Thymic stromal lymphopoietin expression from benign lymphoproliferation to malignant B-cell lymphoma in primary Sjögren's syndrome

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ABSTRACT

Objective. *To investigate the expression of thymic stromal lymphopoietin (TSLP) in primary Sjögren's syndrome (pSS), stratified according to the lymphoproliferative status, from a fully benign (fbSS) stage to myoepithelial sialadenitis (MESA) and to B-cell non-Hodgkin's lymphoma (NHL).*

Methods. *After initial serum studies in large numbers of pSS patients and in controls, TSLP was investigated also in pathologic salivary glands (SG) biopsies from 38 stratified pSS patients (13 fbSS; 13 MESA; 12 NHL) and from 13 controls with non-autoimmune sicca syndrome (nSS) by RT-PCR, immunohistochemistry and immunofluorescence.*

Results. *Significantly higher TSLP serum levels were shown in pSS than controls, increasing from fbSS to MESA and to NHL. In SG biopsies, TSLP-positive B lymphocytes increased with increasing lymphoproliferation, maximally in NHL, consistent with the detection of inducible TSLP long isoform (lfTSLP) mRNA only in MESA and NHL. By contrast, the constitutive TSLP short isoform (sfTSLP) mRNA showed no difference among subgroups. The TSLP expression by glandular epithelium declined with the progression from fbSS to MESA and to NHL.*

Conclusion. *TSLP progressively increases from benign to malignant B-cell lymphoproliferation in pSS. The salivary epithelium expresses TSLP but, with the progression of lymphoproliferation, the B-cells may represent the major source of TSLP, in its long inducible isoform. A possible pathogenetic role of TSLP is herein hypothesised in pSS for the first time. Further analyses on TSLP, also as a biomarker of pSS and related lymphoproliferation, are worthwhile.*

Introduction

Primary Sjögren's syndrome (pSS) is a systemic autoimmune and lymphoproliferative disease characterised by a chronic lymphocytic inflammatory process mainly affecting the salivary glands (SG) and other mucosa-associated lymphoid tissue (MALT) sites, and by a wide range of possible clinical systemic manifestations, including a high risk of B-cell lymphoma development (1-4).

B lymphocyte hyperactivity is a typical hallmark of pSS (3, 5). A wide spectrum of lymphoproliferative pictures occurs in SG in pSS, ranging from fully benign lymphoid infiltrates to myoepithelial sialadenitis (MESA) and, finally, to B-cell non-Hodgkin lymphoma (NHL) (6-9), mainly of the MALT type (9). The salivary epithelium and the infiltrating lymphocytes interplay in perpetuating the autoimmune response and in enhancing lymphoproliferation (10-13), through the production of B-cell stimulating cytokines. Of note, pSS is one important human "model" disease to investigate B-cell lymphomagenesis, since different pathologic tissues can be investigated in different stages of disease progression during a long-term follow-up (1-3, 6-8, 14).

Thymic stromal lymphopoietin (TSLP) is an epithelial lymphopoietic cytokine belonging to the interleukin (IL) 7 family, mainly expressed at interfaces between the body and the environment, showing immunoregulatory properties and acting as a B-cell growth factor (15-18). TSLP binds the TSLP receptor, a heterodimer complex including the IL7 receptor α chain (IL7-Rα) and the TSLP specific receptor chain (TSLPR or CRLF2), widely expressed both on heamatopoietic and non-hae-

matopoietic cells (19). Two isoforms of TSLP have been described, showing opposite biological actions. While the constitutive short form (sfTSLP) regulates the immune tolerance at body barriers (20), the long TSLP isoform (lfTSLP) is inducible and upregulated in several inflammatory diseases (21).

Recent studies highlighted the role of TSLP in the immunopathology of human autoimmune and systemic inflammatory disorders such as allergic diseases (mainly asthma and atopic dermatitis) (22, 23), rheumatoid arthritis (24), psoriasis (25), giant cells arteritis (26), systemic sclerosis (27), celiac and Crohn's disease (28, 29). Moreover, TSLP has been implicated in the pathogenesis of both solid and haematological malignancies (30-32).

Strong evidence supports the key role of TSLP in influencing the growth, differentiation, proliferation and malignant transformation of both mouse and human B lymphocytes, and in promoting humoral autoimmunity (15, 33-35). In particular, TSLP was shown to be involved in the development of severe lymphoproliferation in mice lacking Notch proteins in the skin (36) and in K5-TSLP transgenic mice, where the hyperexpression of TSLP led to the development of a cryoglobulinaemic vasculitis (CV) (33, 37, 38). Furthermore, TSLP involvement has been reported also in human haematological B-cell malignancies and in HCV-related CV (31, 33, 39-41). Strikingly, mixed cryoglobulinaemia occurs in pSS, where it is a strong lymphoma predictor (3, 42, 43).

Based on this background and due to the lack of data in pSS, the present study evaluated the expression of TSLP in pSS-related lymphoproliferation by analysing pathologic tissues showing different degrees of B-cell lymphoproliferation, by means of an integrated *in situ* and molecular study approach. In detail, SG biopsies from pSS patients stratified according to the degree of lymphoproliferation by tissue biopsy (from benign lymphoid infiltrates, to MESA and to NHL) (8) were analysed after having noticed, by preliminary studies, that TSLP serum levels were significantly higher in pSS patients than in controls. The tissue results, also highlighting a progressive increase in TSLP expression with increasing lymphoproliferation to B-cell NHL, suggest a possible pathogenetic role of TSLP in pSS. Investigation in larger series has been recently agreed within the HarmonicSS project (European Union grant 731944; https://harmonicss.eu/), and functional studies are also being developed.

Patients and methods

Patients

• *Preliminary TSLP serum study*

Ninety-one patients with pSS, all fulfilling the 2016 ACR-EULAR classification criteria (44) and all positive for anti-SSA antibodies, were preliminary studied for the expression of serum TSLP levels, in comparison with 80 sex and age-matched healthy blood donors (HBDs) and with 21 patients with non-autoimmune sicca syndrome (nSS). In addition, prospective serum samples collected from the time of MESA diagnosis to the time of NHL development were studied in 3 pSS patients, as well as serum samples from 6 pSS patients with NHL in remission after treatment. Of note, pSS patients with uncontrolled active asthma were excluded (22).

