidences lead to consider this disorder within the spectrum of bone autoinflammatory diseases.[3,4] The aetiology of SAPHO is unclear, but probably recognizes genetic, immunologic and infectious mechanisms. The interaction between infectious agents (e.g. *Propionibacterium acnes*) and the immune system in a genetically predisposed subject may result in dysregulation of neutrophil responses and autoinflammation.[5,6] 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.[7] Due to its rarity (estimated prevalence <1/10.000), and features overlapping with other rheumatic and non-rheumatic disorders, SAPHO syndrome represents a diagnostic challenge for clinicians.[1] In particular, cutaneous manifestations may overlap with psoriasis or other neutrophilic dermatoses,[8] and bone manifestations with SpA and infectious osteitis. Up to date, no reliable biomarkers are available for this disease. Despite extensive bone and skin inflammation during exacerbations, the C reactive protein (CRP) and 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.[9] Therefore, it would be

of extreme importance the identification of new laboratory biomarkers for the diagnosis of the disease and monitoring its activity. At present, no data on salivary proteins in SAPHO syndrome are available. In this work, we assessed whether the immune derangement observed in SAPHO syndrome could be associated to quantitative variations of salivary proteins and peptides. The level of specific salivary proteins/peptides was correlated with clinical and laboratory parameters, in order to have suggestions on potential salivary biomarkers of the disease.

Results

1 Clinical data

Demographics, treatments and status of the disease at the time of the study are listed in Table 1. The main clinical, laboratory and findings are summarized in Table 2. 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 only without cutaneous manifestations, whereas bone manifestations were similar to those observed in the others. Mean disease duration was 10.75 years (± 8.5 SD) and patient #8 had the lowest (2.5 years). White blood cell (WBC) count (mean 8343/mm^3 ± 1980 SD) was normal in all patients, CRP (mean 0.61 mg/dl ± 0.66) was above normal range in 2 patients, and ESR (24 mm/hour ± 16.8 SD) in 4 patients, but both were not related to disease activity status.

2 HPLC-ESI-MS analysis

The following proteins and peptides, as well as several of their post-translationally modified derivatives (phosphorylated,acetylated, cysteinylated, glutathionylated, and methionine oxidized forms) were detected and quantified in saliva from 10 SAPHO and 28 control subjects: salivary cystatins, histatins, acidic proline-rich proteins (aPRPs), statherin, proline-rich peptide P-B, salivary α-defensins 1-4, cystatins A, B, C, β-thymosins 4 and 10, S100A12, , S100A8, S100A9 (short and long isoforms), and S100A7 (D27) proteins. These proteins and peptides have been already identified by us in several previous studies devoted to the characterization of the acidic soluble fraction of the human salivary proteome under physiological and pathological conditions [10-14]. Table 3 reports the Swiss-prot codes, experimental and theoretical average mass values and elution times only of the proteins/peptides and derivatives showing a variation of the level in the two groups under study. Supplemental Table S1 reports the same information for the unvaried proteins/peptides. The results of the statistical analysis performed by comparing the extracted ion current (XIC) peak areas of the proteins measured in all the SAPHO subjects and controls are summarized in Table 4. Statistical analysis was also performed by excluding from the SAPHO group the patient #8, who unlike the others never had skin manifestations (Table 5).

2.1 Salivary cystatins

Cystatin S1 showed a significant lower level in the SAPHO subjects with respect to healthy controls ($p = 0.04$) (Table 4). Statistical analysis performed by excluding the patient #8, confirmed this difference ($p = 0.02$), and also evidenced a significant lower concentration of cystatin SN ($p = 0.02$) (Table 5) in the SAPHO group. Figure 1 shows the XIC peak area distributions of the two proteins in the two groups, patient #8 excluded.

2.2 Histatins

Histatin 1 concentration (p = 0.007) was found deeply lower in saliva of SAPHO patients as compared with controls (Figure 2A), while the level of histatins 3, 5, and 6 was similar in the two groups (Table 4). 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 (Figure 2B-D), and the following p values were determined: 0.004, 0.0001 and 0.0009, for histatins 3, 5, and 6, respectively (Table 5).

2.3 aPRP

Statistical analysis evidenced significant lower levels of diphosphorylated PRP3 ($p < 0.0001$) and P-C peptide (p = 0.005) in saliva of SAPHO compared with the controls (Table 4). Also the levels of 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 5) in the two groups, and the p value for P-C peptide became lower than 0.0001 (Fig. 3A, Table 5).

