Diagnostic potential of soluble and membrane-bound inducible T-cell co-stimulator in Sjögren's disease

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Abstract

Objective

Our aim was to evaluate protein levels of soluble inducible T-cell co-stimulator (ICOS) and membrane-bound ICOS on CD4+ and CD8+ T cells, and its ligand ICOSL on B cells and monocytes in newly diagnosed and established Sjögren's disease (SjD) patients, sicca, and healthy controls. Additionally, we aimed to investigate their associations with SjD clinical features and their diagnostic potential.

Methods

We included 39 newly diagnosed SjD patients, 10 established SjD patients, 30 sicca controls and 27 healthy controls. Soluble ICOS levels were measured in serum using ELISA test and ICOS expression on CD4+ and CD8+ T cells and ICOSL expression on B cells and monocytes were measured in whole peripheral blood using flow cytometry.

Results

Soluble ICOS and ICOS expressed on CD4+ T cells were significantly elevated in newly diagnosed SjD patients. Soluble ICOS showed a positive association with rheumatoid factor. ICOS expression on CD4+ T cells showed positive correlation with gamma globulins, and positive association with anti-Ro antibodies and rheumatoid factor. ROC curveanalysis revealed strong ability of soluble ICOS and ICOS expressed on CD4+ T cells to differentiate between SjD patients at the time they enter diagnostic process and sicca and healthy controls.

Conclusion

Soluble ICOS levels and ICOS expression on CD4+ T cells are elevated in SjD patients when they enter diagnostic process. Notably, this is the first study to report the high diagnostic potential of both markers, supporting their further evaluation as potential biomarkers in SjD.

Key words

Sjögren's disease, inducible T-cell co-stimulator, inducible T-cell co-stimulator ligand

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Introduction

Sjögren's disease (SjD) is a chronic systemic autoimmune disease characterised by lymphocyte infiltration of the exocrine glands, leading to oral and ocular dryness known as sicca symptoms. Besides exocrine gland dysfunction, patients may develop a diverse spectrum of systemic manifestations spanning from mild yet impactful symptoms like fatigue and arthralgia to severe, potentially life-threatening complications such as interstitial lung disease, cryoglobulinaemic vasculitis or lymphoma (1). This heterogeneous presentation and the non-specificity of sicca symptoms, makes the early SjD diagnosis challenging. In addition, as there are no diagnostic criteria for SjD, the diagnosis relays on combination of clinical evaluation, laboratory, and histopathological examination (2). Numerous studies have been conducted in the quest for new biomarkers, but despite these efforts, a significant gap remains in establishing robust biological criteria for diagnosing SjD.

Integrative transcriptomic analyses of salivary glands and peripheral blood of SjD and non-SjD patients or healthy controls, which included differential gene expression analysis, gene co-expression network analysis and pathway analysis, discovered several important hub genes, of which the inducible T-cell co-stimulator (ICOS) gene was identified in all studies (3-5). ICOS is a member of the CD28 superfamily of immune co-receptors and its expression is induced on the cell surface of activated T cells (6). Its binding counterpart is the ICOS ligand (ICOSL), expressed on antigen-presenting cells such as B cells, dendritic cells and macrophages, as well as on non-hematopoietic cells under inflammatory conditions (7). The ICOS-ICOSL signalling pathway plays an important role in adaptive immunity. It promotes the activation and differentiation of T-cells, as well as the production of effector cytokines and is crucial for T cell-dependent B cell responses (8). In addition, crosstalk between T cells and immunofibroblasts via the ICOS-ICOSL pathway in chronic inflammation drives the production of chemokines required for the formation of tertiary lymphoid structure (9), which are known to be present in the salivary glands of SjD patients.

It has been shown that ICOS mRNA expression is increased in both the salivary glands and peripheral blood of SjD patients (3-5). ICOS mRNA expression in the salivary glands was positively associated with xerostomia, focus score, the presence of anti-Ro antibodies, immunoglobulin levels and the ESSDAI score (3). In addition, integrative transcriptomic analysis of peripheral blood from SjD patients identified ICOS mRNA as a potential diagnostic marker (4). ROC curve analysis of ICOS mRNA levels in peripheral blood showed its excellent diagnostic value not only in differentiating between SjD and non-SjD patients, but also in comparing SjD patients with rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) patients (3).

