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Corneal sub-basal nerve plexus assessment and its association with phenotypic features and lymphocyte subsets in Sjögren's Syndrome

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ABSTRACT.

Purpose: **To assess and compare corneal sub-basal nerve plexus morphology with circulating lymphocyte subsets, immunologic status and disease activity in Sjögren syndrome (SjS) patients.**

Methods: **Fifty-five SjS patients, 63 Sicca patients (not fulfilling SjS criteria), 18 rheumatoid arthritis (RA) patients and 20 healthy controls (HC) were included. Systemic disease activity in SjS was assessed with the ESSDAI score. Lymphocyte subpopulations were studied with flow cytometry. Corneal confocal microscopy and ImageJ software were used to characterize corneal sub-basal nerve plexus in terms of nerve density (CNFD), length (CNFL) and tortuosity (CNFT). Conventional dry eye tests were also performed.**

Results: **CNFL and CNFD were lower in SjS, Sicca and RA groups, compared to HC ($p < 0.001$ for both SjS and Sicca); CNFL $p = 0.005$, CNFD $p = 0.018$ in RA). CNFT was higher in SjS, followed by Sicca, RA and HC. A negative correlation was found between ESSDAI score and CNFL ($r=-0.735$, $p = 0.012$). CNFL correlated negatively with $IL21^+CD8^+$T cells ($r=-0.279$, $p = 0.039$) and a positively with total memory ($r = 0.299$, $p = 0.027$), unswitched memory ($r = 0.281$, $p = 0.038$) and $CD24^{Hi}CD27^+$ ($r = 0.278$, $p = 0.040$) B cells. CNFD showed a tendency to significance in its negative correlation with ESSDAI ($r=-0.592$, $p = 0.071$) and in its positive correlation with switched memory B cells ($r = 0.644$, $p = 0.068$).**

Conclusions: **This is the first study aiming to correlate ocular findings with lymphocyte subsets in SjS. The associations founded between CNFL and CNFD and disease activity, $IL21^+$ follicular T cells and some B-cell subsets suggest that corneal nerve damage may parallel systemic disease activity and inflammatory cells' dynamics.**

Key words: corneal confocal microscopy – corneal sub-basal nerve plexus – dry eye – EULAR Sjögren's Syndrome Disease Activity Index – flow cytometry – lymphocyte subsets – Sjögren's Syndrome

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Background

Sjögren's syndrome (SjS) is a chronic systemic autoimmune disease characterized by lymphocytic infiltration and damage of the exocrine glands, predominantly affecting the salivary and lacrimal glands. Dry eye and dry mouth are cardinal features of SjS, usually being the presenting symptoms (Kassan & Moutsopoulos 2004). In SjS, dry eye is characterized by decreased tear flow due to lacrimal gland inflammatory damage (Bron et al. 2017). Lacrimal gland lymphocyte infiltration in SjS correlates with the degree of glandular dysfunction (Tsubota et al. 1996), and the resulting aqueous deficiency leads to tear hyperosmolarity. In turn, hyperosmolarity stimulates ocular surface cells to produce inflammatory cytokines (interleukin IL-1, IL-6 and tumour necrosis factor TNF-α,) and proteases (De Paiva et al. 2006), which leads to additional recruitment of inflammatory cells. The apoptosis of surface epithelial cells (Yeh et al. 2003) and non-apoptotic death of corneal epithelial cells (Sahin et al. 2012) further contributes to this inflammatory vicious cycle.

Both innate (macrophages and NK-cells) and adaptative ($CD4^+$ T cells –

particularly Th1 and Th17) immune responses are involved in SjS pathogenesis (Baudouin 2001). The infiltration of exocrine glands by IL-21-producing $CXCR5^+$ follicular helper T cells (Tfh; Szabo et al. 2013) is crucial to B-cell survival and ectopic formation of germinal-centre(GC)-like structures, and contributes to perpetuating glandular dysfunction. CXCR5 is also expressed in CD8 + T cells, pointing to the existence of a follicular cytotoxic T-cell subset (Tfc; Quigley et al. 2007).

In fact, despite the common inflammatory pathway in all dry eye disease (DED) patients, recent studies reveal a greater upregulation of cytokines in tear fluid in SjS compared to non-SjS dry eye (Lee et al. 2013; Chen et al. 2019). They corroborated the interplay between innate and adaptive immunity in SjS dry eye pathogenesis. B cells also appear to be implicated in ocular manifestations of SjS, although their role is less clear (Stern et al. 2012).

Classical DED evaluations, such as the Shirmer's test, tear break-up time and corneal staining measurement have a low-to-moderate reproducibility and are often influenced by external factors (Nichols et al. 2004). Therefore, new tools for corneal assessment have been used lately, namely *in vivo* confocal microscopy (IVCM), which evaluates corneal sub-basal nerve plexus morphology. Unmyelinated corneal nerves are particularly prone to degeneration, also occurring due to immune or inflammatory conditions (Petropoulos et al. 2019). The damage and subsequent regrowth of these nerves have been described in DED (Belmonte et al. 2017). In SjS, several studies demonstrated significant differences in corneal sub-basal nerve plexus morphology (density, length and tortuosity) compared to healthy individuals (Benítez-del-Castillo et al. 2007; Tuisku et al. 2008; Cardigos et al. 2019).

Our purpose was to evaluate corneal sub-basal nerve plexus and to explore possible correlations between corneal nerves degeneration, immune parameters and disease activity in SjS patients.

Methods

Population

We included adult patients from the Rheumatology Department of *Instituto Português de Reumatologia* and *Hospital Cuf Descobertas*, with confirmed or suspected SjS. Patients fulfilling the AECG criteria formed the SjS group, whereas patients with sicca symptoms not fulfilling AECG criteria formed the 'Sicca' group. AECG criteria consist of 2 clinical items (ocular and oral symptoms), 2 objective items (ocular signs and oral diagnostic tests) and 2 immunological items (presence of focal lymphocytic infiltrates in the minor salivary gland biopsy, and positivity for anti-SSA or anti-SSB antibodies). To fulfil these criteria, at least 4 of the 6 items are necessary, being mandatory to have at least one of the immunological items. However, in a couple of patients, the salivary gland histopathology item was unfulfilled due to lack of minor glands in the biopsy, in which case the presence of anti-SSA/SSB antibodies allowed classification. To prevent overlap between the SjS and Sicca groups, all patients included as Sicca had a screening for anti-SSA/SSB antibodies and a valid minor salivary gland biopsy.

The AECG criteria also define several exclusion criteria: current anticholinergic therapy, hepatitis C and human immunodeficiency virus infection, pre-existing lymphoma, sarcoidosis, graft-versus-host disease, and history of head and neck radiation treatment.

Additional exclusion criteria considered were IgG_4-related disease, other systemic or organ-specific autoimmune diseases, diabetes mellitus or other possible causes of peripheral neuropathy, neurodegenerative diseases, ocular surface disease other than DED, contact lens use, previous ophthalmic surgery and treatment with drugs of known corneal toxicity.

Patients with Rheumatoid Arthritis (RA) without sicca symptoms were included as a control group with another autoimmune disease. A healthy control (HC) group was selected from the Ophthalmology outpatient clinic.

