Phagocyte-specific S100A8/A9 is upregulated in primary Sjögren's syndrome and triggers the secretion of pro-inflammatory cytokines *in vitro*

C. Nicaise[1,2], L. Weichselbaum[3], L. Schandene[4], V. Gangji[3], F. Dehavay[3], J. Bouchat[1], B. Balau[1], T. Vogl[5], M.S. Soyfoo[3]

[1]URPhyM-NARILIS, Université de Namur, Belgium;
[2]Department of Pathology, Hôpital Erasme, Université Libre de Bruxelles, Belgium;
[3]Department of Rheumatology, Hôpital Erasme and Laboratory of Bone and Metabolic Biochemistry, Faculty of Medicine, Université Libre de Bruxelles, Belgium;
[4]Department of Immunology, Hôpital Erasme, Université Libre de Bruxelles, Belgium
[5]Institute of Immunology, University of Münster, Germany.

Abstract

Objective

To determine the role of S100A8/A9 in the pathogenesis of primary Sjögren's syndrome (pSS).

Methods

The serum levels of S100A8/A9 were determined in pSS patients and healthy controls by ELISA. The expression of S100A8/A9 in salivary glands was assessed by immunohistochemistry. The phenotype of $S100A8^+$ and $S100A9^+$ cells was identified using double immunofluorescence. The effects of S100A8/A9 on cytokine production by peripheral blood mononuclear cells (PBMCs) from pSS patients were determined in vitro by flow cytometry. The effects of pro-inflammatory cytokines on S100A8/A9 secretion were additionally investigated in vitro by ELISA in PBMCs from pSS patients and control subjects.

Results

Serum levels of S100A8/A9 were significantly increased in pSS patients compared to healthy controls. The tissular expression of S100A8 and S100A9, identified in professional phagocytes (neutrophils, monocytes and plasmacytoid dendritic cells), was increased in the salivary glands of pSS patients and correlated with focus score.
In vitro, recombinant S100A8/A9 increased the production of IL-1β, IL-6, TNF-α, IFN-γ, IL-10, IL-17A and IL-22 by PBMCs. The S100A8/A9-induced increase in TNF-α production in pSS patients was significant relative to controls. Furthermore, IL-1β, TNF-α, IL-6, and IL-17A stimulated release of S100A8/A9 from PBMCs in pSS patients.

Conclusion

S100A8/A9 is increased in pSS patients contributing to the in vitro increased production of pro-inflammatory cytokines. As such, S100A8/A9 in concert with other cytokines might contribute to the pathogenesis of pSS.

Key words

S100A8/A9, Sjögren's syndrome, salivary glands, DAMP, cytokines

Charles Nicaise, PhD
Laura Weichselbaum, MD
Liliane Schandene, PhD
Valérie Gangji, MD, PhD
Florence Dehavay, MD
Joanna Bouchat, PhD student
Benoit Balau, PhD student
Thomas Vogl, PhD
Muhammad S. Soyfoo, MD, PhD

Please address correspondence to:
Prof. Muhammad S. Soyfoo,
Department of Rheumatology,
Hôpital Erasme,
808 route de Lennik,
1070 Brussels, Belgium.
E-mail: msoyfoo@ulb.ac.be

Received on May 2, 2016; accepted in revised form on July 11, 2016.

Competing interests: none declared.

Introduction

Sjögren's syndrome (SS) is an autoimmune disorder with the pathological hallmark of lymphocytic infiltration of salivary and lacrimal glands (1). The clinical spectrum of the disease varies from the classical dry mouth and eye symptoms to a more severe systemic involvement of several organs. The pathobiology underlying SS is still elusive but several contributing players including the up-regulation of Toll-like receptors, release of pro-inflammatory cytokines, cell death, apoptosis and activation of T and B cells have been shown to contribute to disease initiation and perpetuation (2). The role of damage associated molecular pattern (DAMP) molecules has been forwarded as new potential candidates in fostering autoimmune inflammation in SS (3-5). DAMPs or alarmins are liberated in the extracellular milieu to alert the immune system of imminent danger (6). The immune system responds either by clearing the intruding pathogens and/or by additional secretion of pro-inflammatory cytokines that further activate immigrating phagocytes.

S100A8 and S100A9 are two calcium-binding proteins belonging to the S100 family and are also known as myeloid related protein (MRP8) and MRP14 (7-9). Importantly, S100A8 and S100A9 exist mainly as S100A8/S100A9 heterodimer (termed calprotectin) and are expressed by granulocytes, monocytes and early differentiation states of macrophages. Homo-dimeric as well as tetrameric S100A8 and S100A9 complexes have specific intracellular and extracellular functions (10). As a pleiotropic protein, S100A8/A9 has a widened spectrum of function, playing central roles in inflammatory processes, exerting cytokine-like and chemokine-like features and act as innate amplifiers during infectious and autoimmune diseases (11). More recently, S100A8/A9 have been identified as important DAMPs acting as endogenous ligands of Toll-like receptor 4 (TLR4) and promote systemic endotoxin-induced shock (12).

The involvement of S100A8/A9 in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), juvenile idiopathic arthritis and pSS has been described but its role in the pathogenesis of primary SS (pSS) is still undetermined (13-21). In the present work, we hypothesise the potential role of S100A8/A9 as an alarmin contributing to the pathogenesis of pSS.

Patients and methods

Patients and controls

Blood was collected from 89 pSS patients and 76 healthy controls. All patients fulfilled the revised American-European criteria for pSS (22). Disease activity was evaluated by the European Sjögren's syndrome disease activity index (ESSDAI) (23). Labial salivary gland biopsies from pSS patients (n=38) and controls (sicca patients *i.e.* without immunological profile and not fulfilling criteria for pSS) (n=16) were obtained from the biobank of the department of pathology of the Erasme Hospital. The local ethics committee approved this study.