In contrast to the evidence at the mRNA level, studies investigating ICOS protein expression and its clinical or diagnostic relevance in SjD are much sparser. One study reported an increased expression of ICOS in salivary glands of SjD patients at the protein level, as detected by immunofluorescence microscopy (5). The surface expression of ICOS in $CD4^+$ T cells from peripheral blood was investigated by Garcia-Espinoza *et al.* (10). The group showed that ICOS expression on the surface of $CD4^+$ T cells in peripheral blood of SjD patients is increased compared to healthy controls and that high expression of ICOS is associated with focus score. Protein expression of ICOS was also measured on the surface of T follicular helper cells by Verstappen *et al.* (11). They found that ICOS expression is increased in SjD patients compared to healthy controls. Only two research groups investigated the concentrations of soluble ICOS in the peripheral blood of SjD patients. Both reported elevated levels of soluble ICOS in SjD patients (3, 5).

While some attention has been paid to the expression of ICOS, the expression and clinical significance of its binding partner ICOSL in SjD remains relatively understudied. Nayar *et al.* (9) showed that the expression of ICOSL is elevated in salivary gland tissue of SjD patients with tertiary lymphoid structures. However, to our knowledge, there is cur-

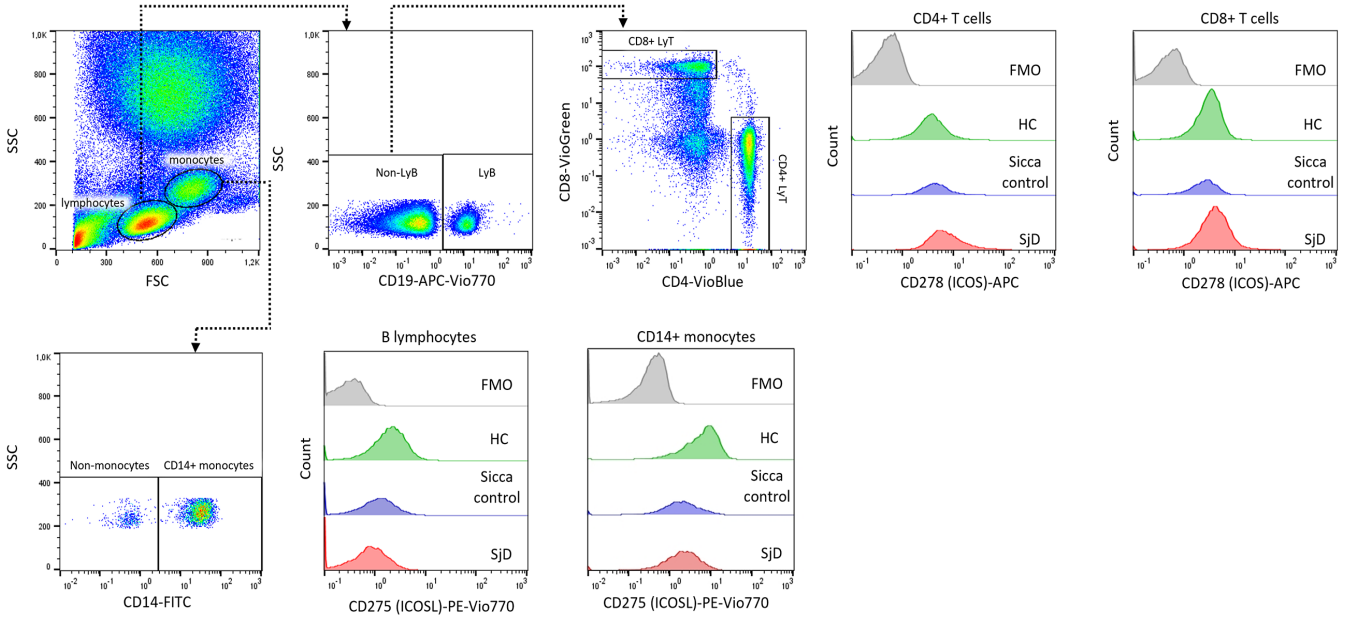

Fig. 1. Gating strategy. Cells were identified based on forward and side scatter parameters and were further divided into B and non-B lymphocyte populations based on CD19 staining. The population of non-B cells was further divided into CD4+ T cells and CD8+ T cells based on CD4 and CD8 staining. Monocytes were identified based on forward and side scatter and based on CD14 staining. FMO represents negative control.
FMO: fluorescence minus one; FSC: forward scatter; HC: healthy controls; MFI: mean fluorescence intensity; SjD: Sjögren's disease; SSC: side scatter.

rently no evidence of ICOSL expression on immune cells in peripheral blood in SjD, nor of its potential clinical or diagnostic significance.