Disease activity in SjS was assessed with the EULAR SjS Disease Activity Index (ESSDAI) (Seror et al. 2010), which consists of 12 domains (constitutional, lymphadenopathy, glandular, articular, cutaneous, pulmonary, renal, peripheral nervous system, central nervous system, muscular, hematologic and biologic), each divided into 3–4 levels of activity. Clinically active disease was defined as activity in any ESSDAI domain, except the hematologic and biologic. Additionally, some patients with musculoskeletal inflammatory involvement were considered as active disease even though they didn't score in the ESSDAI articular domain.

Informed consent was obtained from all participants. The study was approved by the Ethics committees of both recruiting institutions and NOVA Medical School (no.17/2016/CEFCM).

Ocular examinations

All ocular examinations were performed by the same examiner, who was unaware of the subject's condition/group, under standardized conditions of room illumination (low illumination), temperature (20–25°C) and relative air humidity (40–60%; Whitcher et al. 2010).

Conventional DED tests

Conventional ocular tests for DED evaluation followed the previously defined sequence (Lemp 1995). After performing Schirmer's test I, a drop of 0.5% fluorescein was applied and two minutes later, tear break-up time (TBUT)TBUT was measured. A TBUT < 10 seconds was considered abnormal and consistent with dry eye. Finally, corneal staining score (CSS) was assessed by fluorescein staining and classified according to the Oxford grading scale (Bron, Evans & Smith 2003).

IVCM – corneal sub-basal nerve plexus assessment

Corneal sub-basal nerve plexus assessment was performed through IVCM (Heidelberg® Retina Tomograph II, Rostock Cornea Module), according to the protocol established by Tavakoli *et al*(Tavakoli & Malik 2011).

Three to five best-focused, non-overlapping and most representative images were analysed, using ImageJ software, plugin NeuronJ. Central corneal images were selected based on the live imaging given by the camera attached to the microscope and the nerves' vertical orientation(Petroll & Robertson 2015; Kalteniece et al. 2017). As previously reported, sub-basal nerve plexus was characterized in terms of nerve density (CNFD), length (CNFL)

and tortuosity (CNFT). CNFD was defined as the total number of major nerves per square millimetre of corneal tissue; CNFL, was defined as the total length of all nerve fibres and branches, in mm per square millimetre of corneal tissue(Malik et al. 2003); and CNFT was classified as grade 0: almost straight; grade 1: slightly tortuous; grade 2: moderately tortuous; grade 3: quite tortuous; grade 4: very tortuous (Oliveira-Soto & Efron 2001).

Flow cytometry measurements

To perform immunophenotyping, peripheral blood samples were collected into EDTA-containing tubes, processed and analysed within 24 hours after collection.

Pre-validated panels of monoclonal antibodies were used to characterize lymphocyte subpopulations, including CD3[2], CD4[2], CD19[1], CD24[1], CD25[1], CD27[1], CD38[1], CD127[1], CCR6[2], CCR7[1], CXCR3[2], CXCR5[2], Anti-IgD[1] and Anti-IgM[2] ([1]from Biolegend, San Diego, CA, USA; [2]from BD Biosciences, San Jose, CA, USA).

Samples were acquired in a 4-color BD FACS Calibur™ cytometer (BD Biosciences), and cells were analysed with CellQuestPro™ software (BD Biosciences). The staining protocol was described elsewhere (Barcelos et al. 2018). The study addressed several subsets of T cells, including Regulatory T cells (Tregs) and Follicular T cells (Tfh), as well as distinct naïve and memory B-cell subsets.

Each subset was evaluated in percentages and absolute counts, for which a single-platform strategy was used, with BD Trucount tubes™ (BD Biosciences).

Complete gating strategies are described in supplementary data.

Functional assays for the evaluation of IL21-producing T cells

T cells' functional capacities were addressed, particularly IL21 secretion, a cytokine typically associated with Tfh/Tfc subsets. IL17 production was assessed to identify IL21-producing Th17 cells.

Heparinized peripheral blood samples were collected and cells were stimulated with PMA and ionomycin, for 5h at 37°C in a 5% CO_2 atmosphere in the presence of brefeldin A. After stimulation, cells were lysed, washed and incubated with anti-CD3 and anti-CD8 for surface staining. For intracellular stain, cells were treated according to the protocol defined by the manufacturer for the BD Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (BD Biosciences) and marked with anti-IL21 and anti-IL17, after cell fixation and permeabilization. For each patient, stimulated and unstimulated tubes were run in parallel to assure proper stimulation and staining controls. Again, a 4-color BD FACS Calibur™ cytometer (BD Biosciences) and Cell Quest Pro™ software (BD Biosciences) were used for acquisition and analysis. Gating strategies are presented in supplementary data.

Statistical analysis

All data were analysed using GraphPadPrism™ software, version 8 for Windows (GraphPad Software, San Diego, CA, USA). Statistical significance was concluded when *P*-value < 0.05. The normality of distributions was assessed using the D'Agostino and Pearson test. Categorical variables were expressed as numbers and percentages and analysed using Fisher's exact test. Continuous variables were presented as mean (standard deviation) or median (25th–75th percentile), as applicable. The Mann–Whitney *U* test was used to compare every two independent groups. For the assessment of correlations, Spearman correlation coefficients were calculated.

Results

Patients' characteristics

We included fifty-five SjS patients, 63 patients in the Sicca group, 18 RA patients (mean age of 55.3 ± 13.7 years) and 20 subjects in the HC group (mean age of 51.0 ± 6.5 years). SjS and Sicca patients' demographic and clinical features are shown in Table 1.

Ocular assessment

Schirmer's I test mean values were significantly lower in the SjS, Sicca and RA groups, compared to HC ($p < 0.001$) and the frequency of a lower TBUT was also higher in those groups ($p < 0.001$) (Table 2). No significant differences were found between SjS, Sicca and RA patients concerning Schirmer's-I and TBUT. Figure 1A presents data regarding CSS.

CNFD and CNFL were significantly lower in SjS, Sicca and RA groups, compared to HC ($p < 0.001$ for SjS and Sicca in CNFD and CNFL; RA: $p = 0.018$ for CNFD and $p = 0.005$ for CNFL). No differences were found between SjS and Sicca patients regarding IVCM measurements, but CNFD was significantly lower in both groups compared to the RA group ($p \leq 0.040$). CNFL was also significantly lower in Sicca patients compared to RA ($p = 0.044$) (Table 2). SjS, Sicca and RA patients presented higher values of CNFT compared with HCs, without significant differences between them (Fig. 1B).

In SjS patients with higher disease activity (ESSDAI ≥ 5), a negative correlation was found between the ESSDAI and both the CNFL ($r=-0.735$, $p = 0.012$) and the CNFD ($r=-0.592$, $p = 0.071$) (Fig. 2).

Lymphocyte subsets

$CD4^+$ and $CD8^+$ T-cell subsets

Comparing T-cell subsets between SjS patients and HC, we observed that both $CD4^+$T-cell percentages and absolute counts were lower in SjS patients ($p = 0.003$ for percentages; $p < 0.001$ for absolute counts), whereas $CD8^+$ T-cell percentages were higher in SjS ($p = 0.006$) without differences in absolute counts (Table 3, Table S1). Compared to Sicca patients, SjS patients also presented lower $CD4^+$ T-cell percentages ($p = 0.002$) and absolute counts ($p = 0.044$). No differences were found between SjS and RA patients.