• *TSLP tissue study*

For extensive TSLP tissue study, 38 pSS patients were selected on the basis of tissue sample availability within the population that had undergone the preliminary TSLP serum study. All these 38 pSS patients, besides anti-SSA antibody positivity, also presented a SG biopsy positive for pSS (44) as inclusion criterion. They were stratified into three subgroups according to the histopathologic degree (8) of lymphoproliferation in their SG: fully benign MALT infiltrates (fbSS), MESA, and NHL. Of note, all patients had never undergone treatment with immunosuppressants, biotechnological or chemotherapic drugs, and were not receiving any systemic therapy for pSS at the time of sample collection. The disease activity was scored using EULAR Sjögren's Syndrome Disease Activity Index (ESSDAI) (45).

SG biopsies from 13 nSS controls, all showing an absent or very mild and sporadical, non-focal salivary non-specific inflammatory infiltration, were also included.

Local Ethic Committee approval and informed consent from both patients and controls were obtained.

Serum study of TSLP levels

Serum samples were collected and stored at -80°C until their use. Serum levels of TSLP, expressed as pg/mL, were measured by ELISA according to manufacturer's (R&D Systems) protocol. The test is sensitive to detect TSLP levels >3.46 pg/mL. All determinations were performed in duplicate.

RNA isolation from frozen SG and Real Time-PCR

SG samples were collected as part of clinical care for diagnostic purposes. Control RNAs pool, from SG of 24 human healthy controls, was acquired from commercial supplier (Takara).

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) from frozen SG, stored in RNAlater (Invitrogen), from patients and controls. A reverse transcriptase, one-step-PCR assay was developed for the detection and quantitation of sfTSLP and lfTSLP mRNA isoforms.

Frozen SG were re-suspended in 350 µl of buffer RLT and the lysates were processed following the manufacturer's instructions. The RNA purification was performed with silica columns able to bind selectively nucleic acids (RNeasy mini Kit, Qiagen). RNA concentration and purity were measured on the Nanodrop 2000 (Thermo Fisher Scientific) using 1 µl of RNA. The ratio between the absorbance at 260 nm and 280 nm was used to evaluate purity (ratios between 1.8 and 2.0 were considered to be pure). To remove traces of genomic DNA, the isolated RNA was treated with DNAse I (Qiagen, RNase-Free DNase Set), accordingly to the manufacturer's instructions. Then, the mRNA was reverse-transcribed and amplified.

The primers for TSLP isoforms (20) were: sfTSLP forward CGTAAACTTTGCCGCCTATGA, reverse TTCTTCATTGCCTGAGTAGCATTTAT; lfTSLP forward GGGCTGGTGTTAACTTACGACTTCA, reverse

ACTCGGTACTTTTGGTCCCACTCA. β-ACTIN was the endogenous control, according to the manufacturer.

Immunohistochemistry

A single-labeling immunohistochemistry was performed on 3.5-μm-thick formalin-fixed paraffin-embedded sections (FFPE) of SG (as above detailed: minor SG n=25; parotids n=25; submandibular n=1) from the 38 pSS and the 13 nSS subjects. SG slides were deparaffinised and rinsed twice with distilled water. The endogenous peroxidase activity was blocked by immersion in 3.5% hydrogen peroxide for 10 minutes. After three washings in PBS for 5 minutes, the sections were incubated for 1h at 37°C with the primary rabbit anti-human TSLP (Biorbyt) or rabbit anti-human TSLP-R (Biorbyt) antibodies. After three washings in PBS, the FFPE slides were incubated for 30 minutes at room temperature (RT) with a peroxidase-conjugated secondary antibody (EnVision kit, Dako). The enzymatic reaction was detected using the colorimetric DAB system (Dako), according to manufacturer's instructions.

Control stainings to confirm the antibodies specificity were performed both by using an isotype control and also by omitting the primary antibody (Fig. 7). Prostate and liver sections, constitutively TSLP-positive, were used as positive controls (21). The nuclei were counterstained with haematoxylin. Images were obtained using a Leica DMD108 digital micro imaging network (Leica Microsystems), keeping constant the acquisition parameters.

The percentage of positive cells, the intensity of the positivity (as very strong=4, strong=3, moderate=2, weak=1), and/or both (expressed in a composite score: product of percentage of positivity and intensity; ranging from 0 to 400) were evaluated and applied as appropriate.

SG sections were also evaluated for the presence of germinal centre-like ectopic structures (GCs) as previously defined (46).

Immunofluorescence

For immunofluorescence staining, deparaffinised sections were pretreated for epitope retrieval using a pH 6 citrate retrieval solution (Dako), at 98°C for 40 minutes. Then, sections were cooled for 20 minutes at RT and washed three times in PBS. To block non-specific staining, the slides were incubated for 20 minutes at RT in PBS, without Ca^{2+} and Mg^{2+}, containing 10% donkey serum.

Multiple immunofluorescence analyses were performed in all cases and controls. The expression of TSLP, CD19, CD20, CD3, CD68 and Epithelial Related Antigen Ep-CAM was detected using specific primary antibodies and subsequently evaluated by indirect immunofluorescence staining using appropriate secondary antibodies. TSLP expression was detected using an anti-TSLP rabbit polyclonal antibody (Biorbyt), while the expression of CD19 and CD20, CD3, CD68 and Epithelial Related Antigen Ep-CAM was detected using the following mouse-monoclonal antibodies: anti-CD19 clone LE-CD19 (Dako) and anti-CD20 clone L26 (Dako); anti-CD3 clone PC3/188A (Santa Cruz Biotechnology); anti-CD68 clone MAC387 (Dako); anti-Ep-CAM clone MOC31 (Dako); and subsequently evaluated by indirect immunofluorescence staining, using anti-mouse Alexa Fluor A555-labelled (Invitrogen) and anti-rabbit Alexa Fluor 488-labelled (Abcam) secondary antibodies. DAPI was used to detect nuclei. The percentage of double-positive $TSLP^+/CD19^+$, $TSLP^+/CD3^+$, $TSLP^+/CD68^+$ or $TSLP^+/Ep\text{-}CAM^+$ cells was determined calculating the ratio between the number of TSLP-positive cells among the number of total cells for each type, by counting at least 400 positive cells with a 40X oil immersion objective.