ELISA

Serum concentrations of S100A8/A9 were assessed by a sandwich ELISA that allows detection of the S100A8/S100A9 heterodimer as previously described (13). The ELISA was calibrated with native S100A8/S100A9 complexes. Sensitivity of the assay was 0.5 ng/mL.

Cell culture

Peripheral blood mononuclear cells (PBMC) from 10 pSS patients and 8 healthy controls were isolated and stimulated with 1 μg/ml of S100A8 and S100A9 to determine cytokine production (IL-1β, IL-2, IL-4, IL-5, IFN-γ, TNF-α, IL-6, IL-9, IL-10, IL-12, IL-13, IL-17A, IL-22) by flow cytometry (eBioscience, Vienna, Austria). PBMC from 6 pSS patients and 6 healthy controls were also stimulated with TNF-α, IL-1β, IL-6 and IL-17 and LPS to determine S100A8/A9 release.

Immunohistochemistry and immunofluorescence

Immediately after removal, salivary glands (SG) were fixed in 4 % buffered formaldehyde, paraffin-embedded and sectioned (5 μm-thick). Immunostaining against human S100A8

Table I. Clinical and demographic characteristics of pSS patients.

Clinical features	
Number of patients	89
Age (years)	53 ± 1.5
Gender (F/M)	74/15
Disease duration (years)	6.3 ± 0.76
ESSDAI	2.55 ± 0.22
Medications	n
Hydroxychloroquine	45
Steroids	7
Azathioprine	3
Methotrexate	2
Rituximab	1
Clinical features	
Anti SSA+	52
Anti SSB+	31
Articular	54
Pulmonary	3
Vasculitis	5
Renal	4
Peripheral neuropathy	18
Fatigue	58
Salivary gland swelling	10
Haematologic	4
Biological features	
ESR (mm/h)	17.3 ± 1.8
CRP (mg/dl)	0.5 ± 0.1
IgG (g/dl)	1.4 ± 0.6
IgA (g/dl)	0.23 ± 0.01
IgM (g/dl)	0.1 ± 0.01
Neutrophil (/mm^3)	3699 ± 199
Lymphocytes (/mm^3)	1644 ± 71
Monocytes (/mm^3)	468 ± 20
Complement C4 (mg/dl)	18.1 ± 1.35
Complement CH50 (mg/dl)	116 ± 25.7

Data are expressed as means ± SEM.

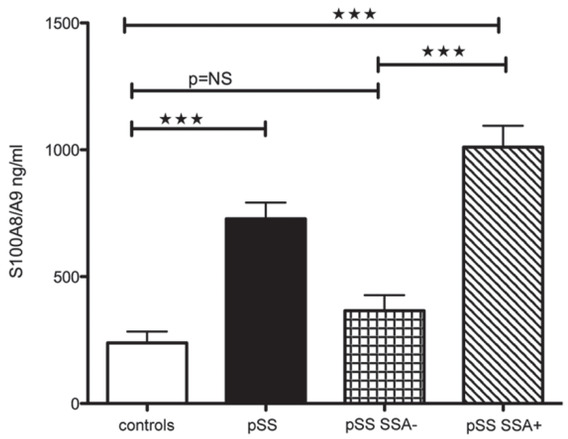

Fig. 1. Serum levels of S100A8/A9 in pSS patients (n=89) are significantly increased relative to controls (n=76); $p<0.001$. In pSS patients, the increase in S100A8/A9 was significant only in pSS patients with anti-SSA (and/or SSB) antibodies ($p<0.001$) relative to healthy patients while in pSS patients without anti-SSA (and/or SSB), serum S100A8/A9 levels were comparable to controls. Bars represent values of mean ± standard error of the mean. ***$p<0.001$.

(HPA024372; Sigma-Aldrich) and S100A9 (HPA004193; Sigma-Aldrich) were performed as previously described using diaminobenzidine as chromogen (24). The numbers of S100A8$^+$ and S100A9$^+$ cells infiltrating the salivary glands were blindly counted by two investigators (C.N. and F.D.) using a semi-quantitative score (score 0: <5 cells/mm^2; score 1: <15 cells/mm^2; score 2 <50 cells/mm^2; score 3 ≥50cells/mm^2). For double immunofluorescence, random salivary gland sections from control (n=5) and pSS patients (n=5) were dewaxed, rehydrated, permeabilised in Tris-buffered saline (TBS), and blocked with 5% normal goat serum for 15 minutes at room temperature, preceded by a heat-induced antigen retrieval (10 minutes at 95°C) in 10 mM citrate buffer pH6.0. Sections were next incubated with the primary antibodies diluted in TBS with 1% goat serum, overnight at room temperature. The following primary antibodies were used: rabbit anti-human S100A8 (HPA024372; Sigma-Aldrich), 1:100; mouse anti-human S100A8 (MAB4570; R&Dsystems), 1:100; rabbit anti-human S100A9 (HPA004193; Sigma-Aldrich), 1:100; mouse anti-human MPO (392105; R&Dsystems), 1:50; mouse anti-human CD68 (M0814; Dako), 1:50; mouse anti-human αSMA (18-0106; Life Technologies), 1:100; mouse anti-human CD123 (MAB301; R&Dsystems) 1/25; and mouse anti-human cytokeratins (clone AE1/AE3, Dako), 1/100. The secondary antibodies goat anti-rabbit IgG conjugated to Alexa Fluor 488 (1:200; Life Technologies) and goat anti-mouse IgG conjugated to Alexa Fluor 568 (1:200; Life Technologies) were added for 1 hour at room temperature. Slides were washed once again in TBS and mounted using Prolong Gold anti-fade reagent plus DAPI (P36931; Life Technologies). In all experiments, negative controls were carried out by omitting the primary antibody. Images were acquired on an upright epifluorescent microscope (Olympus BX63) equipped with ORCA-ER camera (Hamamatsu).