Tregs absolute counts were lower in all patients' groups when compared to HC, although with similar percentages. In SjS patients, a negative correlation was found between the ESSDAI score and Tregs numbers ($r=-0.341$, $p = 0.011$), which was stronger when considering patients with moderate/high disease activity (ESSDAI ≥ 5) ($r=-0.862$, $p = 0.001$) (Fig. 3).

Regarding follicular T-cell subsets, no differences were found in the percentages of $CXCR5^+CD4^+$Tfh cells when comparing SjS patients with any of the other groups.

Table 1. Patients´ characteristics

	SjS $n = 55$	Sicca $n = 63$
Age (years, mean ± SD)	57.8 (11.8)	60.8 (10.9)
Age at diagnosis (years, mean ± SD)	51.8 (11.6)	57.5 (10.8)
Symptom duration (years, mean ± SD)	11.7 (7.7)	9.9 (5.0)
Ocular symptoms, *n* (%)	52 (94.5)	60 (95.2)
Oral symptoms, *n* (%)	53 (96.4)	61 (96.8)
Ocular signs, *n* (%)	34 (61.8)	33 (52.4)
Oral signs, *n* (%)	40 (72.7)	43 (68.3)
Extraglandular disease (ever), *n* (%)	23 (41.8)	23 (36.5)
Clinically active disease, *n* (%)	27 (49.1)	9 (14.3)
ESSDAI ≥ 5, *n* (%)	9 (16.4)	NA
Joint symptoms, *n* (%)	24 (43.6)	32 (50.8)
Skin, *n* (%)	16 (29.1)	17 (27.0)
Other Extraglandular involvement, *n* (%)	8 (14.5)	0 (0)
Raynaud's phenomenon, *n* (%)	8 (14.5)	16 (25.4)
Focus score ≥ 1, *n* (%)	42 (79.2)*	0 (0)
Anti-SSA, *n* (%)	37 (67.3)	0 (0)
Anti-SSB, *n* (%)	11 (37.5)†	0 (0)
ANA ≥ 1/320, *n* (%)	44 (80.0)	35 (55.6)
ANA ≥ 1/640, *n* (%)	31 (56.4)	16 (25.4)
RF, *n* (%)	23 (47.9)†	18 (29.5)‡
Gammaglobulin ≥ 1.6 g/dl, *n* (%)	12 (21.8)	3 (4.8)§
Therapy (any), *n* (%)	30 (54.5)	29 (46.0)
Glucocorticoids, *n* (%)	18 (32.7)	17 (27.0)
Hydroxychloroquine, *n* (%)	19 (34.5)	19 (30.2)
Immunossupressants, *n* (%)	9 (16.4)	8 (12.7)

ANA = antinuclear antibody; Anti-SSA/SSB = anti-Sjögren's syndrome A/B antibody; RF = rheumatoid factor; SjS = Sjögren's syndrome.
* $n = 53$.
† $n = 48$.
‡ $n = 58$.
§ $n = 62$.

However, Tfh1 cells percentages ($CXCR3^+CCR6^-CXCR5^+CD4^+$) were increased in SjS when compared to Sicca (p = 0.009), RA (p = 0.064) and HC (p = 0.011) (Table 3, Table S1). Although SjS patients presented lower absolute numbers of Tfh cells compared to both RA (p = 0.068) and HC (p < 0.001), the absolute counts of the Tfh1 subset were similar between the 3 groups of patients (SjS, Sicca, RA), but lower in SjS (p = 0.006) and Sicca (p = 0.009) when compared to HC.

Exploring the association between Tfh subsets and disease activity, we found a positive correlation between the ESSDAI and Tfh1 percentages, (r = 0.295, p = 0.029), which was more significant in patients with active disease (r = 0.607, r = 0.001), and among those, stronger when considering patients with ESSDAI ≥ 5 (r = 0.914, r = 0.001) (Fig. 3. Conversely, a negative correlation was found between the ESSDAI and Tfh17 percentages (r=−0.365, p = 0.006), which was also stronger in patients with active disease (r=−0.482, p = 0.013) or with ESSDAI ≥ 5 (r=−0.705, p = 0.023).

As for $CXCR5^+CD8^+$Tfc cells, no differences were found in percentages and absolute counts when comparing SjS with Sicca, RA or HC.

IL21 production by follicular T cells

In the functional evaluation for IL21 production, SjS patients were found to have increased percentages of IL21-secreting $CD4^+$T cells when compared to Sicca (p = 0.007), RA (p = 0.064) and HC (p = 0.013), although without significant differences in absolute

Table 2. Ophthalmic assessment. comparison between SJS, SICCA, RA AND HC

					Group comparisons (p-values)†					
	SjS $n = 55$	Sicca, $n = 63$	Rheumatoid Arthritis, $n = 18$	Healthy Controls, $n = 20$	SjS versus Sicca	SjS versus HC	SjS versus RA	Sicca versus RA	Sicca versus HC	RA versus HC
Oral and ocular objective features*										
Ocular assessment										
Schirmer test I (mm)	6 (6.05)	7.22 (5.61)	7.00 (6.00)	21.55 (7.74)	0.178	**<0.001**	0.501	0.78	**<0.001**	**<0.001**
TBUT (low, %)	70.9	52.4	77.8	0	0.058‡		0.763‡	0.063‡		
Tear meniscus height (mm)	0.34 (0.21)	0.42 (0.23)	0.29 (0.22)	0.45 (0.07)	0.068	0.064	0.342	**0.033**	0.694	**0.009**
Tear meniscus area (mm^2)	0.06 (0.06)	0.07 (0.05)	0.03 (0.03)	0.07 (0.02)	0.100	0.060	0.128	**0.007**	0.773	**0.001**
Sub-basal nerve plexus density (mm/mm^2)	10.26 (6.49)	9.08 (3.01)	11.97 (4.79)	15.35 (5.14)	0.657	**<0.001**	**0.040**	**0.010**	**<0.001**	**0.018**
Sub-basal nerve plexus length (mm/mm^2)	28.13		(12.94)	26.23 (9.13)	32.97 (12.46)	43.92 (12.92)	0.822	**<0.001**	0.120	**0.044**
<0.001	**0.005**									

* Mean and SD, unless otherwise stated.
† Mann–Whitney test, unless otherwise stated.
‡ Fisher's exact test.
Bold numbers represent significant differences (p < 0.05).