Statistical analysis

Analyses were performed using GraphPad Prism software (v. 7.02; GraphPad Software). Student *t*-test or the non-parametric Mann-Whitney and Kolmogorov-Smirnov tests were used to calculate the statistical significance between groups. Paired samples were analysed with the t-test or Wilcoxon signed-rank test, as appropriate. Dichotomous variables were compared by Fisher's exact test when requested. Correlation analyses were performed by using the Pearson correlation coefficient. A p-value <0.05 was considered significant.

Results

Patients

In the preliminary serum study including 91 anti-SSA positive pSS patients, 86 were female (94.5%), showing a mean age of 57.2 years (range 25–80).

The main features of the 38 pSS patients selected among them for the present extensive TSLP tissue study are shown in Table I. The three stratified pSS subgroups were composed as follows: 1) fbSS: n=13: 12/13 minor SG and 1/13 parotid; 2) MESA: n=13: 11/13 parotids and 2/13 minor SG; NHL: n=12; 11/12 parotids and 1/12 submandibular. The SG biopsies from 13 nSS subjects (female n=12, 93.3%; mean age: 53.3 years, 37–70) included in the study were: 11/13 minor SG and 2/13 parotids.

Serum TSLP analyses

• TSLP serum levels are elevated in pSS and increase with advancing lymphoproliferation, with the presence of germinal centres in SG and with increasing disease activity

In the preliminary study including 91 pSS patients and 101 controls (80 HBDs, 21 nSS), serum TSLP resulted significantly increased in pSS (mean 47.19 pg/mL, range 0-324.89) compared to nSS (mean 2.74 pg/mL, range 0–15.9; $p<0.0001$) and to HBDs controls (mean 0.59 pg/mL, range 0–11.09; $p<0.0001$) (Fig. 1A). The significance was the same also excluding the NHL patients (n=12) (Fig. 1B). Of note, among the 80 HBDs, TSLP was detectable only in 4 subjects at very low levels.

In the 38 pSS stratified subjects, serum TSLP showed a progressive, significant increase from fbSS (mean 23.1 pg/mL; range 0–53.06) to MESA (mean 58.34 pg/mL; range 20.62-128.52) (MESA *vs*. fbSS $p=0.0006$) and finally to NHL, where the increase was very pronounced (mean 151.96 pg/mL; range 58.16–324.89) (NHL *vs*. fbSS $p<0.0001$; NHL *vs*. MESA $p=0.0044$) (Fig. 1C).

Furthermore, by the analysis of prospective sera, an increase in TSLP levels from MESA to NHL was no-

Table I. Main features of stratified pSS patients.

	fbSS n=13	MESA n=13	NHL n=12	*p*-value
Age, years: mean (range)	57 (41-76)	55.1 (28-80)	59.7 (39-77)	ns
Female: n° (%)	12 (92.3%)	12 (92.3%)	11 (91.7%)	ns
Duration of disease, months: mean (range)	30.2 (9-65)	48.4 (14-125)	83.6 (14-185)	0.007
ESSDAI: median (range)	2 (1-12)	7 (1-12)	18 (14-26)	<0.0001
Persistent salivary gland swelling: n° (%)	2 (15.4%)	10 (76.9%)	12 (100%)	<0.0001
Anti-SSA antibodies: n° (%)	13 (100%)	13 (100%)	12 (100%)	ns
Anti-SSB antibodies: n° (%)	6 (46.2%)	9 (69.2%)	9 (75%)	ns
Rheumatoid Factor: n° (%)	8 (61.5%)	10 (76.9%)	10 (83.3%)	ns
Cryoglobulinaemia: n° (%)	0 (0%)	5 (38.5%)	7 (58.3%)	0.001
Low C3: n° (%)	2 (15.4%)	4 (30.8%)	3 (25%)	ns
Low C4: n° (%)	1 (7.7%)	5 (38.5%)	5 (41.7%)	ns
Hypergammaglobulinaemia: n° (%)	2 (15.4%)	3 (23.1%)	3 (25%)	ns
Biopsy positive for pSS diagnosis: n° (%)	13 (100%)	13 (100%)	12 (100%)	ns