2.4 P-B peptide

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 Figure 3B.

2.5 S100A12

S100A12 protein showed a higher frequency in the SAPHO group (6/10) with respect to the controls (6/28) (Table 4). 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 5.

3 Correlations between HPLC-ESI-MS data and clinical and laboratory parameters

The XIC peak areas of the salivary proteins/peptides 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, Figure 4A-D). Moreover, CRP showed a negative correlation with S100A12 ($p = 0.02$; $R = -0.7$, Figure 4E). The cystatin SN abundance decreased in relation to the disease duration ($p = 0.03$; $R = -0.7$, Figure 4F). 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 ($p = 0.04$, $R = -0.7$, Figure 5A), and positively with the total white cells count ($p = 0.04$, $R = 0.7$, Figure 5B). 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 (Figure 5C-D).

Discussion

The variety of skin and bone manifestations of SAPHO syndrome hinder a timely diagnosis and the prompt start of an adequate treatment[7]. Although several studies investigated the origin of the disease and the relationship with mandibular osteomyelitis, the etiology is still unknown.[15,16] The involvement of different salivary proteins and peptides in maintaining the mouth homeostasis, the protection against microbial infections, and activation of the immunity system has been highlighted in several previous studies. For instance, it is recognized that aPRPs, play an important role in the modulation of calcium phosphate action in the oral cavity, in the creation of

a protective environment for the teeth, and in the modulation of bacteria adhesion to the oral surfaces.[17] Moreover, it has been shown that PRPs are cleaved by exo- and endo-proteases generating several small peptides,[14,18-20] and it is interesting the detection of fragments containing the GGRPQ C-terminal sequence among them.[20,21] Indeed, Huang et al. demonstrated that the selective binding of GPPPQGGRPQ peptide inhibits colonization and growth of *P. acnes*, suggesting that salivary peptides can participate to the innate immune system through the inhibition of specific microorganisms.[22] GPPPQGGRPQ peptide matches with the 148-157 sequence of PRP-1 and with the 26-35 sequence of P-C peptide. Interestingly, we observed a lower concentration of PRP-1, as well as of P-C peptide, and PRP-3 (generated by PRP-1 proteolysis before secretion), in SAPHO patients compared with healthy controls. This 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.[23] 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.[24]

It has been shown that salivary cystatins can suppress some viral infections,[25,26] whereas their ability to inhibit bacterial cysteine proteases is debatable.[27-29] Among them, cystatin SN plays a key role in controlling the proteolytic activity of *Trypanosoma cruzi*.[30] It also exhibits antifungal activity against *Candida albicans,* and candidiasis onset in autoimmune polyendocrine syndrome type 1 has been correlated to cystatin SN deficiency.[31] It has been suggested that cystatins, released during inflammatory processes, may regulate the proteolytic activity from the host. For instance, cystatin SN and SA inhibit human lysosomal cathepsins implicated in the destruction of periodontal tissues.[32] Cystatin SA has been implicated in the induction of cytokines by human

gingival fibroblasts.[33] 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. In this respect, it should be underlined that patient #8 showed the lower disease duration and the major cystatin SN concentration.

Another class of salivary proteins, playing a protective role in the mouth, is represented by histatins, small cationic peptides rich in histidine.[34,35] Their action consists in the protection of the tooth structure,[36] anti-fungal activity,[37,38] inhibitory effect on several oral bacteria,[39] and wound healing.[40] Histatin-derived peptides have been demonstrated to be active against various microbes, such as *Propionibacterium acnes*.[41] 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.[5,45] Indeed, histatin 3 has been demonstrated 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.[42] The same cytokine suppression was also demonstrated for histatin 5, both in gingival fibroblasts and in dendritic cells stimulated by *P. gingivalis*.[43,44]