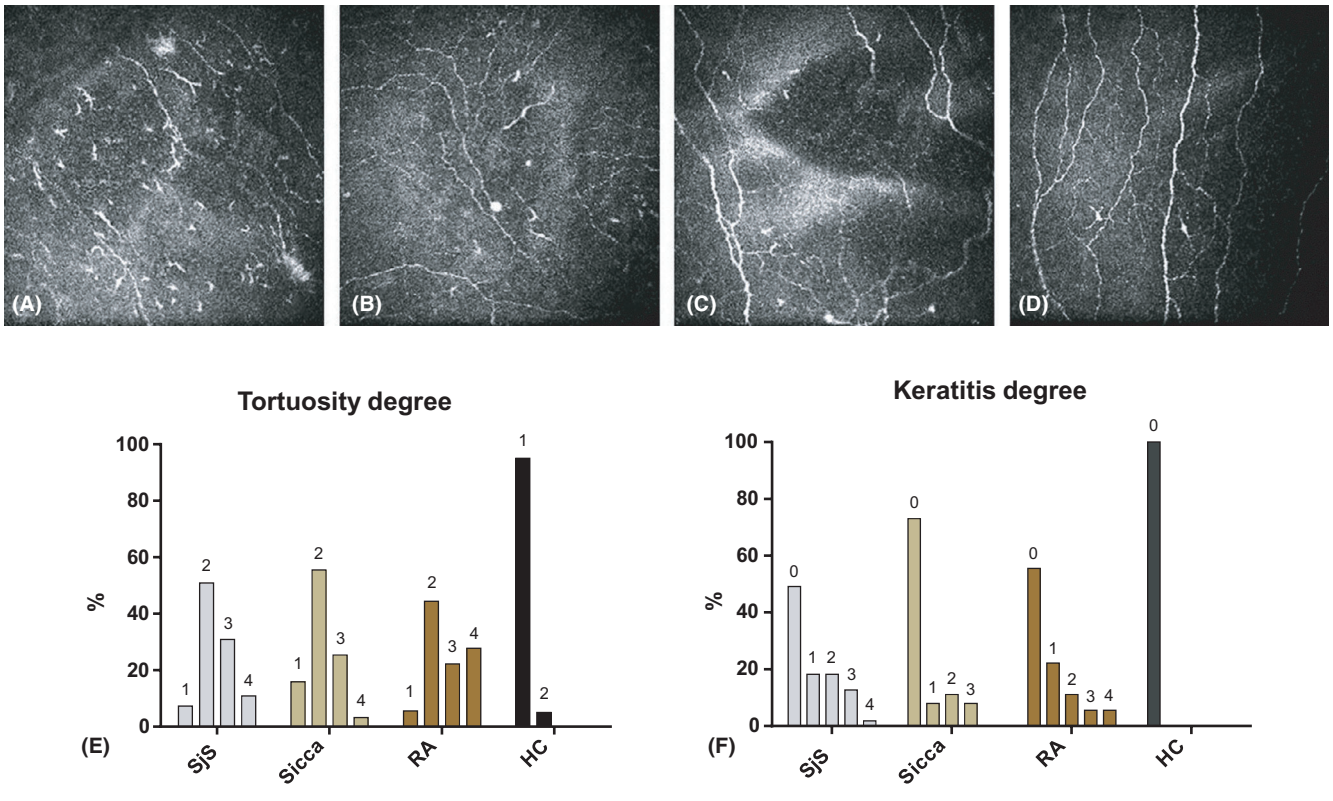

Fig. 1. Ocular Examinations. In vivo confocal microscopy images of the corneal sub-basal nerve plexus in a patient with SjS (A), in a Sicca group patient (B), in a patient with RA (C) and in a HC subject (D), showing reduced nerve density and length and increased nerve tortuosity in the former three compared to the healthy control. Heidelberg Retina Tomograph II, Rostock Cornea Module images 400 x 400µm. Bar plot with the distribution of corneal nerve tortuosity (E) and keratitis (F) degrees within each group. SjS, Sjögren's syndrome. RA, Rheumatoid Arthritis. HC, Healthy Controls

counts. IL21-secreting $CD8^+$T-cell percentages were also increased in SjS patients when compared to Sicca ($p = 0.071$), RA ($p = 0.002$) and HC ($p = 0.022$), with a corresponding increase in absolute counts which reached significance when comparing SjS with RA patients ($p = 0.016$). Data are shown in Table 3 and Table S1.

Moreover, when considering SjS patients with clinically active disease, a positive correlation was found between the ESSDAI score and either $IL21^+CD4^+$ ($r = 0.446$, $p = 0.023$) and $IL21^+CD8^+$ T-cell ($r = 0.470$, $p = 0.016$) percentages (Fig. 3).

B-cell subsets

The percentages of IgD^+CD27^-B cells (naïve) were higher in SjS patients when compared to RA ($p = 0.044$) and HC ($p = 0.065$), whereas memory B-cell ($CD27^+$) percentages were lower in SjS comparing to HC ($p = 0.029$).

Transitional $IgM^{++}CD38^{++}$B cells were increased in SjS patients with a numerical trend to significance when compared to Sicca ($p = 0.064$).

Among B-cell subsets known for being enriched in regulatory cells, $CD24^{Hi}CD38^{Hi}$B cells were increased in SjS patients, with significant differences when compared to Sicca ($p = 0.026$) and RA ($p = 0.037$). Conversely, a significant decrease in $CD24^{Hi}CD27^+$B cells was found in SjS patients, when compared to Sicca ($p = 0.045$), and HC ($p = 0.003$). Results are summarized in Table 3 and Table S1.

Finally, we explored the association between B-cell subsets and disease activity in SjS patients and found significant negative correlations between the ESSDAI and absolute counts of memory B cells ($r=-0.316$, $p = 0.019$), switched memory B cells ($r=-0.315$, $p = 0.019$) and $CD24^{Hi}CD27^+$B cells ($r=-0.270$, $p = 0.047$) (Fig. 4). A stronger negative correlation was found between the ESSDAI and switched memory B cells ($r=-0.651$, $p = 0.041$) and $CD24^{Hi}CD27^+$B cells ($r=-0.644$, $p = 0.045$) when considering only patients with ESSDAI ≥ 5.

Association between ophthalmic parameters and lymphocyte subsets

We then explored the association between ocular and immune parameters.

Regarding B cells, positive correlations were found between CNFL and absolute counts of total memory ($r = 0.299$, $p = 0.027$), unswitched memory ($r = 0.281$, $p = 0.038$) and $CD24^{Hi}CD27^+$ B cells ($r = 0.278$, $p = 0.040$), and a tendency for a positive correlation between CNFD and switched memory B-cell counts ($r = 0.644$, $p = 0.068$) (Fig. 5).

Considering T cells, a negative correlation was found between CNFL and $IL21^+CD8^+$ T-cell counts ($r=-0.279$, $p = 0.039$). Moreover, when considering only patients with ESSDAI ≥ 5, a tendency for a positive correlation between CNFL and Tregs counts was found ($r = 0.644$, $p = 0.069$), as well as a strong negative correlation between Tfh1 and both CNFL and CNFD ($r=-0.760$, $p = 0.017$ and r-0.700, $p = 0.043$, respectively (Fig. 5).