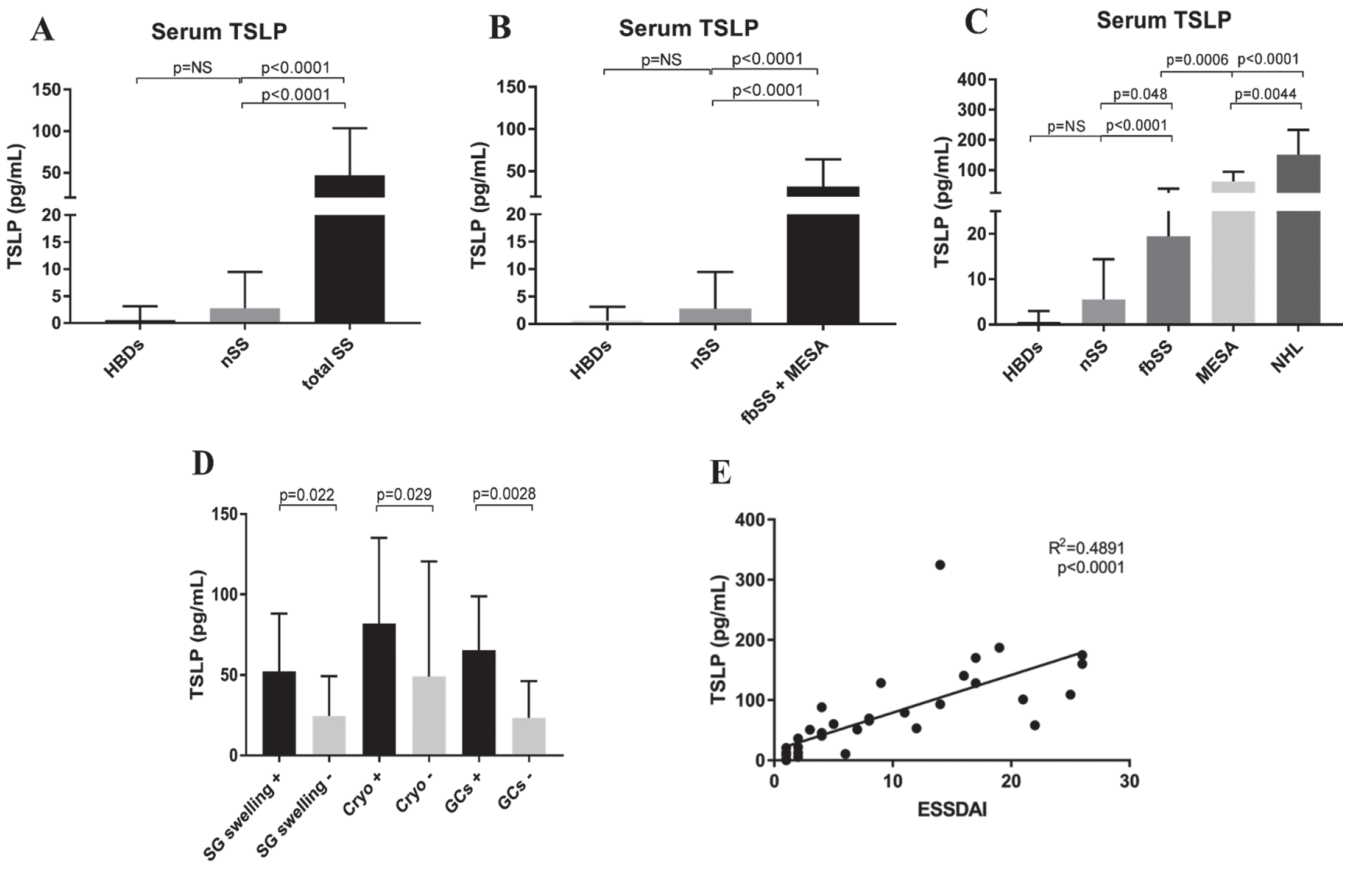

Fig. 1. Serum levels of TSLP.
A: Serum levels of TSLP are higher in pSS patients compared to HBDs and nSS controls.
B: This is observed also when pSS patients with NHL are not included in the analysis.
C: Serum TSLP shows a progressive significant increase from fbSS to MESA, and finally to NHL.
D: pSS patients with persistent parotid swelling (SG swelling +), with mixed cryoglobulinaemia (Cryo +), or with GCs-like structures in SG biopsy (GCs +) show significant higher TSLP serum levels compared to pSS patients not showing these features.
E: Serum levels of TSLP correlate with the disease activity assessed by ESSDAI (r=0.703).

ticed in all the 3 pSS studied patients, ranging from 37.6% to 704.6% (mean of serum TSLP increase: 298.4%). By contrast, in sera from pSS patients with NHL in complete remission after treatment, TSLP serum levels were similar to those of fbSS patients or completely negative in all the 6 pSS studied cases.

Relevant pSS clinical features linked to lymphoproliferation were investigated in detail. Patients with the two better recognised pre-lymphomatous pSS conditions, *i.e.* persistent SG swelling and mixed cryoglobulinaemia (3, 42, 43), showed significantly higher TSLP serum levels compared to pSS without glandular enlargement (p=0.022) and to pSS without cryoglobulinaemia (p=0.029) (Fig. 1D), respectively. pSS patients with GCs-like structures in SG biopsy, which have been associated

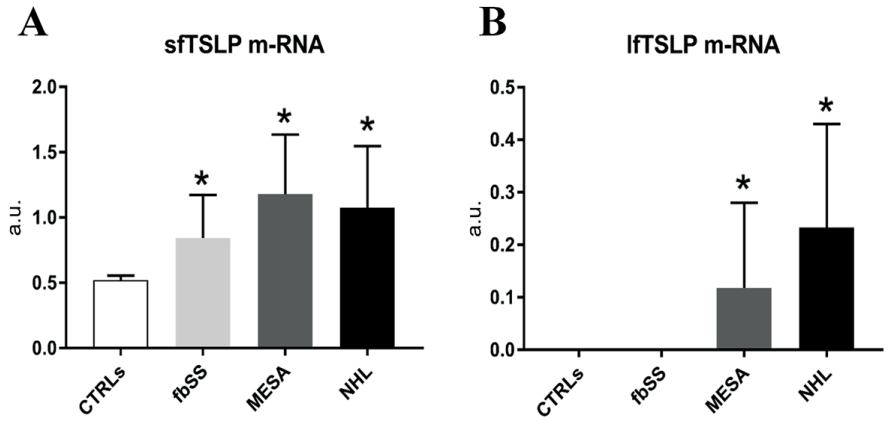

Fig. 2. Levels of m-RNA expression of TSLP isoforms in salivary glands.
A: SfTSLP mRNA levels in SG are higher in pSS compared to healthy controls (CTRLs) SG, with no difference between pSS subgroups.
B: LfTSLP mRNA is detected only in SG of pSS with MESA or with NHL. **p*<0.05, compared to CTRLs.

with an increased risk of NHL evolution (46), showed significantly higher serum TSLP levels (p=0.0028), compared to pSS without GCs-like structures (Fig. 1D). Finally, a significant correlation between higher TSLP serum levels and a higher ESSDAI, also linked to lymphoma in pSS (47), was found (r=0.703; R^2=0.4891; p<0.0001) (Fig. 1E).

TSLP tissue mRNA expression

• *Constitutive sfTSLP is upregulated in the SG of all the subsets of pSS, while inducible lfTSLP is expressed limitedly to pSS with MESA or NHL*

The constitutive sfTSLP mRNA was significantly increased in all pSS SG compared to healthy controls (CTRLs) SG, with no significant differences between pSS subgroups (Fig. 2A). By contrast, the inducible lfTSLP mRNA resulted expressed only in SG of pSS with MESA and NHL, while it was absent in both fbSS SG and, in accordance with the literature (20), in CTRLs SG (Fig. 2B).

Immunohistochemistry

• *Salivary gland epithelial cells express TSLP and the expression declines with the progression of lymphoproliferation, from fbSS to MESA and to NHL*

The SG epithelium stained TSLP-positive. However, a significant decline of the percentage of TSLP-positive epithelial cells, of the intensity of staining and of both (reported as TSLP-score in Fig. 3A) was found from fbSS (Fig. 3D), where the TSLP expression was similar to nSS (Fig. 3C), to MESA (p=0.046) (Fig. 3E) and finally to NHL (fbSS *vs*. NHL: p=0.0147; MESA *vs*. NHL: p=0.04) (Fig. 3F-G).