In this study, we analysed protein levels of soluble ICOS, membrane-bound ICOS and ICOSL in newly diagnosed and established SjD patients. Furthermore, we investigated their associations with SjD clinical characteristics and evaluated their diagnostic potential.

Materials and methods

Study design and study population

We included 69 patients with suspected SjD and 10 patients with an established SjD. All patients had sicca symptoms. Patients with suspected SjD underwent routine diagnostic procedures for SjD and were then stratified into two groups: the 'newly diagnosed SjD patients' group (n=39), which comprised patients that fulfilled the 2016 ACR/EULAR SjD classification criteria (2), and the 'Sicca controls' group (n=30) which consisted of patients in whom the SjD diagnosis was ruled out during the diagnostic process. Patients with a known diagnosis of SjD were included in an "established SjD " group (n=10). Blood samples were collected from all patient groups for serologic and flow cytometric analyses. Additionally, blood samples were collected from 27 healthy control subjects, who tested negative for antinuclear (ANA) and extractable nuclear antigen (ENA) autoantibodies and had normal levels of the acute phase protein serum amyloid A (SAA). Informed consent was obtained from all participants prior to enrolment in the study. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Republic of Slovenia (no. 0120-395/2022/3).

Clinical and demographic data collection

Patients' and healthy controls' demographic data, such as gender and age were collected. Patients' clinical data were collected, including the presence of sicca symptoms and minor salivary gland (MSG) histology characteristics, such as focus score, presence of germinal centres (GC), and lymphoepithelial lesions (LELs). The presence of anti-Ro and anti-La antibodies, ANA, rheumatoid factor (RF), and levels of gamma globulins were recorded. Xerostomia and xerophthalmia were evaluated by an unstimulated salivary flow (USF) rate and with Schirmer's test. Patients underwent salivary gland ultrasonography (SGUS) to evaluate morphological changes in major salivary glands using the OMERACT scoring system (12). Systemic disease activity was evaluated using the ESSDAI (13). Symptom burden of dryness, pain and fatigue was measured by the ESSPRI questionnaire (14). Treatment status with immunomodulatory drugs was recorded.

ELISA

Peripheral blood was collected in a vacutainer tube without additives. After centrifugation (1800 x g, 10 min, 23°C) serum was collected and stored at -80°C. After thawing, the concentrations of soluble ICOS were determined using sandwich ELISA Kit for ICOS (Cloud-Clone Corp., Texas, USA) with the detection range from 15.6–1000 pg/mL and detection limit of 6.2 pg/mL. Analysis was conducted according to the manufacturer's protocol.

Whole blood staining for flow cytometry

Venous blood was collected into heparin-containing vacutainer tubes and processed within two hours. Whole blood (100 µL) was stained with a cocktail of fluorochrome-conjugated antibodies for 15 min at 4°C in the dark. ICOS expression was assessed using anti-CD19-APC-Vio770 (1:400, clone REA675,

Miltenyi Biotech, Bergisch Gladbach, Germany) to identify B-cells and non-B cells. $CD4^+$ and $CD8^+$ T cells were identified using anti-CD4-VioBlue (1:150, clone REA623, Bergisch Gladbach, Germany) and anti-CD8-VioGreen (1:100, clone REA734, Miltenyi Biotech, Bergisch Gladbach, Germany), respectively. ICOS expression was evaluated using anti-CD278 (ICOS)-APC (1:44, clone REA192, Miltenyi Biotech, Bergisch Gladbach, Germany). ICOSL expression was analyzed using anti-CD19-APC-Vio770 (1:400, clone REA675, Miltenyi Biotech, Bergisch Gladbach, Germany) for B-lymphocyte identification, anti-CD14-FITC (1:200, clone REA599, Miltenyi Biotech, Bergisch Gladbach, Germany) for monocyte identification, and anti-CD275 (ICOSL)-PE-Vio770 (1:100, clone REA991, Miltenyi Biotech, Bergisch Gladbach, Germany) to assess ICOSL expression on B cells and monocytes. The samples were washed and lysed with a red blood cell lysis solution (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's protocol. After washing and resuspending the cells in 0.5% BSA-FACS buffer, the samples were measured using the MACSQuant® Analyzer 10 (Miltenyi Biotech, Bergisch Gladbach, Germany) and analyzed using FlowJo V10 software (Becton Dickinson, NJ, USA). Leukocyte subtypes were identified by their size and granularity (FSC/SSC), and lymphocyte subtypes based on the presence or absence of CD19 marker, and the surface expression of CD4 and CD8. Monocytes were identified based on CD14 expression. Fluorescence minus one (FMO) was used as a negative control (Fig. 1).