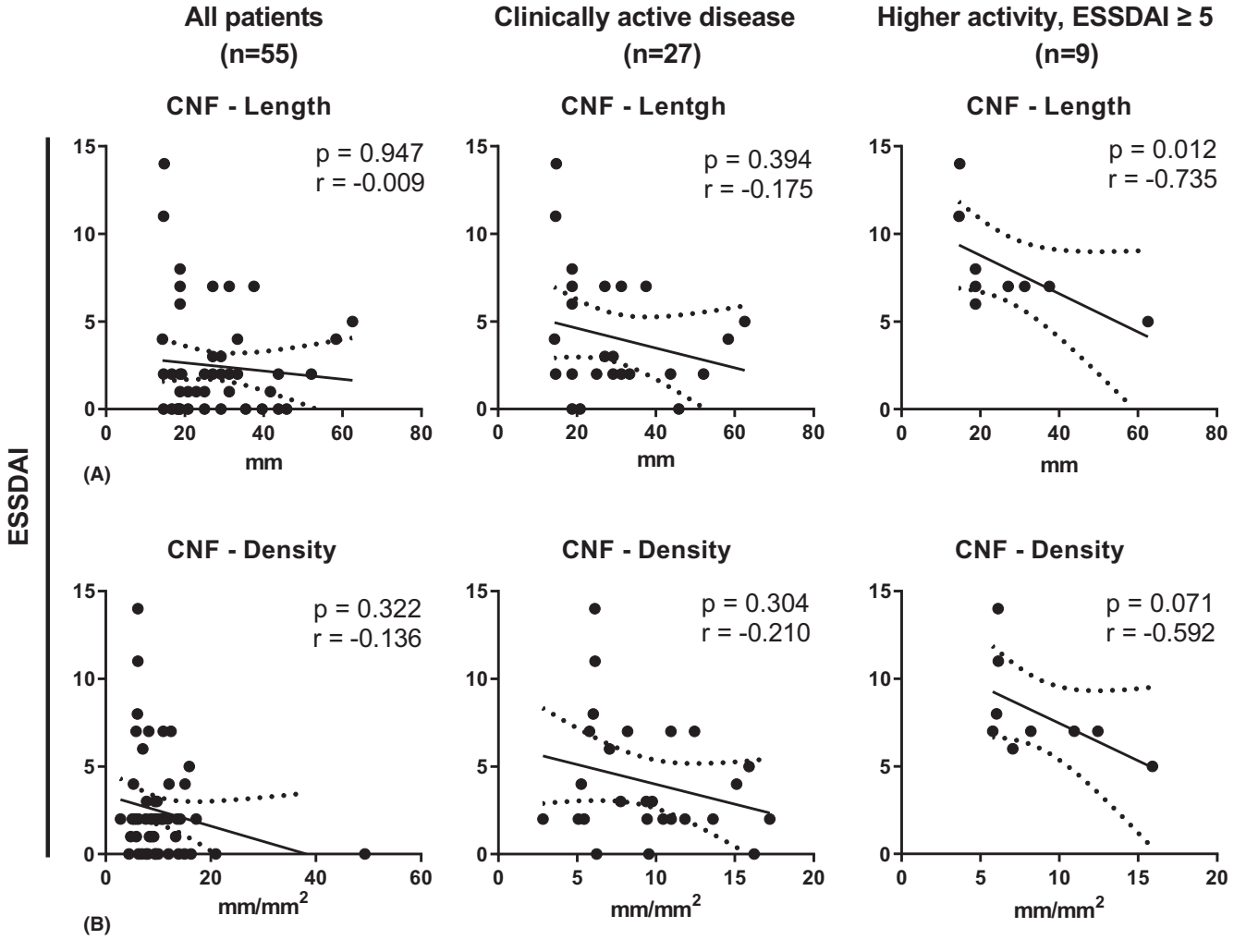

Fig. 2. ESSDAI score correlations with CNF Length (A) and CNF Density (B) in all patients, patients with clinically active disease, and patients with ESSDAI score ≥ 5. Spearman correlation coefficients, 95% confidence interval and p-values are indicated. CNF, Corneal Nerve Fibre

Discussion

Our study aimed to characterize ocular objective findings in SjS patients, particularly the study of corneal innervation through confocal microscopy, and to explore their association with clinical and immunological features of the disease. Thus, changes in circulating lymphocyte subsets were studied to assess their relationship with ocular and clinical manifestations, as well as disease activity.

Although we found no differences in the percentages of circulating $CXCR5^+$ follicular T cells in SjS patients, our study supports the evidence of an increased follicular compartment in SjS when considering $IL21^+CD4^+$ and $IL21^+CD8^+$ T-cell subsets. A modest positive correlation was found between ESSDAI and $IL21^+CD8^+$ T cells in the whole SjS group. However, greater correlations were found between disease activity and both Tfh and Tfc cells when considering only patients with clinically active disease, which was even more robust considering patients with higher disease activity (ESSDAI ≥ 5).

We also found, among $CXCR5^+CD4^+$ T cells, a higher proportion of the Tfh1 subset in SjS, which also correlated with ESSDAI. This observation may be in concordance with the accepted Th1-derived responses (Saito et al. 2018) in the pathogenesis of SjS.

Our data on the association between disease activity and follicular T cells not only are in accordance with the proposed role of Tfh cells in the pathogenesis of SjS (Verstappen et al. 2017; Pontarini, Lucchesi & Bombardieri 2018) but also suggest, for the first time, the involvement of Tfc.

We confirmed a decrease in absolute numbers of circulating T cells, which is a well-described feature of SjS (Talal et al. 1974). This could indicate retention of these cells at target organs, as supported by previous studies showing a T-cell predominance in lymphocytic infiltrates of exocrine glands (Singh & Cohen 2012). The noteworthy exception was the higher numbers of $IL21^+CD8^+$ T cells, which showed a numerical trend to significance, compared to the other study groups, reinforcing the suggestion of a role of these cells in SjS pathology.

Our cohort also evidenced the distinctive B-cell profile classically considered for SjS – lower levels of memory subsets and increased naïve and transitional compartments, as previously reported (Roberts et al. 2014; Barcelos et al. 2018). These B-cell disturbances in SjS may represent the presence of a more immature component in transit to target organs and a shift in maturation towards the plasma cell lineage. Also, the increased frequency of circulating

Table 3. Comparison of lymphocyte subsets and ratios (percentages) in SJS, SICCA, RA and healthy controls