The inflammatory infiltrate of fbSS and MESA and the infiltrating cells of NHL stained TSLP-positive (Fig. 3B), with a less intense staining (grade 1–2) in

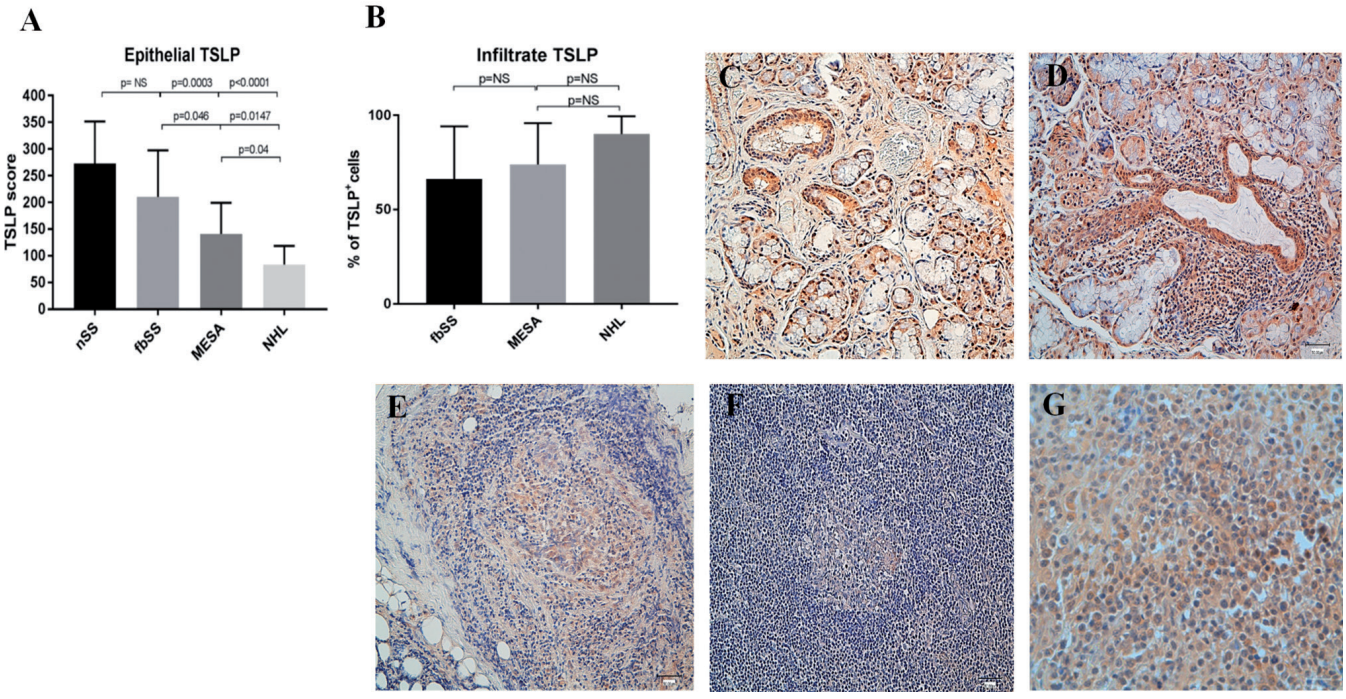

Fig. 3. TSLP expression in pSS salivary glands assessed by immunohistochemistry.
A: Histogram of epithelial TSLP expression showing similar levels in fbSS and nSS, and a significant decline from fbSS to MESA and to NHL.
B: Histogram of TSLP expression by the infiltrate showing high percentages of TSLP-positive cells in all the pSS subgroups.
C-G: Immunohistochemistry for TSLP in **C.** nSS, **D.** fbSS, **E.** MESA and **F-G.** NHL: TSLP epithelial staining declines, as well as the TSLP-positive cells percentage increases in the inflammatory/neoplastic cells from D to F-G, with a staining intensity progressively weaker (**G:** almost all the cells in NHL stain weakly positive). Original magnification: 20x; G: 40x. Scale bar 20um.