The anti-inflammatory role of the histatins is also supported by the negative correlation found between the level of histatin 3 and ESR, being the latter a marker of inflammation. However, a positive correlation between salivary histatins and serum CRP level, which was within the normal range in most of the SAPHO patients, was observed in this study. This result, in contrast with the anti-inflammatory role of histatins previously discussed, highlights 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.[46] However, the high abundance of S100A12 in SAPHO patients suggests a probable involvement of this protein in the inflammatory status typical of the disease. S100A12 is constitutively expressed by innate immune cells, primarily by activated granulocytes,[47] and induced in several cell types,[48] where it has been implicated in intracellular and extracellular functions, involving the calcium ions as second messenger.[49,50] Moreover, antimicrobial and antifungal activities have been attributed to S100A12,[51] it has been associated to chronic inflammatory conditions[52] such as cystic fibrosis,[53] rheumatoid arthritis,[54] psoriasis,[54] and autoinflammatory diseases.[55] In particular, S100A12 has been found up-regulated in inflamed synovial tissue of patients affected by rheumatoid and psoriatic arthritis[53] and in saliva of HIV-1 positive patients with dysregulation of neutrophil response.[56] Interestingly, S100A12 has been shown to be a sensitive parameter to detect subclinical inflammation in familial Mediterranean fever.[57] Its inflammatory properties are related to the chemoattractant capacity for monocytes and mast cells,[58] to the ability to operate as a Damage Associated Molecular Pattern (DAMP) molecule,[59] and to induce NFκB mediated expression of pro-inflammatory cytokines IL-1β, IL-18, IL-6 and TNF-α.[60] The binding of S100A12 to TLR-4 and RAGE receptors on monocytes and granulocytes has been demonstrated to amplify and perpetuate inflammation.[61] The antagonization of IL-1β or TNF-α induced by these events represents an effective treatment for SAPHO syndrome.[7]

Experimental

1 Study subjects

10 SAPHO patients (38.0 ± 11.1 years old) with a protracted disease course, fulfilling criteria of Benhamou et al.,[62] were consecutively enrolled from the Internal Medicine and Immunology outpatients clinic of Cagliari University. 28 female healthy (33.5 ± 10.3 years old) were enrolled as controls. The informed consent procedures are consistent with the latest stipulations established by the Declaration of Helsinki. Ethics Committee approval was obtained for this study.

2 Salivary samples

Unstimulated whole saliva was collected from patients and healthy controls using a soft plastic aspirator in ice bath. The donors did not eat or drink 2 hours before the collection, established between 10.00 and 12.00 a.m. Immediately after collection, each salivary simple was diluted in 1:1 v/v ratio with a 0.2% solution of 2,2,2-trifluoroacetic acid (TFA) containing 50 μM leuenkephalin, and the solution was centrifuged at 20000 g for 15 min (4°C). The acidic supernatant was separated from the precipitated and either immediately analyzed by HPLC-ESI-MS apparatus or stored at -80°C until the analysis. Leuenkephalin peptide allowed to control the accuracy of the dilution and thus to normalize salivary levels of proteins and peptides.

3 Blood samples

Blood lymphocytes subsets defined as “TH1” (CD4+IFNγ+), “TH17” (CD4+IFNγ- IL-17+), “TH1/TH17” (CD4+IFNγ+ IL-17+) were defined by analysis of intracellular cytokines production using flow-cytometry, using a protocol previously described.[42] WBC counts, serum CRP and ESR were determined by routine methods on the same day of saliva collection.

4 Reagents and instruments

All analytical grade common chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MI, USA). HPLC-ESI-IT-MS analyses were performed by a Surveyor HPLC system connected by a T splitter to a photo diode-array detector and to an LCQ Advantage mass spectrometer (ThermoFisher Scientific, San Jose, CA, USA) equipped with an electrospray ionization source (ESI). The chromatographic column was a reversed phase Vydac (Hesperia, CA, USA) C8 column (dimensions 150 x 2.1 mm) with 5 μm particle diameter.

5 RP-HPLC-ESI-MS analysis

The solutions utilized for the chromatographic separation were: 0.056% TFA acidic solution (eluent A) and 0.05% TFA in acetonitrile-water 80:20 (eluent B).The gradient applied for the analysis was linear from 0% to 54% of B in 39 minutes and from 54% to 100% of B in 10 minutes, at a flow rate of 0.30 ml/min. The T splitter addressed a flow-rate of 0.20 ml/min toward the diode array detector and 0.10 ml/min 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. Mass spectra were collected every 3 ms in the positive ion mode, MS spray voltage was 5.0 kV and the capillary temperature was 260 °C. The diode array detector was set at 214 and 276 nm.