Statistical analysis

Significance was assessed by one-way analysis of variance followed by multiple comparisons for multiple comparisons and the *t*-test for comparison between 2 different groups. Spearman's rank coefficient was used to determine the correlation with different biological variables and ESSDAI. The Kendall tau Rank Correlation was used to compute the correlation between S100A8/A9 tissular immunoreactivites and Chisholm score (Wessa, 2012, Kendall tau Rank Correlation (v. 1.0.11) in Free Statistics Software (v. 1.1.23-r7), Office for Research Development and Education, URL http://www.wessa.net/rwasp_kendall.wasp/). $p<0.05$ was considered as statistically significant.

Results

In our study we included 89 pSS patients and 76 control subjects. The relevant clinical data of pSS patients are summarised in Table I. The control subjects for the dosage of serum S100A8/A9 were healthy patients (without any sicca symptoms) and consisted of 50 females and 26 males (18±2.9 years).

The serum levels of S100A8/A9 were significantly increased in pSS patients compared to controls ($p<0.001$, Fig. 1). The increase in S100A8/A9 observed in pSS patients was significant in pSS patient subgroups with anti-SSA (and/or anti-SSB, data not shown) antibodies compared to pSS patients without anti-SSA antibodies or controls ($p<0.001$). We next determined if serum S100A8/A9 levels could be a marker of disease activity of pSS. There was no significant correlation between serum S100A8/A9 levels and ESSDAI (r=0.11 and $p=0.299$, data not shown). We also determined whether serum levels of S100A8/A9 could correlate with high disease activity (ESSDAI≥ 5). Serum S100A8/A9 levels were not significantly increased in pSS patients with high disease activity compared to pSS patients with low disease activity ($p=0.14$) and did not correlate with disease activity ($p=0.25$). There