Statistical analysis

First, identification of outliers was conducted using Grubbs method and identified outliers were excluded from further statistical analysis. Normality of the Gaussian distribution was tested using the Shapiro-Wilk test. Multiple group comparisons were performed using Kruskal-Wallis test with *post-hoc* Dunn's multiple comparisons test. Two group comparisons were performed using Mann-Whitney U-test. Correlation analyses were conducted using Spearman's correlation test. Diagnostic value of ICOS expression was determined by ROC curve analysis. GraphPad Prism 10.3.1 software (GraphPad Software, San Diego, CA, USA) was used for data processing. *p*-values lower than 0.05 were considered significant.

Table I. Baseline demographic, serological and clinical characteristics of study participants.

		Healthy controls (n=27)	Sicca controls (n=30)	Newly diagnosed SjD (n=39)	Established SjD (n=10)
Age	Median (IQR)	60 (18)	52 (25)	61 (26)	65 (12)*
Gender	M/F (%)	22/78	20/80	13/87	0/100
Anti-Ro	% positive	0	7	69	60
Anti-La	% positive	0	3	28	20
ANA	% positive	0	23	82	100
RF	% positive	0	10	46	30
Gamma globulins (g/L)	Median (IQR)	NA	11 (4)	15 (6)**	13 (10)
Focus score	% (>1/<1/NA)	NA	0/50/50	90/5/5	90/10/0
USF	% (pos/neg/NA)	NA	6/17/77	69/26/5	80/10/10
Schirmmer's test	% (pos/neg/NA)	NA	17/13/70	69/26/5	50/40/10
SGUS	% (pos/neg/NA)	NA	13/84/3	74/26/0	30/10/60
ESSDAI	Median (IQR)	NA	NA	2 (5)	3 (7)
ESSPR	Median (IQR)	NA	NA	6 (3)	6 (5)
Thearpy					
No therapy	%	0	68	59	58
NSAIR	%	0	26	11	8
Glucocorticoids	%	0	3	14	17
Antimalarials	%	0	0	7	0
Immunosupresives	%	0	3	5	0
Other DMARDs***	%	0	0	5	17

ANA: antinuclear antibodies; DMARDs: disease modifying anti rheumatic drugs; NA: not analysed; SjD: Sjögren's disease; SGUS: salivary gland ultrasound; NSAIR: non-steroidal anti rheumatics.
* established SjD patients were significantly older than sicca controls;
** newly diagnosed SjD patients had significantly higher levels of gamma globulins than sicca controls;
*** one newly SjD patient was receiving certolizumab pegol and one was receiving sulfasalazine; one established SjD patient was receiving rituximab, and one was receiving nintedanib.

Results

Demographic and clinical characteristics of participants

Our study included four groups of participants: 39 newly diagnosed SjD patients, 10 established SjD patients, 30 sicca controls and 27 healthy controls. The groups were age and sex matched except for the established SjD group, in which participants were older compared to those in the sicca control group. The demographic and clinical data are presented in Table I.

Levels of solube ICOS and ICOS expression on periphjeral blood T cells

Levels of soluble ICOS were elevated in newly diagnosed SjD patients compared to healthy (median (IQR) pg/mL 57 (62) *vs*. 26 (12), $p<0.0001$) and sicca controls (median (IQR) pg/mL 57 (62) *vs*. 31 (30), $p=0.0496$) (Fig. 2). In established SjD patients, levels of soluble ICOS were elevated only compared to healthy controls (median pg/mL (IQR) 50 (99) *vs*. 26 (12), $p=0.0415$) but not compared to sicca controls (median (IQR) 50 (99) *vs*. 31 (30), $p>0.9999$).

Surface expression of ICOS on $CD4^+$ T cells was increased in newly diagnosed SjD patients and in established SjD patients compared to sicca controls (median (IQR) MFI 8.590 (3.400) and 8.465 (4.210) *vs*. 5.585 (2.583), $p=0.0024$ and $p=0.0082$). There was a clear trend of increased expression of ICOS on $CD4^+$ T cells in newly diagnosed and established SjD patients compared to healthy controls, however, the difference did not achieve statistical significance (median (IQR) MFI 8.590 (3.400) and 8.465 (4.210) *vs*. 6.690 (2.410), $p=0.1138$ and $p=0.1752$).