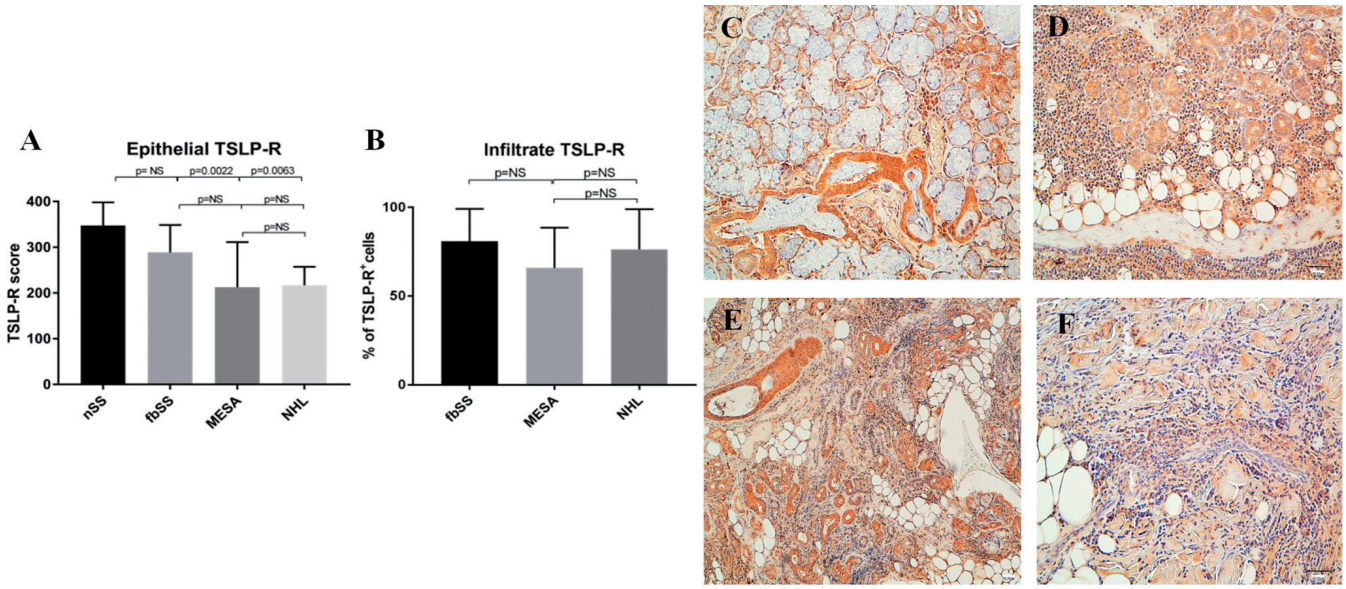

Fig. 4. TSLP-R expression in pSS salivary glands assessed by immunohistochemistry.
A: Histogram of TSLP-R expression by the salivary epithelium showing a similar expression of TSLP-R between fbSS and nSS. The residual epithelium in MESA and NHL showed a similar TSLP-R staining compared to fbSS.
B: Histogram of TSLP-R expression by the inflammatory infiltrate showing no difference in the percentage of TSLP-R-positive cells between the three pSS subgroups.
C-F: Immunohistochemistry for TSLP-R in **C.** nSS, **D.** fbSS, **E.** MESA and **F.** NHL: TSLP-R is highly expressed both by the glandular epithelium and by the inflammatory or neoplastic cells. Scale bar 20 um. Original magnification 20x.

MESA (Fig. 3E) and in NHL (Fig. 3F-G). This positivity was subsequently confirmed by immunofluorescence (see the Immunofluorescence paragraph).

• *TSLP-R is expressed in all the pSS subsets*

TSLP-R was expressed in the SG of all the pSS subgroups, both in the epithelial and in the inflammatory cells (Fig. 4A-F). Of note, also the infiltrating cells of NHL showed a positive staining (Fig. 4F).

Specifically, epithelial TSLP-R staining (Fig. 4A) was similar in fbSS (Fig. 4D), MESA (Fig. 4E) and NHL (Fig. 4F). The inflammatory or neoplastic infiltrate of fbSS, MESA and NHL, respectively, stained TSLP-R-positive (Fig. 4B, D-F) with no difference between the three pSS subgroups.

Immunofluorescence

• *TSLP-positive B-cells in salivary glands progressively increase with the progression of lymphoproliferation from fbSS to MESA and to B-cell NHL*

A progressive, significant increase in the percentage of TSLP-positive B-cells, calculated as the ratio between the number of TSLP-positive B-cells among the total number of infiltrating B-cells, was observed in SG biopsies by double staining immunofluorescence (Fig. 5A) from fbSS (mean 32.3%, range 0–80%) (Fig. 5B-D) to MESA (mean 53.4%, range 26–90%) (p=0.0344) (Fig. 5E-G) and to NHL (mean 92.9%, range 80–100%) (NHL *vs*. fbSS: p=0.0017; NHL *vs*. MESA: p=0.0023) (Fig. 5H-L).

Strikingly, the two pSS patients with MESA showing the more pronounced TSLP-positive B-cell infiltrate (90% of B-cells) both developed a B-cell NHL in the follow-up. Furthermore, in one pSS patient with a benign histopathologic picture in minor SG but with a concomitant NHL of the MALT type in the breast, the percentage of TSLP-positive B-cells infiltrating the minor SG was very high (80%).

Of note, along with the evolution from fbSS to B-cell NHL, TSLP-positive B-cells acquired an almost exclusive nuclear expression of TSLP (Fig. 6C), while in fbSS the TSLP staining was completely detected in the cytoplasmic compartment (Fig. 6A). Interestingly, in MESA samples both the compartments stained TSLP-positive (Fig. 6B). In contrast with the B-cells, TSLP positivity was detected only in a minority of the total infiltrating T cells by double immunofluorescence, both in fbSS (mean 7.5%, range 3–11%) and in MESA (mean 11%, range 2–18%) SG, with no significant difference between these subgroups. In NHL, T cells were almost completely absent and did not significantly express TSLP (mean 0,4%, range 0–1%). No difference of TSLP localisation in different cellular compartments of the T cells was observed. Rare macrophages did not show expression TSLP in all the pSS subgroups (data not shown).

Discussion

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterised by oral and ocular dryness, by many other possible glandular and extraglandular manifestations, and by a high risk of B-cell NHL evolution, which is the main cause of decreased survival. B-cell expansion represents a key event in the pathophysiology of pSS and, currently, is a main target of developing therapies (1-4, 8). Due to the large variability of pSS, an improved stratification of the disease is needed (6-8). This is relevant also because pSS represents an impor-