6 Data Analysis

The proteins and peptides quantified in the present study have been already identified by us in previous studies,[10-14] thus, their characterization was not here reported. Experimental mass values were obtained by deconvolution of averaged ESI-MS spectra automatically performed by using MagTran 1.0 software.[63] Experimental masses were compared with theoretical average mass values available at the Swiss-Prot Data Bank (http://us.expasy.org/tools), and they are reported in

Table 3, and S1. The specific multiply-charged ions generated by the protein at the ion source were searched in the chromatographic profiles by XIC procedure. The label-free quantification was based on the area of the XIC peaks that is proportional to the concentration of the protein and may be used to perform relative quantifications of the same protein in different samples under constant analytical conditions.[64] The m/z values used to quantify each protein/peptide were selected by excluding values common to other closely eluting proteins (± 0.5 m/z) and XIC peak areas were considered adequate when the signal to noise ratio was at least 5.

7 Statistical Analysis

The software GraphPad Prism (version 4.0) was used for calculated means and standard deviations of protein XIC peak areas and for statistical analyses. A comparison between patients and controls was performed by the Mann-Whitney and Unpaired t test with and without Welch's correction, depending on the data distribution (skewed or normal) and the variances (unequal or homogeneous). Clinical data were correlated with XIC areas by the test of Spearman. Statistical analysis was considered to be significant when the p value was <0.05 (two tailed).

Conclusions

Due to the small number of subjects available, the statistical power of this study suffers of some limitations; though, the rarity of this disease reduces the chance of recruiting a larger group of patients. In addition, we are aware about the relative heterogeneity in some parameters of our SAPHO patients (e.g. treatments or disease duration) which could represent confusing factors for a correct interpretation of our data. Therefore, the validation of our observations requires a larger study including additional information as well as longitudinal sampling and analysis. Despite these limitations, the present study bears some novel points. In fact, this is the first study that

investigates the salivary proteome and peptidome in SAPHO syndrome and some interesting results have been obtained. A significant reduction of cystatins, histatins, and aPRPs, which are involved in the protection against infections, may be related with a reduced ability of SAPHO patients to contrast colonization from bacteria, such as *P. acnes*, often associated as possible exogenous trigger, particularly in the first phase of the disease. Moreover, the role played by histatins in the modulation of inflammatory processes, and in the regulation of cytokine's induction suggests that the lower levels of these peptides may be connected with the dysregulation of innate immunity and neutrophil response found in SAPHO syndrome, although their involvement in the pathogenesis has still to be fully elucidated. The inverse correlation between levels of cystatin SN and the duration of the disease may preliminarily suggest a decrease over time in production of some of these proteins during the chronic phase of the disease. However, it is so far unknown whether the significant reduction of the salivary proteins and peptides analyzed in our study is primary or secondary. Finally, the high S100A12 expression in saliva of SAPHO patients and the association of this protein with inflammation and innate immunity promote further studies focused on its possible application as salivary biomarker of SAPHO syndrome.

Acknowledgements

The authors acknowledge the financial support of the Cagliari University, the Regione Sardegna, the Catholic University of Rome, the Italian National Research Council (CNR), and the Nando Peretti Foundation.

Conflict of interest:

No conflict of interest relevant to this article to disclose from any of the authors.

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Table 1. Demographics, historical disease status, current treatment and current disease status of each SAPHO patient at the time of the study.

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, Spondylodiscitis	Biological drug (ADA[a])	REM[b] Partial remission	ACT[c] (SC[d])
2	8	Psoriasis	Sternocostoclavicular osteitis, Hyperostosis	NSAIDs[e]	REM Partial remission	ACT (SC)
3	16	Palmoplantar pustulosis	Sternocostoclavicular hyperostosis and osteitis, Zigomatic and parietal bone, Multiple foci of spondylodiscitis	Biological drug (ADA)	ACT No remission	ACT (SC. SI[f], Spine)
4	7	Palmoplantar pustulosis	Sacroiliitis, Sternoclavicular hyperostosis	Biological drug (ADA)	REM Partial remission	ACT (SC, SI)
5	4	Palmoplantar pustulosis	Sternoclavicular osteitis	Biological drug (ADA)	REM Partial remission	ACT (Jaw, SC)
6	7	Palmoplantar pustulosis	Arthritis, Synovitis, Sternocostal hyperostosis, Femur osteitis	Biological drug (ADA)	REM Remission	REM (None)
7	3	Palmoplantar pustulosis	Sacroiliitis, Sternocostoclavicular osteitis	Biological drug (ADA)	REM Partial remission	ACT (SC)
8	2,5	None	Sacroiliitis, Sternoclavicular hyperostosis, Clavicular edema	Biological drug (ADA)	- Remission	REM (None)
9	23	Palmoplantar	Sternocostoclavicular	NSAIDs	ACT	ACT (SC, SI)