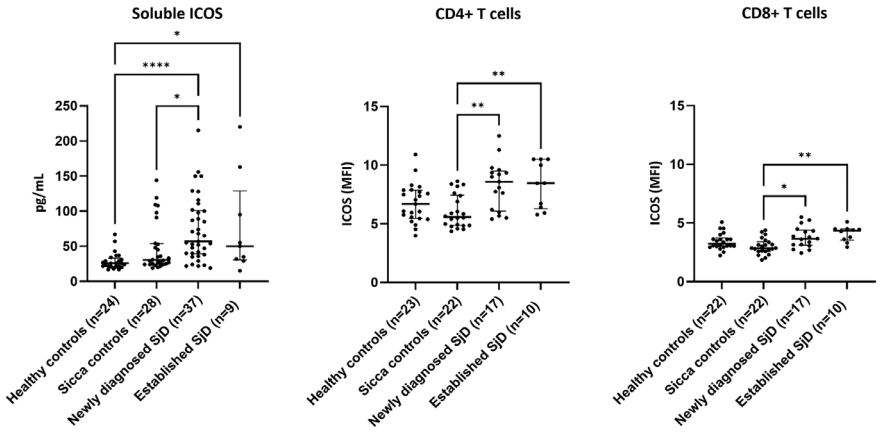

Fig. 2. Levels of soluble ICOS in serum and ICOS expression in CD4+ and CD8+ T cells in patients and controls.
ICOS: inducible T-cell co-stimulator; MFI: mean fluorescence intensity; SjD: Sjögren's disease.

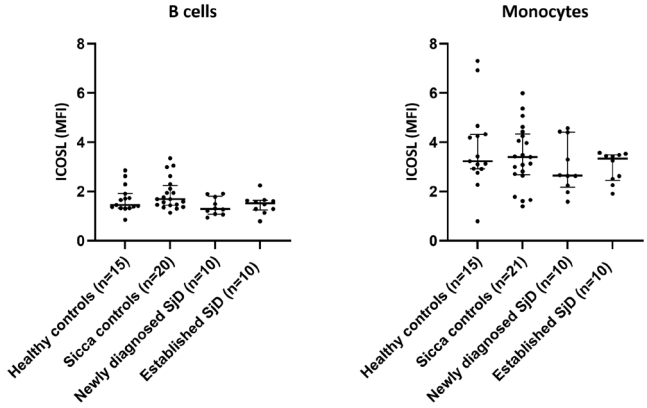

Fig. 3. ICOSL expression in B cells and monocytes in patients and controls.
ICOSL: inducible T-cell co-stimulatory ligand; MFI: mean fluorescence intensity; SjD: Sjögren's disease.

Surface expression of ICOS on CD8+ T cells was increased in newly diagnosed SjD patients and in established SjD patients only compared to sicca (median (IQR) MFI 3.660 (1.305) and 4.350 (0.958) *vs*. 2.855 (0.823), p=0.0234 and p=0.0018), but not compared to healthy controls (median (IQR) MFI 3.660 (1.305) and 4.350 (0.958) *vs*. 3.230 (0.717), p>0.9999 and p=0.1750).

ICOSL expression on surface of peripheral blood B cells and monocytes

ICOSL expression on B cells and monocytes was slightly lower in newly diagnosed SjD patients compared to sicca controls; however, the difference was not statistically significant (Fig. 3).

Correlation analysis

Spearman's correlation analysis, performed on a population of newly diagnosed SjD patients, showed a strong correlation between ICOS expression on CD4+ T cells and ICOS expression on CD8+ T cells (Table II).

Spearman's correlation analysis of measured parameters with clinical characteristics, such as focus score, number of germinal centres, USF, Schirmer's test, gamma globulin levels, ESSDAI, and ESSPRI showed positive correlation of ICOS expression on CD4+ T cells with gamma globulin levels. Levels of soluble ICOS, ICOS expression on CD8+ T cells and ICOSL expression on B cells and monocytes did not correlate with any of the tested clinical parameters within SjD group.

Comparison of measured parameters within subgroups created based on anti-Ro, anti-La and RF positivity, presence of germinal centres and lymphoepithelial lesions and presence of morphological changes visible with SGUS showed that levels of soluble ICOS were significantly higher in SjD patients with present RF (Fig. 4). ICOS expression on CD4+ T cells was significantly higher in SjD patients who presented serological abnormalities such as anti-Ro and RF. ICOS expression on CD8+ T cells was significantly higher in SjD patients with present anti-Ro antibodies. None of the parameters were significantly associated with MSG histological characteristics, such as germinal centres and lymphoepithelial lesions.