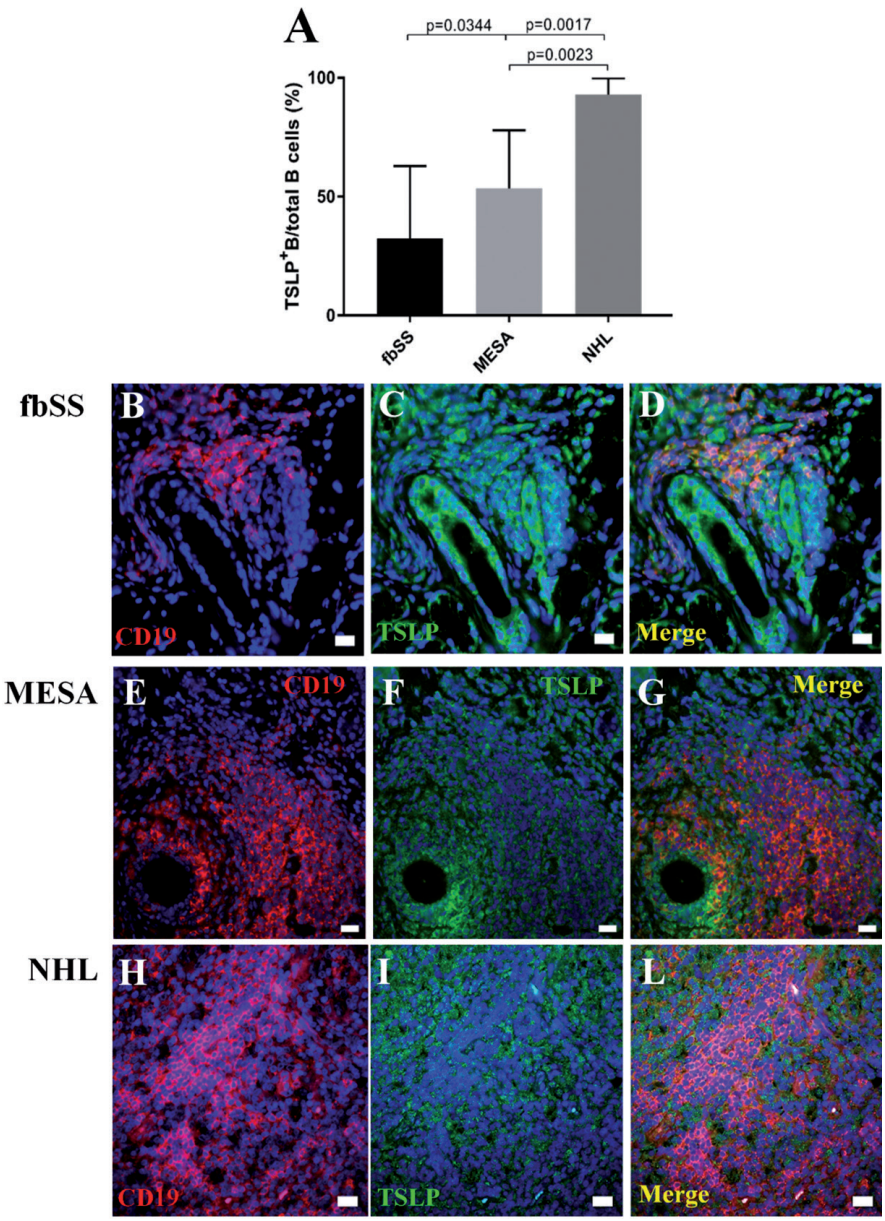

Fig. 5. TSLP expression by B lymphocytes infiltrating pSS salivary glands assessed by immunofluorescence double staining.
A: Histogram showing that the percentage of SG TSLP-positive B-cells (among the total B cells) significantly increases with the progression from fbSS to MESA and to NHL.
B-D: Immunofluorescence staining in fbSS: **B.** CD19 single staining (red), **C.** TSLP single staining (green), **D.** CD19 and TSLP double staining.
E-G: Immunofluorescence staining in MESA: **E.** CD19 single staining (red), **F.** TSLP single staining (green), **G.** CD19 and TSLP double staining.
H-L: Immunofluorescence staining in NHL: **H.** CD19 single staining (red), **I.** TSLP single staining (green), **L**:CD19 and TSLP double staining. Original magnification 20x. Scale bar 20 um. In blue: DAPI.

tant human "model" disease for B-cell lymphomagenesis in general.

In this study, the expression of TSLP in pSS-related B-cell lymphoproliferation was investigated and disclosed for the first time in definite pSS patients, stratified according to their SG histopathologic features. Three different subgroups were studied, including not only the extremes of fully benign and malignant pSS-related lymphoproliferation, but also MESA, *i.e.* an intermediate stage of B-cell lymphoproliferation between them. Preliminary unpublished data by our Group on serum TSLP levels in pSS were encouraging to this end. According to the current accepted models of lymphomagenesis, MALT B-cell NHL follows the emergence of B-cell clonal expansion (48). Cytokines, chemokines and local growth factors, produced by the salivary epithelium and the inflammatory infiltrate, contribute to this process (10-13).

TSLP is involved in maintaining immune tolerance and in regulating lymphocyte homeostasis (15-17, 33-35). A role of TSLP has been also reported in numerous diseases, including allergic, autoimmune, inflammatory and malignant disorders (22-32, 36-38), leading to the concept of both regulatory and harmful TSLP effects in different contexts. The evaluation of such a dual role of TSLP improved after the identification of two biologically opposite TSLP isoforms, the short form (sfTSLP) and the long form (lfTSLP), controlled by two different promoter regions and presently not distinguishable by immunoassays because of a complete C-terminal extremity overlapping (20, 21). The sfTSLP is constitutively expressed at the surface epithelial barriers, and thus also by the normal SG epithelium (20). It shows homeostatic functions and antimicrobial activity and is downregulated or totally undetectable in several pathologic disorders (21). By contrast, the lfTSLP is induced under pro-inflammatory conditions, while it is very low/undetectable at the steady state (20, 21, 49). The contribution of TSLP to B-cell malignancy and B-cell autoimmune response has been reported in mice models (33, 36-38) and in human B acute lymphoblastic leukaemia, Hodgkin lymphoma, multiple myeloma and HCV-related CV (31, 32, 39-41).

In this study, TSLP serum levels were shown to increase from fully benign lymphoproliferation to prelymphomatous conditions (*i.e.* persistent glandular swelling and mixed cryoglobulinaemia) and finally to malignant B-cell lymphopoliferation in stratified pSS. Furthermore, an increase in serum TSLP levels from MESA to NHL was also noticed in prospective sera from single patients, while such an increase was never observed when lymphoma was in complete remission. Finally, significantly higher TSLP serum levels were observed in two conditions associated with lymphoma progression,