		pustulosis	hyperostosis and osteitis, Sacroiliitis, Femur osteitis		No remission	
10	10	Severe acne	Arthritis (elbow, knee), Sternocostoclavicular hyperostosis and osteitis	Biological drug (ADA)	REM Remission	REM (None)

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

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

Pt #	Blood TH-17 >Normal Y/N TH-17[a]	TH-1[b]	TH-1/TH-17	Neutrophilia >Normal Y/N WBC[c] cells/mm3 Neu[d] cells/mm3	CRP[e] >Normal Y/N Serum value	ESR[f] >Normal Y/N Serum value
1	Y 2.91	17.97	0.19	N 4900 2470	N 0,39	Y 32
2	N 0.13	4.55	0.13	N 8000 4560	N 0,10	N 11
3	Y 3.05	12.31	0,00	N 7200 3900	N 0,47	N 10
4	Y 3.07	25.19	0.24	N 8300 3194	N 0,11	Y 33
5	Y 2.77	9.85	0.32	N 9400 5640	N 0,4	N 18
6	Y 2.86	21.29	0.10	N 10500 6300	N 0,5	N 9
7	Y 2.1	20.00	0,00	N 9700 6200	N 0,24	N 30
8	n.d	n.d	n.d	N 7800 3300	Y 1,8	Y 40
9	n.d	n.d	n.d	N 6200 3521	N 0,2	Y 55
10	n.d	n.d	n.d	N 11430 5270	Y 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 (Neu) (normal range: 1800 - 7500/µl).
[e] C reactive protein (normal range: 0-0.5).
[f] Erythrocyte sedimentation rate (normal range: <30).

Table 3. Swiss-Prot code, experimental and theoretical average mass values and elution times of proteins and peptides investigated showing level variation in the two groups**.**

Proteins	Swiss-Prot code	Experimental av mass (Theoretical av mass)	Elution time (min)
Salivary cystatin family			
Cystatin S	P01036	14186 ± 2 (14185)	36.5-37.1
Cystatin S1	P01036	14266 ± 2 (14265)	36.6-37.1
Cystatin S2	P01036	14346 ± 2 (14345)	36.8-37.2
Cystatin SN	P01037	14312 ± 2 (14313)	34.8-35.2
Cystatin SA	P09228	14347 ± 2 (14346)	38.4-38.9
Histatin family			
Histatin 1	P15515	4928.2 ± 0.5 (4928.2)	23.3-23.8
Histatin 1 nonphos	P15515	4848.2 ± 0.5 (4848.2)	23.4-23.8
Histatin 3	P15516	4062.2 ± 0.4 (4062.4)	17.6-17.9
Histatin 6 (1/25)	P15516	3192.4 ± 0.3 (3192.5)	14.0-14.4
Histatin 5 (1/24)	P15516	3036.5 ± 0.3 (3036.3)	14.2-14.7
S100A12	P80511	10444 ± 2 (10444)	39.5-40.2
Salivary acidic proline-rich phosphoproteins			
PRP-1 diphos	P02810	15515 ± 2 (15514-15515)	22.9-23.3
PRP-1 monophos	P02810	15435 ± 2 (15434-15435)	23.9-24.3
PRP-1 nonphos	P02810	15355 ± 2 (15354-15355)	24.2-24.7
PRP-1 triphos	P02810	15595 ± 2 (15594-15595)	22.6-22.9
PRP-3 diphos	P02810	11161 ± 1 (11161-11162)	23.3-23.8
PRP-3 monophos	P02810	11081 ± 1 (11081-11082)	23.8-24.2
PRP-3 nonphos	P02810	11001 ± 1 (11001-11002)	24.8-25.1
PRP-3 diphos Des-Arg_{106}	P02810	11004 ± 1 (11005-11006)	23.5-23.8
P-C	P02810	4370.9 ± 0.4 (4370.8)	13.6-14.5
P-B peptide	P02814	5792.9 ± 0.5 (5792.7)	29.4-30.5

Table 4. Results of the statistical analysis performed on S-type cystatins, histatins, aPRPs, P-B peptide and S100A12. XIC peak areas (mean ± SD ($x10^7$), frequency and p value obtained by T-test are reported.