Expression of ICOSL on B cells and monocytes was not significantly different among any of the analysed subgroups (data not shown).

None of the measured parameters differed significantly between treatment naive individuals and those receiving immunomodulatory therapy (data not shown).

Diagnostic performance of soluble ICOS and membrane-bound ICOS and ICOSL

The ability of all measured parameters to discriminate between newly diagnosed SjD patients, sicca patients and healthy controls was assessed using receiver operating characteristic curve analysis (Fig. 5 and Table III). Soluble ICOS and ICOS expressed on CD4+ T cells showed very good diagnostic ability to discriminate between SjD patients and healthy controls (AUC=0.8587; p<0.0001 and AUC=0.7289; p=0.0143) and also between SjD patients and sicca controls (AUC=0.6957; p=0.0072 and AUC=0.8302; p=0.0005). Soluble ICOS showed better discrimination between SjD patients and healthy controls, while ICOS expressed on CD4+ T cells provided better discrimination between SjD patients and sicca controls. In contrast, ICOS expression on CD8+ T cells and ICOSL expression on B cells and monocytes showed lower or no diagnostic ability to discriminate between SjD and sicca or healthy controls.

Discussion

In this study, we investigated soluble ICOS levels, ICOS expression on CD4+ and CD8+ T cells and ICOSL expression on B cells and monocytes in newly diagnosed and established SjD patients,

Table II. Spearman's correlation between measured parameters and clinical characteristics. Analysis was performed on a population of newly diagnosed SjD patients.

	Soluble ICOS (pg/mL)		ICOS on CD4+ T cells (MFI)		ICOS on CD8+ T cells (MFI)		ICOSL on B cells (MFI)		ICOSL on monocytes (MFI)	
	r	p-value	r	p-value	r	p-value	r	p-value	r	p-value
Soluble ICOS (pg/mL)	1.000		0.294	0.2681	-0.071	0.7967	-0.212	0.5603	-0.286	0.4202
ICOS on CD4+ T cells (MFI)	0.294	0.2681	1.000		**0.797**	**0.0002***	-0.321	0.3679	0.517	0.1293
ICOS on CD8+ T cells (MFI)	-0.071	0.7967	**0.797**	**0.0002***	1.000		-0.273	0.4483	0.243	0.4964
ICOSL on B cells (MFI)	-0.212	0.5603	-0.321	0.3679	-0.273	0.4483	1.000		0.328	0.3527
ICOSL on monocytes (MFI)	-0.286	0.4202	0.517	0.1293	0.243	0.4964	0.328	0.3527	1.000	
Focus score	0.253	0.1368	0.221	0.4069	0.009	0.9757	-0.134	0.7311	0.328	0.3846
Number of geminal centers	0.306	0.0889	0.412	0.1278	0.290	0.2915	-0.209	0.6208	0.154	0.7146
USF test (mL/min)	-0.296	0.0840	0.162	0.5296	0.149	0.5643	0.427	0.2222	0.000	1.0000
Schrimmer right eye (mm/5 min)	-0.191	0.2723	0.132	0.6124	0.135	0.6024	0.201	0.5761	0.134	0.7099
Schrimmer left eye (mm/5 min)	-0.034	0.8450	0.201	0.4355	0.147	0.5709	0.272	0.4432	0.282	0.4232
Gamma globulins (g/L)	0.172	0.3226	**0.641**	**0.0066***	**0.474**	**0.0563**	-0.406	0.2443	0.244	0.4913
ESSDAI	0.204	0.2334	0.319	0.2099	0.218	0.3984	-0.474	0.1675	0.053	0.8829
ESSPRI	0.146	0.4576	-0.322	0.3044	-0.291	0.3567	-0.216	0.6357	0.126	0.7929

ICOS: inducible T-cell co-stimulatory; MFI: mean fluorescence intensity; ICOSL: inducible T-cell co-stimulatory ligand; USF: unstimulated salivary flow.
* statistical significance.