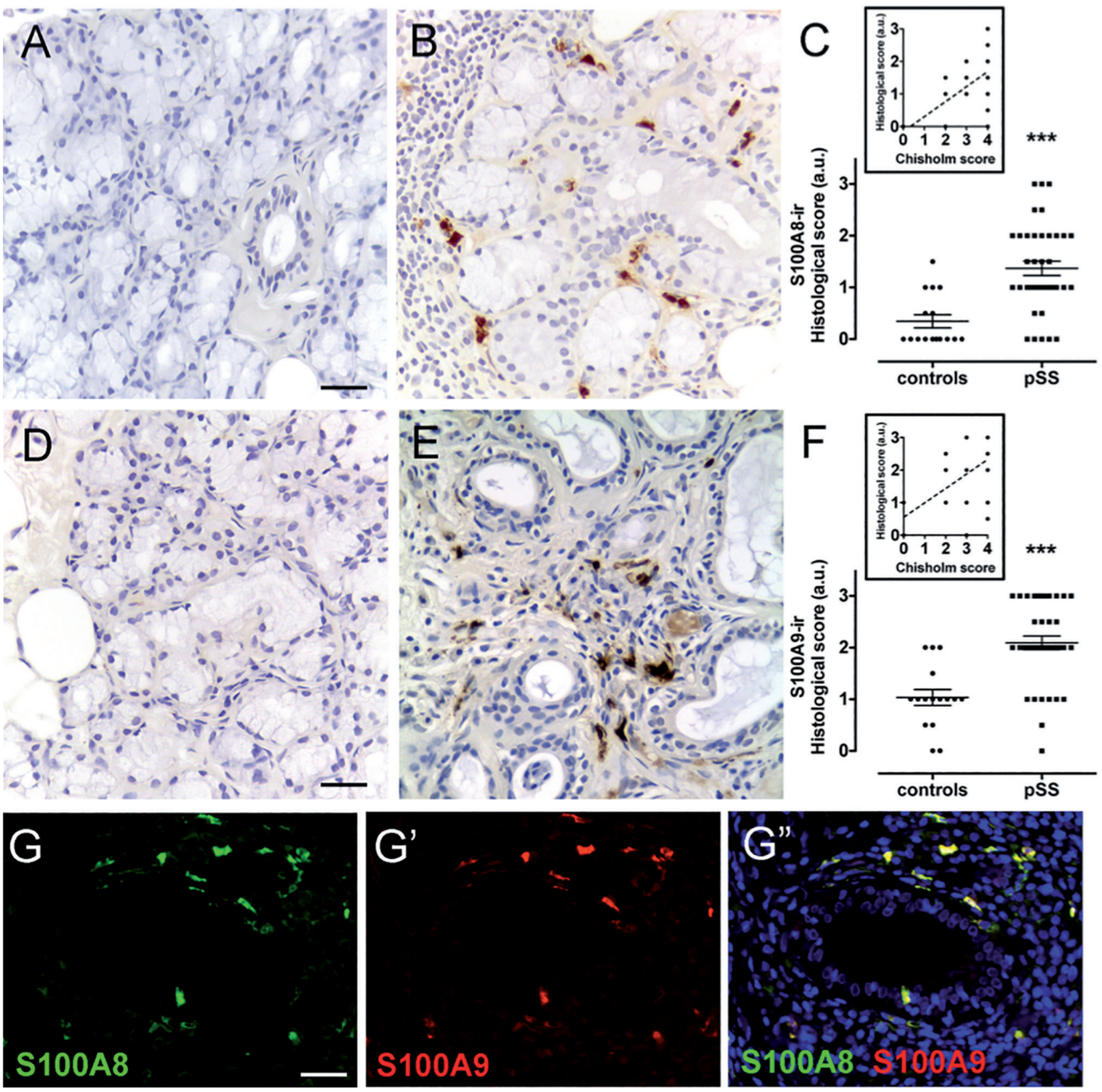

Fig. 2. S100A8 immunohistochemistry in labial salivary glands of controls (**A**) and pSS patients (**B**). Quantification of S100A8 immunoreactivity (S100A8-ir) shows a significantly higher density of S100A8$^+$ cells in SG from pSS patients compared to controls (p<0.001) (**C**). Inset in C represents the correlation between Chisholm score and S100A8 histological score. **D-F**. S100A9 immunohistochemistry in labial salivary glands of controls (**D**) and pSS patients (**E**). Quantification of S100A9 immunoreactivity (S100A9-ir) shows a significantly higher density of S100A9$^+$ cells in SG from pSS patients compared to controls (p<0.001) (**C**). Inset in F represents the correlation between Chisholm focus score and S100A9 histological score. S100A8 and S100A9 immunoreactivities were confined to the cytoplasmic compartment of infiltrating cells, although some S100A9 extracellular staining could be detected for the biopsies with the highest Chisolm score. Results are expressed as mean ± standard error of the mean. ***p<0.001. S100A8 or S100A9 immunoreactivites were semi-quantified on 16 and 38 slides respectively for controls and pSS patients. As shown in panel G", S100A8$^+$ cells (green, G) co-express S100A9 (red, G'). Scale bar in A, B, D, E and G represents 50 µm.

was no significant correlation of serum S100A8/A9 levels with the histological focus scores in the salivary glands (SG) in pSS patients (data not shown). Furthermore, correlations with different clinical laboratory variables were made. We could determine significant correlations of serum S100A8/A9 levels with lymphocyte counts (r= -0.21; p=0.01), serum IgA (r= 0.368; p=0.007) and C-reactive protein (r=0.30; p=0.01) while other clinical variables such as erythrocyte sedimentation rate, circulating monocytes, neutrophils and other serum immunoglobulins did not correlate significantly with serum S100A8/A9 levels.

Next, we determined the immunoreactivities for S100A8 and S100A9 in the salivary glands from pSS patients (n=38) and from sicca patients (n=16).