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[a]
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	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

[a] ns = not significant

Table 5. Results of the statistical analysis performed on S-type cystatins, histatins, aPRPs, P-B peptide and S100A12. XIC peak areas (mean ± SD ($x10^7$), frequency and p value obtained by T-tests performed excluding patient #8 were reported.

Proteins	**SAPHO patients**		**Healthy controls**		**p value**
	mean ± SD	**Frequency**	**mean ± SD**	**Frequency**	
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	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**

Legends to figures.

Figure 1. Distribution of XIC peak area of cystatins S1 (panel A) and SN (B) in SAPHO group by excluding patient #8 (grey circles) and in healthy controls (empty circles). * $p \leq 0.05$.

Figure 2. Distribution of XIC peak area of histatin (Hst) 1 in SAPHO and healthy control groups (panel A), Hst-3 (B), Hst-5 (C) and Hst-6 (D) in SAPHO group by excluding patient #8 and in healthy controls. Grey and empty circles used, respectively, for SAPHO patients and healthy controls. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Figure 3. Distribution of XIC peak area of P-C peptide (panel A) and P-B peptide (B) in SAPHO group by excluding patient #8 (grey circles) and in healthy controls (empty circles). ** $p \leq 0.01$; *** $p \leq 0.001$

Figure 4. Correlation analysis between CRP and Hst-1 (panel A), Hst-3 (B), Hst-5 (C), Hst-6 (D), S100A12 (E), and between the cystatin SN and the disease duration (F) in SAPHO patients.

Figure 5. Correlation analysis between Hst-3 and ESR (panel A), WBC (B) and Neutrophil count (Neu) (C), and between Hst-5 and Neu (D) in SAPHO patients excluding the patient #8.

Figure 1.

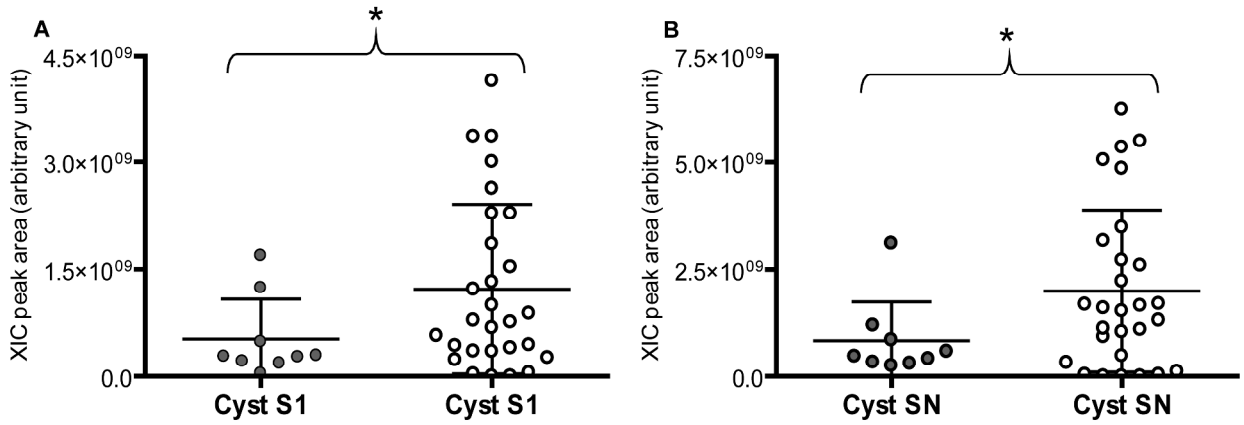

Figure 2.