Lymphocyte	SjS	Sicca	Rheumatoid Arthritis	Healthy Controls	Group comparisons (p-values)					
%. median (25th-75th percentile)	$n = 55$	$n = 63$	$n = 18$	$n = 20$	SjS versus Sicca	SjS versus RA	SjS versus HC	Sicca versus HC	Sicca versus RA	RA versus HC
Lymphocyte	31.5 (24.5–37.2)	32.2 (25.5–36.4)	23.9 (18.8–30.6)	35.8 (29.3–39.8)	0.723	**0.022**	**0.039**	**0.043**	**0.004**	**<.001**
B cells	9.7 (6.7–13.4)	11.2 (8.5–14.8)	6.1 (4.1–7.1)	10.8 (8.4–14.4)	0.106	**<0.001**	0.321	0.803	**<0.001**	**<0.001**
T cells	75.0 (69.1–78.4)	71.6 (67.2–77.1)	80.3 (72.6–83.4)	74.7 (70.1–79.3)	0.116	**0.044**	0.684	0.079	**0.004**	0.092
CD4+	61.2 (53.0–67.3)	65.9 (60.5–70.2)	65.3 (49.0–72.6)	68.4 (60.8–75.8)	**0.002**	0.278	**0.003**	0.378	0.553	0.274
Th1	39.0 (29.0–46.2)	39.8 (30.4–47.3)	33.8 (28.9–47.2)	36.8 (30.2–47.1)	0.701	0.541	0.885	0.626	0.421	0.647
Th17	20.6 (13.9–29.2)	22.4 (16.4–28.7)	23.4 (14.8–29.0)	24.4 (16.3–32.9)	0.427	0.794	0.286	0.601	0.878	0.567
Tregs	8.2 (6.5–10.5)	7.6 (7.0–8.9)	7.6 (6.2–9.3)	7.2 (6.7–8.7)	0.144	0.348	0.241	0.948	0.675	0.981
CD8+	38.4 (32.0–47.0)	34.1 (29.8–39.5)	34.8 (27.4–51.0)	31.6 (23.0–39.2)	**0.005**	0.355	**0.006**	0.345	0.554	0.262
CXCR5 + CD4+ T cells	18.6 (14.7–23.7)	18.2 (14.9–22.3)	20.0 (15.6–24.8)	20.6 (16.9–22.7)	0.804	0.572	0.55	0.291	0.362	0.935
Expression of CCR3 and CCR6*										
Tfh1 (CCR3 + CCR6-)	36.2 (30.3–40.8)	31.7 (28.9–36.4)	29.1 (25.7–39.0)	30.6 (25.2–35.2)	**0.009**	0.064	**0.011**	0.283	0.435	0.649
Tfh17 (CCR3-CCR6+)	21.4 (18.1–26.8)	23.0 (19.0–27.0)	24.4 (16.4–29.0)	20.9 (18.3–30.4)	0.287	0.475	0.646	0.794	0.798	0.934
Surface expression of CCR7†										
Tfh Naïve (CCR7+)	18.1 (14.6–23.3)	18.0 (14.8–21.7)	19.7 (15.3–23.9)	20.2 (16.6–22.0)	0.980	0.515	0.515	0.298	0.425	0.981
Tfh Differentiated (CCR7-)	0.39 (0.26–0.89)	0.3 (0.18–0.57)	0.42 (0.20–0.71)	0.36 (0.22–0.67)	**0.025**	0.887	0.558	0.238	0.21	0.834
CXCR5 + CD8+ T cells										
Tfc Naïve (CCR7+)	1.9 (1.2–2.8)	2.0 (1.4–3.4)	1.2 (0.8–2.5)	2.0 (1.3–2.8)	0.369	0.087	0.715	0.694	**0.021**	0.075
Tfc Differentiated (CCR7-)	0.72 (0.44–1.30)	0.67 (0.45–1.10)	0.59 (0.25–1.25)	0.75 (0.51–0.99)	0.795	0.561	0.960	0.718	0.494	0.608
IL-21 producing CD4 + T cells										
IL21+	12.5 (8.4–15.0)	9.6 (7.5–12.7)	9.5 (8.2–11.7)	9.5 (6.3–10.9)	**0.007**	0.064	**0.013**	0.454	0.837	0.628
IL21 + IL17+	0.67 (0.54–0.96)	0.73 (0.55–1.00)	0.63 (0.33–0.85)	0.68 (0.24–1.07)	0.724	0.313	0.448	0.445	0.177	0.902
IL-21-producing CD8 + T cells										
IL21+	4.1 (2.5–5.7)	3.1 (2.1–4.4)	2.4 (1.2–3.2)	2.6 (1.0–4.4)	0.071	**0.002**	**0.022**	0.200	**0.018**	0.447
IL21 + IL17+	0.29 (0.13–0.46)	0.23 (0.16–0.60)	0.16 (0.09–0.54)	0.33 (0.15–0.75)	0.935	0.323	0.290	0.396	0.128	0.113
B-cell subsets										
IgD and CD27 surface expression										
Naïve	66.6 (52.0–77.5)	67.1 (47.8–75.2)	55.8 (27.6–66.3)	53.8 (42.6–74.2)	0.555	**0.044**	0.065	0.235	0.072	0.618
Memory	29.5 (20.1–44.2)	31.1 (22.7–45.4)	32.2 (24.2–59.7)	44.3 (23.6–54.7)	0.399	0.214	**0.029**	0.169	0.488	0.618
Unswitched memory	13.6 (8.7–22.0)	17.5 (10.9–25.5)	16.3 (10.3–28.4)	21.6 (13.7–32.2)	0.166	0.488	**0.028**	0.195	0.833	0.249
Switched memory	14.5 (9.89–21.3)	14.7 (10.7–21.7)	17.4 (13.7–30.7)	18.7 (13.7–27.5)	0.802	0.127	0.133	0.119	0.155	0.979
Transitional CD24HiCD38Hi	5.7 (3.1–9.5)	4.0 (2.2–5.8)	3.0 (1.0–6.1)	3.2 (2.5–6.5)	**0.026**	**0.037**	0.119	0.778	0.255	0.389
CD24HiCD27+	16.9 (11.9–25.6)	21.4 (13.9–35.8)	23.2 (12.1–38.6)	32.0 (20.0–42.5)	**0.045**	0.281	**0.003**	0.139	0.855	0.287
Plasmablasts IgM-/+ CD38++	2.0 (0.9–3.5)	1.2 (0.8–1.9)	1.4 (1.3–2.9)	1.4 (1.0–1.9)	**0.006**	0.459	0.063	0.507	0.052	0.373

* Percentages among Tfh. CCR3 + CCR6+ and CCR3-CCR6- cells not shown.
† Includes Tfh1, Tfh17 and other subsets.
Bold numbers represent significant differences ($p < 0.05$).

follicular T cells in SjS may represent the escape of some cells from target organs where they are undergoing expansion and supporting GC-like structures.

A hallmark feature of SJS is decreased tear flow, but several other conditions may also impair lacrimal gland function (Conrady, Joos & Patel 2016). Ocular assessment by classical tests (Schirmer's-II, TBUT) revealed that SjS, Sicca and RA patients had lower levels compared to HC.

It is known that tear composition is affected by the lacrimal gland inflammatory environment and that corneal integrity and innervation are compromised in DED (Tsubota et al. 2017). Therefore, the development of novel and more specific tests to assess ocular involvement in SjS is an active field of research.

In this study, SjS and Sicca patients presented a significantly lower CNFD and CNFL, as well as a higher CNFT, compared to HC, which has already been described (Tuisku et al. 2008; Belmonte et al. 2017; Cardigos et al. 2019). Interestingly, our non-sicca RA group presented lower CNFL and CNFD than HC, and higher CNFT, suggesting an influence of the