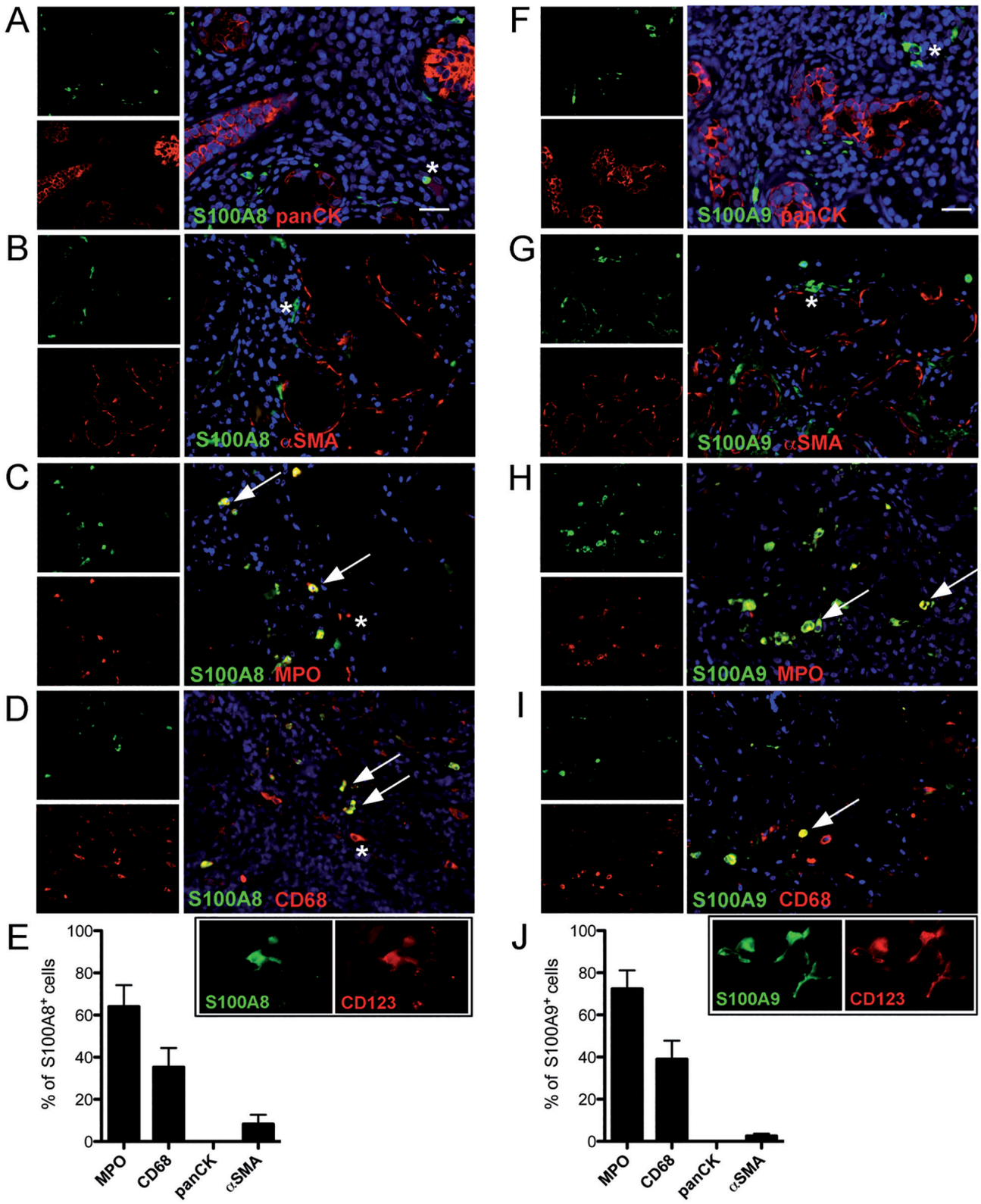

Fig. 3. The phenotype of intratissular S100A8$^+$ and S100A9 $^+$ cells was identified using double immunofluorescence against pan-cytokeratin (panCK), αSMA, MPO, CD68 and CD123 markers. PanCK-immunoreactive acinar or ductal epithelial cells did not express S100A8 (asterix in A) or S100A9 (asterix in F). Rare S100A8$^+$ (B) or S100A9$^+$ (G) cells were labelled for αSMA. Many S100A8$^+$ or S100A9$^+$ cells co-expressed MPO (white arrows in C and H), although colocalisation was not full and some MPO positive cells were not S100A8$^+$ (asterix in C). S100A8$^+$ or S100A9$^+$ cells also co-labelled with CD68 (white arrows in D and I) or CD123 markers (insets in D and I). Scale bars represent 50 µm. n=5 controls and n=5 pSS salivary glands; no difference was detected in the phenotype of S100A8/A9-immunoreactive cells between control and pSS samples. Graphs E and J show the percentage of cells double positive for S100A8/A9 markers and MPO, CD68, panCK or αSMA.

There was no immunoreactivity for S100A8 and S100A9 in the acinar structures and ducts of SG of both pSS and controls (Fig. 2A-D). While absent in control patients, S100A8$^+$ and S100A9$^+$ cells were infiltrating the connective tissue around the acini (Fig. 2B and E). Based on the density of immunoreactive cells per field, we semi-quantified S100A8 and S100A9 immunoreactivity in whole-salivary glands. Immunoreactivity scores for S100A8 (Fig. 2C) and S100A9 (Fig. 2F) were significantly enhanced in Sjögren salivary glands compared to controls (p<0.001 and p<0.001 respectively). Moreover, according to Kendall tau Rank Correlation, S100A8 and S100A9 immunoreactivities were positively correlated to Chisholm score in Sjögren patients (insets in Fig. 2C and F, r=0.43, p<0.001 and r=0.41, p<0.01, respectively). Remarkably, all S100A8$^+$ cells co-expressed S100A9 protein and inversely (Fig. 2G). Using double immunofluorescence, we then sought to identify the S100A8/A9 cells infiltrating the salivary tissue. S100A8$^+$ or S100A9$^+$ cells never expressed pan-cytokeratin marker and were rarely immunoreactive for αSMA (Fig. 3A-B and F-G). However, markers of phagocytic cells, including MPO for neutrophils, CD68 for monocytes/macrophages and CD123 for plasmacytoid dendritic cells, were highly correlated with S100A8 and S100A9 expressions (Fig. 3C-D and H-I). More than 60% of S100A8/A9-expressing cells were MPO$^+$, followed by about 37% of CD68$^+$ cells and about 3% of αSMA$^+$ cells (Fig. 3E and J). Among the phagocytic cells immunoreactive for S100A8/A9, we could identify CD123$^+$ cells, most likely plasmacytoid dendritic cells (insets in Fig. 3D and I).

To determine if the release of S100A8/A9 triggers an immune response, we investigated the extent to which S100A8/A9 was able to induce cytokine secretion by PBMCs from pSS patients. S100A8/A9 in a dose-related manner (from 1ng/ml to 1μg/ml) significantly stimulated the production of IL-1β, IL-6, TNF-α, IFN-γ, IL-10, IL-17A and IL-22. In pSS patients, S100A8/A9 at 1μg/ml induced more than 100-fold the secretion of IL-1β, IL-6, TNF-α (Fig. 4A). Furthermore, we investigated whether the S100A8/A9 stimulated cytokine production by PBMCs from pSS patients differed from PBMCs from controls. Similarly to the results observed with pSS patients, S100A8/A9 stimulated the production of IL-1β, IL-6, TNF-α, IFN-γ, IL-10, IL-17A and IL-22. The S100A8/A9-induced cytokine secretion was not significantly different between pSS patients and controls except for a significantly greater increase in TNF-α production in pSS patients (Fig. 4B).

We also determined if the stimulation by IL-1β, TNF-, IL-17A, IL-6 and LPS could trigger the secretion of S100A8/A9 thereby constituting a vicious inflammatory loop perpetuating disease progression. IL-1β, TNF-α, IL-6, LPS and IL-17A significantly (p<0.01) increased the secretion of S100A8/A9 in PBMCs from pSS patients. Similarly, the stimulation of PBMCs with