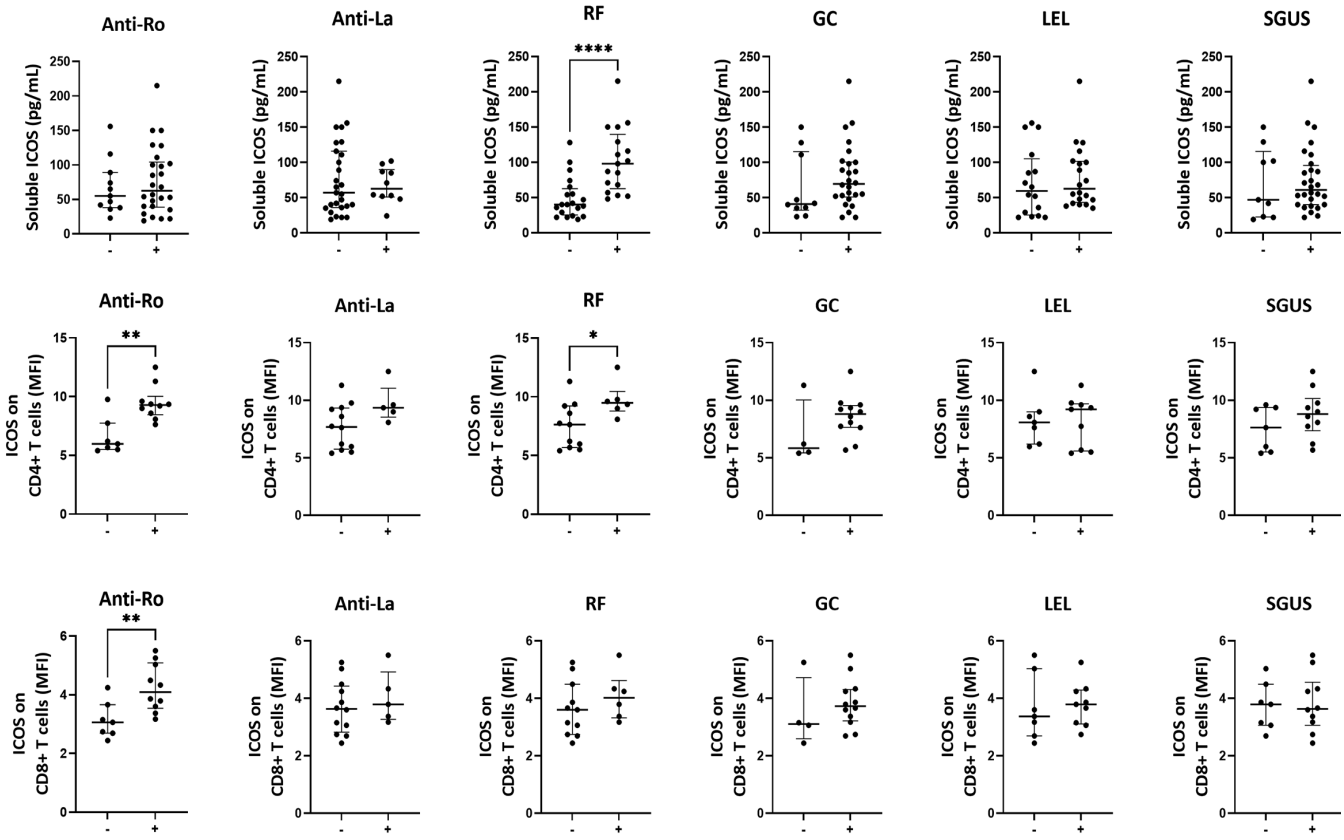

Fig. 4. Comparison of measured parameters between subgroups. Analysis was performed on a population of newly diagnosed SjD patients.
GC: germinal centers; LEL: lymphoepithelial lesions; MFI: mean fluorescence intensity; RF: rheumatoid factor; SGUS: salivary gland ultrasound.

individuals with sicca symptoms and healthy controls. While previous research has primarily focused on the mRNA expression of these molecules in SjD, this study focused on evaluation of protein levels of soluble and membrane-bound ICOS and ICOSL and their clinical relevance. To our knowledge, this study is the first to evaluate their diagnostic potential in SjD patients.