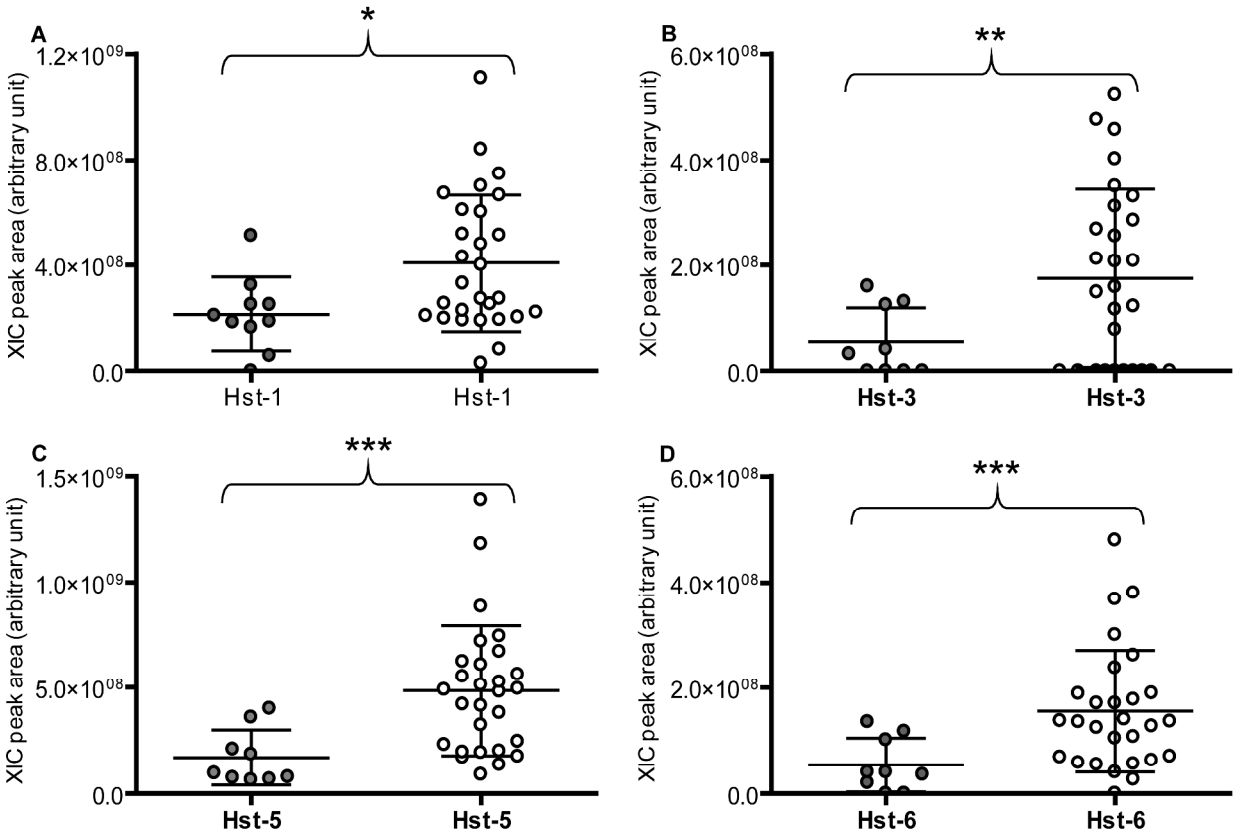

Figure 3.