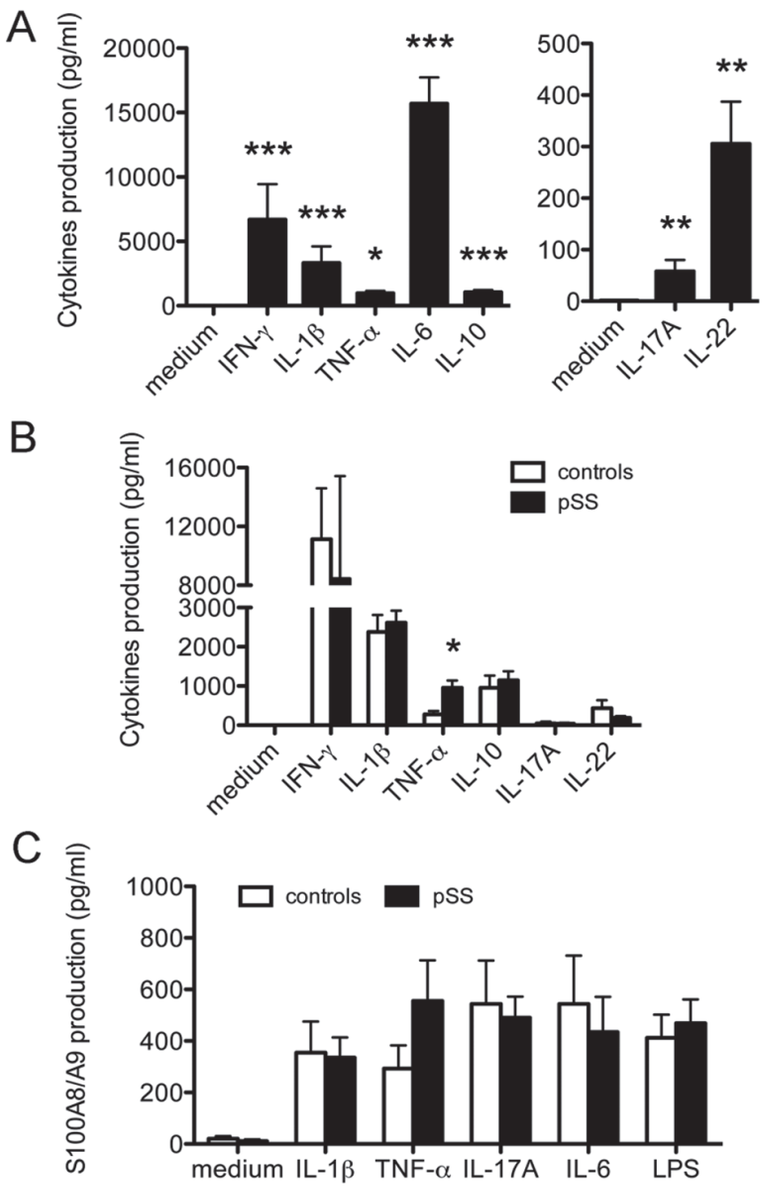

Fig. 4. Stimulation of PBMCs from 10 pSS patients with 1 μg/ml of recombinant S100A8/A9 induced the significant release of cytokines (IFN-γ, IL-1β, TNF-α, IL-6, IL-10, IL-17A, IL-22) compared to unstimulated condition (medium) (**A**). S100A8/A9-induced cytokines production in PBMCs from 10 pSS patients relative to age and sex-matched controls (**B**). IL-1β, TNF-α, IL-6, Lipopolysaccaride (LPS) and IL-17A induced release of S100A8/A9 in PBMCs from 6 pSS patients and 6 age and sex-matched controls (**C**). Bars represent values of mean ± standard error of the mean.
*p<0.05, **p<0.01, ***p<0.001.

IL-1β, TNF-α, IL-6, LPS and IL-17A significantly stimulated S100A8/A9 production in healthy controls. The S100A8/A9-induced secretion by IL-1β, TNF-α, IL-6, LPS and IL-17A was not significantly different between pSS patients and controls (Fig. 4C).

Discussion

In this study, we show that the expression of S100A8/A9 is upregulated in pSS patients, both in serum and in the salivary tissue and that S100A8/A9 displays both intracellular and extracellular functions and is able to trigger the secretion of pro-inflammatory cytokines by PBMCs *in vitro*. Several lines of evidence underline the involvement of S100A8/A9 in triggering inflammatory processes (7, 11). In SLE and RA for example, serum S100A8/A9 levels have been shown to correlate with disease activity and are thus potential markers to monitor disease activity and response to treatment. In SLE, S100A8/A9 has also been shown to be valuable markers to differentiate between disease flare and concomitant infection (13). S100A8/A9 secreted by activated phagocytes in inflammatory conditions portrays as a better marker of disease activity as compared to acute phase reactants synthesised by the liver (13, 25). A pivotal role of the S100 proteins inflammatory processes has been highlighted by the identification of a new inflammatory syndrome characterised by extremely high levels of these molecules (26). Increased serum and salivary S100A8/A9 levels have been shown in pSS patients compared to controls (16, 20, 27). A recent study by Nordal *et al*. showed significantly increased levels of S100A8/A9 as well as S100A12 in a cohort of 141 pSS patients relative to controls. In another study, however, serum S100A8A/A9 levels did not differ significantly from healthy patients while salivary S100A8/A9 levels were significantly increased in SS patients (28). Moreover, faecal S100A8/A9 was significantly increased in pSS patients and correlated with ESSDAI relative to controls (29).

We identified the S100A8/A9$^+$ cells infiltrating the pSS salivary glands as being mostly polymorphonuclear neutrophils and macrophages. Some S100A8/A9-immunoreactive cells were sparsely positive for markers of myofibroblasts and plasmacytoid dendritic cells, this should be further confirmed by using other specific cell markers such as vimentin or BDCA2 and BDCA4, respectively. The involvement of plasmacytoid dendritic cells has recently been forwarded in the pathophysiology of pSS or other autoimmune disease (30, 31).