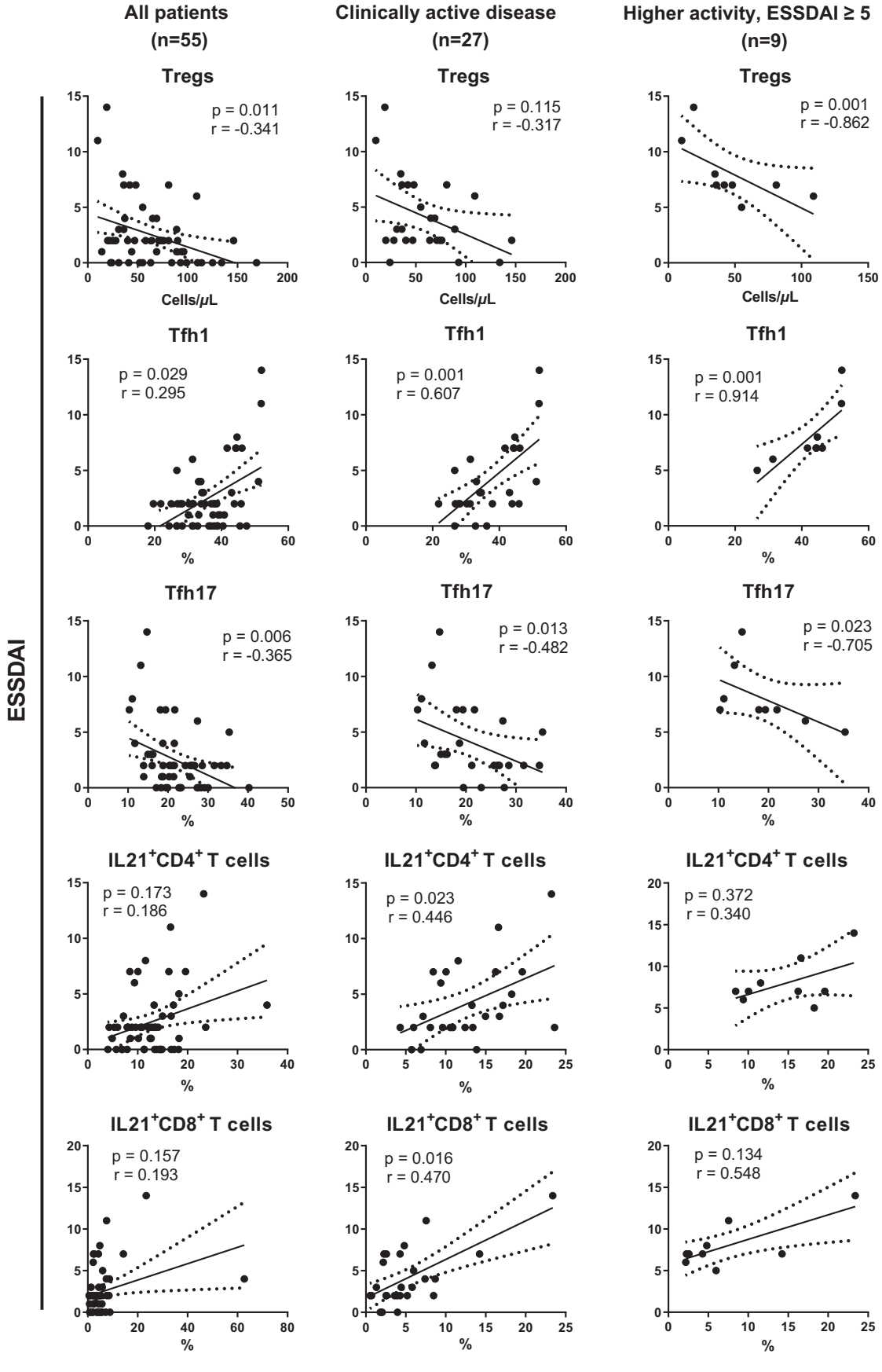

Fig. 3. ESSDAI score correlations with T-cell subsets in all patients (n = 55), patients with clinically active disease and patients with ESSDAI score ≥ 5 (n = 9). Spearman correlation coefficients, 95% confidence interval, and p-values are indicated

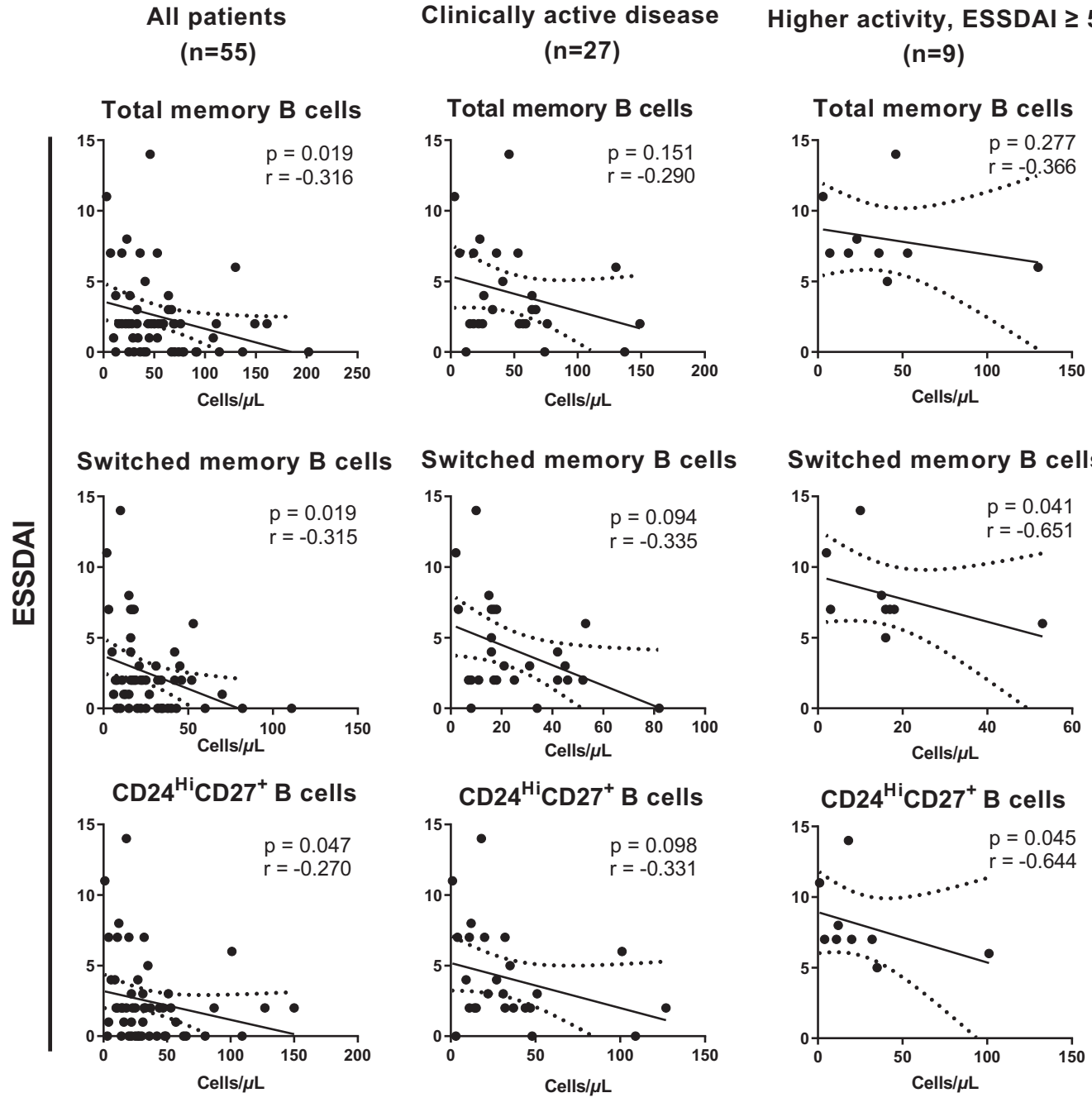

Fig. 4. ESSDAI score correlations with B-cell subsets in all patients, patients with clinically active disease and patients with ESSDAI score ≥ 5. Spearman correlation coefficients, 95% confidence interval and p-values are indicated

inflammatory environment in corneal nerve damage and regrowth. Despite that, the differences found in corneal nerve morphology between both pSS and Sicca patients and RA patients may represent the impact of DED on cornea innervation.