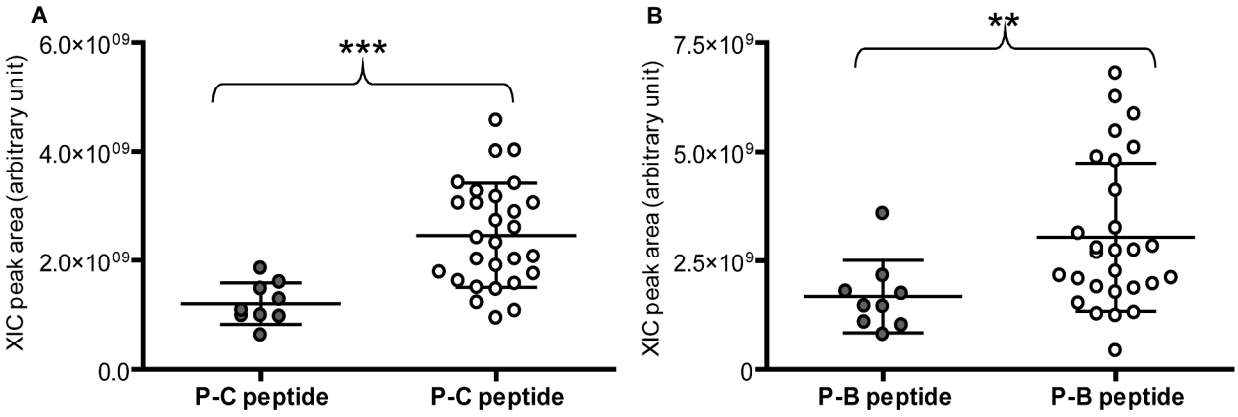

Figure 4

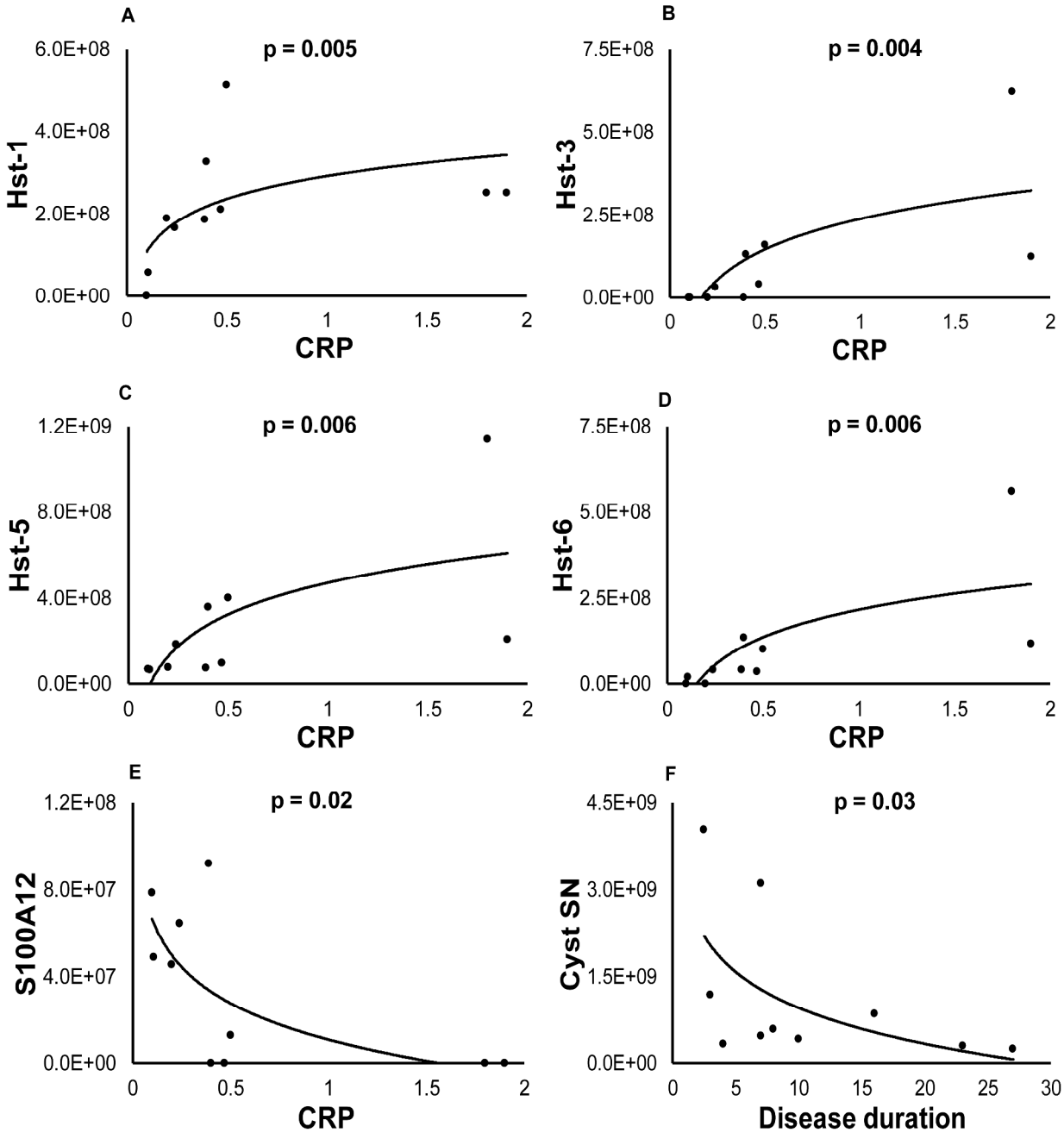

Figure 5.