In vitro, we showed that PBMCs exposition to recombinant S100A8/A9 promotes the release of pro-inflammatory cytokines. Indeed, S100A8/A9 triggers the release of TNF-α, IL-6, IFN-γ, IL-10, IL-1β, IL-17A and IL-22. One of the main pathophysiological features of pSS is the release of pro-inflammatory cytokines contributing to the initiation and perpetuation of disease. As such, S100A8/A9 once secreted can stimulate immune cells to produce inflammatory mediators such as TNF-α thereby promoting further infiltration of epithelial cells and ensuing damage. Apart from TNF-α, no significant difference was observed between pSS and controls patients for other S100A8/A9-induced cytokines. This could suggest that S100A8/A9-induced activation of circulating immune cells is not hampered. Furthermore, the production of pro-inflammatory cytokines can in vicious loop fashion trigger the secretion of S100A8/A9 thereby entertaining the inflammatory cycle perpetuating damage. These observations have also been shown in psoriasis (32). Our *in vitro* results are consistent with other observations including lupus glomerulonephritis and inflammatory arthritis where following the secretion of S100A8/A9 and activation of biochemical cascade there is transduction of several pro inflammatory cytokines (such as TNF-α). Furthermore, S100A8/A9, in a concerted fashion, through paracrine and autocrine effects, amplify the inflammatory response (33-36). One question remains open in respect to the effect of S100A8/A9 on the epithelial cells. To remind, the increase in serum S100A8/A9 was only signifcant for the pSS subgroup with anti-SSA and/or antiSSB antibodies. Multiple hypothesis might explain this phenomenon: autoantibodies-activated phagocytes would be more prone to extracellularly release S100A8/A9; or inversely S100A8/A9$^+$ phagocytes are a population of antigen-presenting cells that stimulate the humoral response in a way that favors the production of autoantibodies; or we cannot rule out a pro-apoptotic effect of extracellular S100A8/A9 on glandular epithelial cells, inducing uncontrolled release and exposition of autoantigens to imunne system. Indeed, pro-apoptotic and cytotoxic effects have been described when S100A8/A9 was applied onto cancer cells (37).

In conclusion, our study emphasises that phagocyte-specific S100A8/A9 is upregulated in pSS and that it triggers the secretion of pro-inflammatory cytokines *in vitro* that could further contribute to disease perpetuation.

Key message

S100A8/A9 is increased in Sjögren's syndrome and could play an important role in perpetuating inflammation

Acknowledgments

We thank Daniel Van Vlaender, Kathleen De Swert for technical assistance and Dr Marie-Cécile Nollevaux (Dept. of Pathology, UCL Mont-Godinne) for kindly providing αSMA antibody.

References

1. FOX RI: Sjögren's syndrome. *Lancet* 2005; 366: 321-31.
2. VOULGARELIS M, TZIOUFAS AG: Pathogenetic mechanisms in the initiation and perpetuation of Sjögren's syndrome. *Nat Rev Rheumatol* 2010; 6: 529-37.
3. EK M, POPOVIC K, HARRIS HE, NAUCLER CS, WAHREN-HERLENIUS M: Increased extracellular levels of the novel proinflammatory cytokine high mobility group box chromosomal protein 1 in minor salivary glands of patients with Sjögren's syndrome. *Arthritis Rheum* 2006; 54: 2289-94.
4. DUPIRE G, NICAISE C, GANGJI V, SOYFOO MS: Increased serum levels of high-mobility group box 1 (HMGB1) in primary Sjögren's syndrome. *Scand J Rheumatol* 2012; 41: 120-3.
5. AWADA A, NICAISE C, ENA S *et al*.: Potential involvement of the IL-33-ST2 axis in the pathogenesis of primary Sjögren's syndrome. *Ann Rheum Dis* 2014; 73: 1259-63.
6. CHAN JK, ROTH J, OPPENHEIM JJ *et al*.: Alarmins: awaiting a clinical response. *J Clin Invest* 2012; 122: 2711-9.
7. ROTH J, VOGL T, SORG C, SUNDERKOTTER C: Phagocyte-specific S100 proteins: a novel