Although corneal nerves characterization could be a promising instrument to evaluate ocular signs of SjS, the relationship between their changes and disease phenotype, systemic disease activity, and lymphocytic profile had not been explored before. Exploring the association between disease activity and corneal nerve parameters, we found a strong negative correlation between the ESSDAI and CNFL and CNFD in patients with higher disease activity (ESSDAI ≥ 5). In fact, patients with higher ESSDAI presented worst ocular outcomes, that is lower CNFD and CNFL. These data suggest that corneal nerve damage may parallel systemic disease activity, driven by the immune environment in these patients, since patients with higher disease activity presented higher levels of Tfc cells, but lower Tregs, and even $CD24^{Hi}CD27^{+}$B-cell subsets. Ocular dysfunction, along with disease activity, could then relate to the lack of the protective immunosuppressive subsets, such as Tregs and regulatory B cells within the $CD24^{Hi}CD27^{+}$ compartment.

The absence of a more evident association between disease activity and corneal nerve parameters in the whole SjS population may be due to the

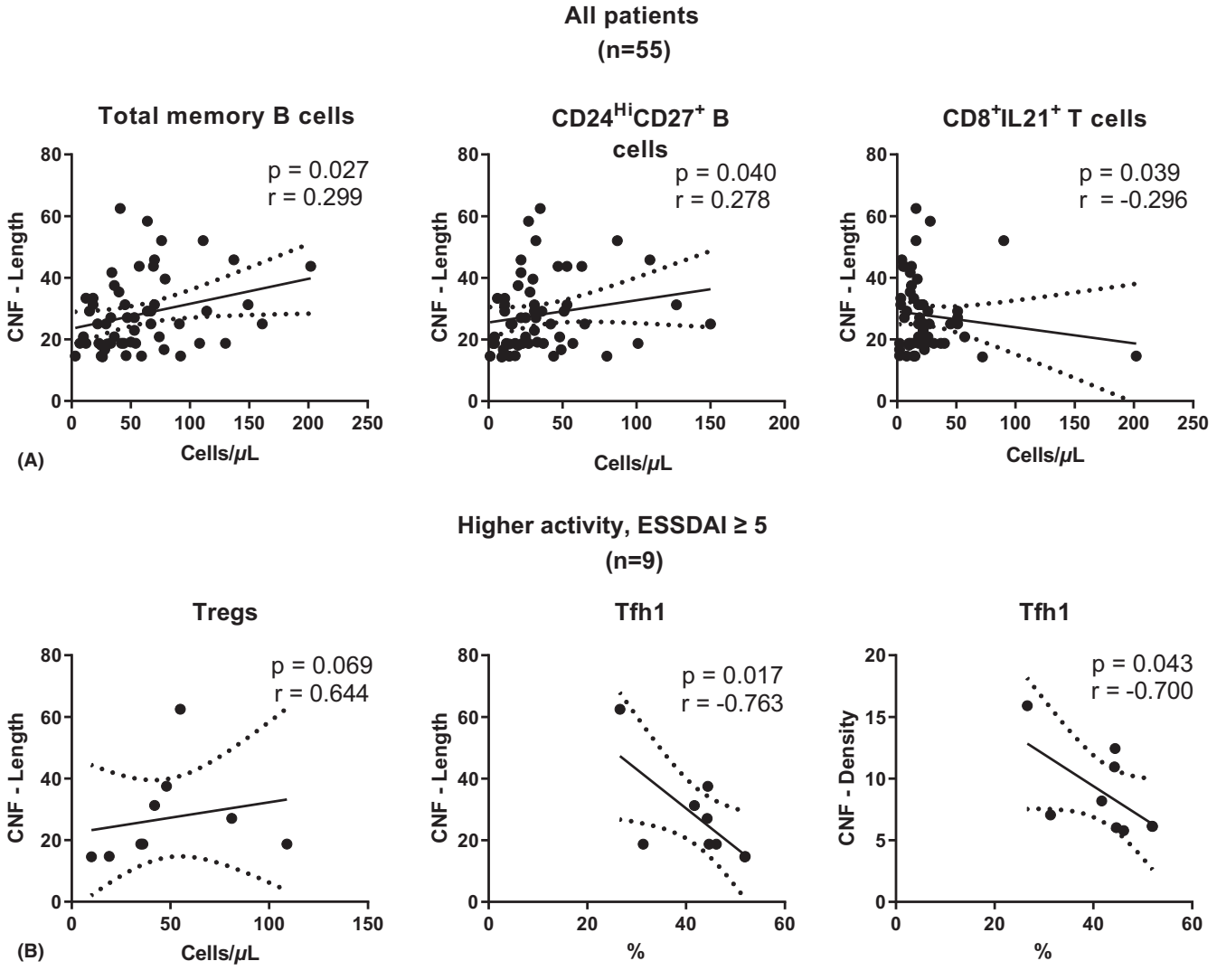

Fig. 5. CNF Length and Density correlations with T- and B-cell subsets in all patients (A), and in patients with ESSDAI score ≥ 5 (B). Spearman correlation coefficients, 95% confidence interval and p-values are indicated. CNF, Corneal Nerve Fibre

presence of many stable patients with long-standing disease and undergoing systemic therapy, who had inactive disease or very low ESSDAI scores. The assessment of SjS patients with less evolved disease and not undergoing therapy could better clarify the association between corneal nerve parameters, disease activity and lymphocyte subset distribution. Moreover, the possibility to assess local tissues could also help to clarify the immune cell movements between different target sites.

Regarding our study limitations, our study included patients with variable disease duration, which could influence some findings. The effect of therapies in lymphocyte populations is also elusive and unquantified. SjS patients (as well as Sicca and RA patients) were under different therapeutic strategies and this might affect lymphocytic populations and, consequently, the correlations observed between lymphocytic populations and ocular features. Also, we evaluated exclusively peripheral blood lymphocyte subsets, even though the salivary and lachrymal glands are major target organs.

A future study would aim to verify the induction of lymphocyte subsets by ocular surface cells, to demonstrate if ocular cells can induce autoimmune epithelitis the same way exocrine glands do, demonstrating that ocular cells are also active participants in the destructive autoimmune cycle of SjS (Manoussakis & Kapsogeorgou 2010).

This is the first study conducted to correlate ocular findings with lymphocyte subsets in SjS. DED damage to the sub-basal corneal nerve plexus was evidenced, particularly in patients with SjS, which was greater with longer duration and disease activity. Moreover, the association between disease activity and some B-cell subsets with CNFD and CNFL favours the hypothesis that corneal nerve damage may parallel systemic disease activity. Evaluating ocular findings, particularly the cornea, not only for SjS diagnosis but also to assess treatment efficacy might be increasingly explored considering the easy access to this tissue. Further prospective longitudinal studies may clarify these results.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Gating strategy for the identification of distinct T and B-cell subsets.
Fig S2. Identification of follicular T cell subsets.
Table S1. Comparison of lymphocyte subsets and ratios (absolute counts) in Sjögren (SjS), Sicca, Rheumatoid Arthritis (RA) and healthy controls (HC).