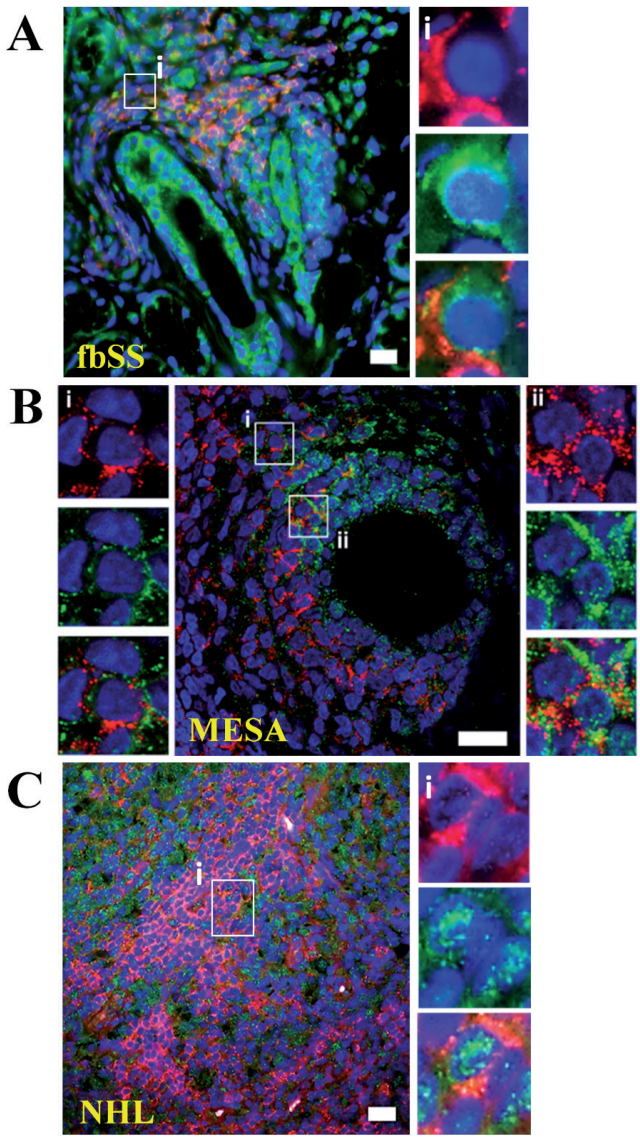

Fig. 6. TSLP is differently localised in cell compartments of B lymphocytes in salivary glands with different degrees of pSS lymphoproliferation. **A**: Cytoplasmic TSLP expression by B cells of fbSS (A-i): CD19 (red) and TSLP (green) co-localise in B-cell cytoplasmic compartment. **B**: Cytoplasmic and mixed (nuclear/cytoplasmic) TSLP localisation in B cells in two different areas (B-i and B-ii) of MESA: TSLP (green) is expressed in i) cytoplasmic and in ii) both nuclear and cytoplasmic compartment of B cells; in red CD19. **C**: Nuclear TSLP expression by B cells of NHL (C-i): TSLP (green) is mainly expressed in B-cell nuclei; in red CD19. Original magnification 20x. Scale bar 20 um. In blue: DAPI.

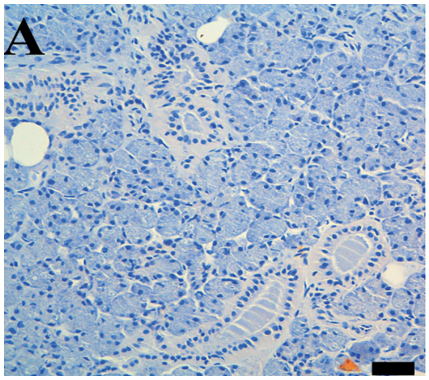

Fig. 7. A. Negative control of TSLP staining by immunohistochemistry in salivary gland. Scale bar 20 um. Original magnification 20x.

i.e. the presence of GCs-like ectopic structures in SG (50), and a higher pSS activity (47). Overall, an association between TSLP and pSS-related lymphoproliferation was suggested by all these TSLP serum studies. These studies need in any case to be replicated and validated in larger series, as planned in the HarmonicSS project (European Union grant 731944; https://harmonicss.eu/),

Rather, this study aimed to analyse the expression of TSLP directly in the affected tissue in different subgroups of pSS patients, showing different degrees of B-cell lymphoproliferation. In this way, a possible role of TSLP in pSS-related B-cell lymphoproliferation can be better highlighted.

The most impressive evidence, to us, was the significant increase in the percentage of TSLP-positive B-cells, among total B-cells, along with the progression of pSS B-cell lymphoproliferation. This percentage was maximal in malignant lymphoma, where almost all the B-cells were TSLP-positive. RT-PCR, differentiating between the expression of constitutive sfTSLP and inducible lfTSLP, was important for data interpretation. The presence of lfTSLP mRNA was detected in pSS stages characterised by a higher B-cell lymphoproliferation, *i.e.* MESA and lymphoma. Then, *in situ* studies, coupled with molecular analyses, suggest that the TSLP increase in pSS, associated with increasing lymphoproliferation, may be mainly linked to the B-cells, rather than to production by epithelial cells or by other sources (such as T cells or macrophages). A B-cell autocrine loop can be hypothesised, based on the expression also of the TSLP-R by the tissue lymphomatous cells in this study (Fig. 4B, F), and additional functional analyses are planned.

Of major interest, to us, was also the evidence that the malignant B lymphocytes in NHL biopsies expressed TSLP almost exclusively in the nuclei, whereas TSLP expression was limited to the cytoplasmic compartment of B-cells in fbSS, and in both the compartments in prelymphomatous MESA samples (Fig. 6). Since the import or export of proteins from the nucleus to cytoplasm, or vice-versa, has been linked to cell deregulation and to malignant lymphoma, representing a potential target of treatment (51-53), additional investigation is worthwhile in pSS also for this issue. B-cell expression of TSLP in advanced pSS-related lymphoproliferation does not exclude a possible role also of TSLP produced by the epithelium and the inflammatory infiltrate in earlier stages of pSS. A significantly increased expression of constitutive sfTSLP mRNA was shown in SG of pSS with respect to healthy controls. In addition, an intense TSLP staining in both the epithelium and the inflammatory infiltrate was shown in the fully benign stage of pSS. These two observations could mirror a TSLP production in response to an antigenic stimulation in pSS MALT lesions. An initial infectious trigger, hypothesised in the pathophysiology of pSS, as well as of B-cell lymphoma in general (48, 54, 55), could induce TSLP

expression for a barrier protective response and TSLP, on the other hand, could also support the initial phases of B-cell expansion.

In conclusion, this study shows for the first time that TSLP increases with advancing lymphoproliferation in pSS, based on the investigation of selected tissue samples from well characterised and stratified patients.

A possible pathogenetic involvement of TSLP in pSS-related B-cell expansion and lymphoma evolution could be then hypothesised. Further research is needed, also including TSLP as a possible novel biomarker of pSS.

Key messages

- TSLP increases in pSS-related advancing B-cell lymphoproliferation.
- Salivary gland-infiltrating B-cells express TSLP, maximally in pSS-related lymphoma.
- TSLP might be involved in the pSS evolution into B-cell malignancy.

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