group of proinflammatory molecules. *Trends Immunol* 2003; 24: 155-8.
8. DALE I, FAGERHOL MK, NAESGAARD I: Purification and partial characterization of a highly immunogenic human leukocyte protein, the L1 antigen. *Eur J Biochem* 1983; 134: 1-6.
9. FAGERHOL MK: Nomenclature for proteins: is calprotectin a proper name for the elusive myelomonocytic protein? *Clin Mol Pathol* 1996; 49: M74-9.
10. KESSEL C, HOLZINGER D, FOELL D: Phagocyte-derived S100 proteins in autoinflammation: putative role in pathogenesis and usefulness as biomarkers. *Clin Immunol* 2013; 147: 229-41.
11. EHRCHEN JM, SUNDERKOTTER C, FOELL D, VOGL T, ROTH J: The endogenous Toll-like receptor 4 agonist S100A8/S100A9 (calprotectin) as innate amplifier of infection, autoimmunity, and cancer. *J Leukoc Biol* 2009; 86: 557-66.
12. VOGL T, TENBROCK K, LUDWIG S *et al*.: Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. *Nat Med* 2007; 13: 1042-9.
13. SOYFOO MS, ROTH J, VOGL T, POCHET R, DECAUX G: Phagocyte-specific S100A8/A9 protein levels during disease exacerbations and infections in systemic lupus erythematosus. *J Rheumatol* 2009; 36: 2190-4.
14. FROSCH M, STREY A, VOGL T *et al*.: Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis Rheum* 2000; 43: 628-37.
15. FERRO F, VAGELLI R, BRUNI C *et al*.: One year in review 2016: Sjögren's syndrome. *Clin Exp Rheumatol* 2016; 34: 161-71.
16. BALDINI C, GIUSTI L, CIREGIA F *et al*.: Proteomic analysis of saliva: a unique tool to distinguish primary Sjögren's syndrome from secondary Sjögren's syndrome and other sicca syndromes. *Arthritis Res Ther* 2011; 13: R194.
17. GIUSTI L, BALDINI C, BAZZICHI L, BOMBARDIERI S, LUCACCHINI A: Proteomic diagnosis of Sjögren's syndrome. *Expert Rev Proteomics* 2007; 4: 757-67.
18. HU S, WANG J, MEIJER J *et al*.: Salivary proteomic and genomic biomarkers for primary Sjögren's syndrome. *Arthritis Rheum* 2007; 56: 3588-600.
19. FLEISSIG Y, DEUTSCH O, REICHENBERG E *et al*.: Different proteomic protein patterns in saliva of Sjögren's syndrome patients. *Oral Dis* 2009; 15: 61-8.
20. NORDAL HH, BRUN JG, HALSE AK, MADLAND TM, FAGERHOL MK, JONSSON R: Calprotectin (S100A8/A9), S100A12, and EDTA-resistant S100A12 complexes (ERAC) in primary Sjögren's syndrome. *Scand J Rheumatol* 2014; 43: 76-8.
21. HAMMER HB, ODEGARD S, FAGERHOL MK *et al*.: Calprotectin (a major leucocyte protein) is strongly and independently correlated with joint inflammation and damage in rheumatoid arthritis. *Ann Rheum Dis* 2007; 66: 1093-7.
22. VITALI C, BOMBARDIERI S, JONSSON R *et al*.: Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis* 2002; 61: 554-8.
23. SEROR R, RAVAUD P, BOWMAN SJ *et al*.: EULAR Sjogren's syndrome disease activity index: development of a consensus systemic disease activity index for primary Sjögren's syndrome. *Ann Rheum Dis* 2010; 69: 1103-9.
24. SOYFOO MS, DE VRIESE C, DEBAIX H *et al*.: Modified aquaporin 5 expression and distribution in submandibular glands from NOD mice displaying autoimmune exocrinopathy. *Arthritis Rheum* 2007; 56:2566-74.
25. INCIARTE-MUNDO J, VICTORIA HERNANDEZ M, RUIZ-ESQUIDE V *et al*.: Serum Calprotectin Versus Acute-Phase Reactants in the Discrimination of Inflammatory Disease Activity in Rheumatoid Arthritis Patients Receiving Tumor Necrosis Factor Inhibitors. *Arthritis Care Res* (Hoboken) 2016; 68: 899-906.
26. SAMPSON B, FAGERHOL MK, SUNDERKOTTER C *et al*.: Hyperzincaemia and hypercalprotectinaemia: a new disorder of zinc metabolism. *Lancet* 2002; 360: 1742-5.
27. CUIDA M, HALSE AK, JOHANNESSEN AC, TYNNING T, JONSSON R: Indicators of salivary gland inflammation in primary Sjögren's syndrome. *Eur J Oral Sci* 1997; 105: 228-33.
28. BRUN JG, CUIDA M, JACOBSEN H *et al*.: Sjögren's syndrome in inflammatory rheumatic diseases: analysis of the leukocyte protein calprotectin in plasma and saliva. *Scand J Rheumatol* 1994; 23: 114-8.
29. ANDREASSON K, SAXNE T, SCHEJA A, BARTOSIK I, MANDL T, HESSELSTRAND R: Faecal levels of calprotectin in systemic sclerosis are stable over time and are higher compared to primary Sjögren's syndrome and rheumatoid arthritis. *Arthritis Res Ther* 2014; 16: R46.
30. LOOD C, STENSTROM M, TYDEN H *et al*.: Protein synthesis of the pro-inflammatory S100A8/A9 complex in plasmacytoid dendritic cells and cell surface S100A8/A9 on leukocyte subpopulations in systemic lupus erythematosus. *Arthritis Res Ther* 2011; 13: R60.
31. GOTTENBERG JE, CAGNARD N, LUCCHESI C *et al*.:. Activation of IFN pathways and plasmacytoid dendritic cell recruitment in target organs of primary Sjögren's syndrome. *Proc Natl Acad Sci USA* 2006; 103: 2770-5.
32. NUKUI T, EHAMA R, SAKAGUCHI M *et al*.: S100A8/A9, a key mediator for positive feedback growth stimulation of normal human keratinocytes. *J Cell Biochem* 2008; 104:453-64.
33. PEPPER RJ, WANG HH, RAJAKARUNA GK *et al*.: S100A8/A9 (calprotectin) is critical for development of glomerulonephritis and promotes inflammatory leukocyte-renal cell interactions. *Am J Pathol* 2015; 185: 1264-74.
34. VAN LENT PL, GREVERS L, BLOM AB *et al*.: Myeloid-related proteins S100A8/S100A9 regulate joint inflammation and cartilage destruction during antigen-induced arthritis. *Ann Rheum Dis* 2008; 67: 1750-8.
35. VAN LENT PL, GREVERS LC, BLOM AB *et al*.: Stimulation of chondrocyte-mediated cartilage destruction by S100A8 in experimental murine arthritis. *Arthritis Rheum* 2008; 58: 3776-87.
36. CESARO A, ANCERIZ N, PLANTE A, PAGE N, TARDIF MR, TESSIER PA: An inflammation loop orchestrated by S100A9 and calprotectin is critical for development of arthritis. *PLoS One* 2012; 7: e45478.
37. GHAVAMI S, KERKHOFF C, LOS M, HASHEMI M, SORG C, KARAMI-TEHRANI F: Mechanism of apoptosis induced by S100A8/A9 in colon cancer cell lines: the role of ROS and the effect of metal ions. *J Leukoc Biol* 2004; 76: 169-75.