First, our results show that soluble ICOS was significantly elevated in the serum of newly diagnosed SjD patients compared to sicca and healthy controls. This finding is consistent with the findings of Luo *et al*. (3) and Li *et al*. (5) who both reported elevated levels of soluble ICOS in plasma/serum of SjD

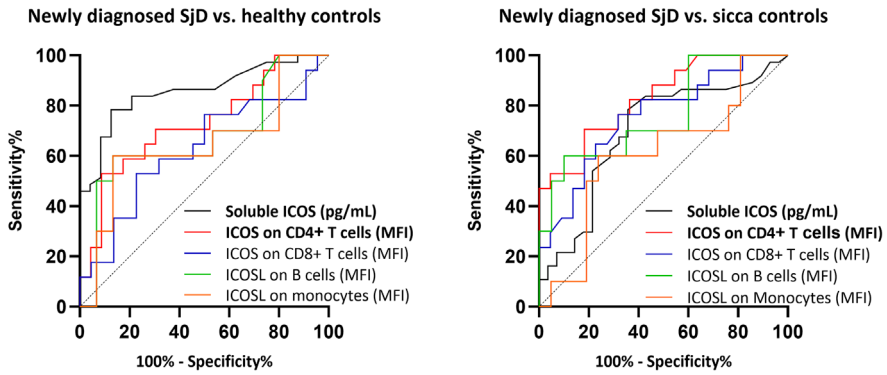

Fig. 5. ROC curve analysis of measured parameters.
AUC: area under the curve; CI: confidence interval; ICOS: inducible T-cell co-stimulatory; MFI: mean fluorescence intensity; SjD: Sjögren's disease.

patients. However, none of these groups investigated associations between soluble ICOS levels and clinical characteristics. In our study, soluble ICOS was positively associated with the presence of RF, a serological marker previously linked to higher disease activity in SjD, as measured by the ESSDAI (15). Although soluble ICOS did not correlate with other clinical features traditionally associated with severe disease manifestations, such as hypergammaglobulinaemia, anti-Ro antibodies, or positive SGUS (16-19), its association with RF suggests a potential role in identifying patients at increased risk for more active disease. Furthermore, the lack of difference in soluble ICOS levels between anti-Ro-positive and anti-Ro-negative patients highlights its diagnostic advantage. Since anti-Ro antibodies represent the principal serological component of the current ACR/EULAR classification criteria, additional biomarkers that are not restricted to anti-Ro positive subsets are highly desirable, particularly for the accurate classification of anti-Ro negative patients.

Second, we found that ICOS expression on CD4+ T cells is significantly elevated in newly diagnosed SjD patients compared to sicca controls and showed a non-significant trend toward elevation compared to healthy controls. This aligns with the findings of Garcia-Espinoza *et al*. (10) who observed elevated ICOS expression on peripheral blood CD4+ T cells, and Versatppen *et al*. (11) who reported similar findings in circulating T follicular cells. Garcia-Espinoza *et al*. (10) further examined associations between ICOS expression and clinical parameters, reporting a positive association with focus score, but no association with anti-Ro/La positivity or immunoglobulin levels. In contrast, we observed a positive correlation with gamma globulin levels, and a positive association with anti-Ro, and RF positivity. Additionally, we observed a clear, even though non-significant, trend toward elevated ICOS expression on CD4+ T cells in SjD patients with present germinal centres. Elevated ICOS expression on CD4+ T cells together with the positive association with anti-Ro, anti-La, RF and gamma globulin levels, all markers of B-cell hyperactivity, supports established role of the ICOS-ICOSL signalling pathway in T:B cell interactions, germinal centre formation and subsequent B-cell hyperactivity (8), a known feature of SjD. Moreover, the observed association with gamma globulin levels, anti-Ro and RF positivity we may speculate that this parameter holds greater potential for identification of patients with higher risk for severe disease manifestation compared to the levels of soluble ICOS.

Third, we showed that ICOS expression is also elevated on CD8+ T cells in newly diagnosed SjD patients compared to sicca controls. To date, no other reports on ICOS expression on CD8+ T cells in SjD patients exist. This may be due to the fact that ICOS has been recognised mainly in the context of CD4+ T lymphocyte function. However, in recent years, it has been shown, that ICOS stimulation is critical for development of CD8+ tissue-resident memory T cells (Trm) (20). Trm are a subset of CD8+ T cells maintained in non-lymphoid tissues that contribute to tissue surveillance and direct pathogen elimination (21). Notably, Trm cells have been shown to be expanded in the salivary glands of SjD patients, where they are believed to contribute to the persistent inflammation and tissue damage (22). These findings raise the possibility that ICOS may be involved in CD8+ T cell activity in SjD, potentially contributing to the presence or function of Trm cells in affected tissues, although further studies are needed to clarify this role.

In the analysis of correlations and associations of ICOS expressed on CD8+ T cells, we observed only correlation of this parameter with anti-Ro presence, which implies its limited potential for identification of patients holding greater risk for sever disease manifestation.

Fourth, we demonstrated that ICOSL expression on B cells and monocytes was lower in newly diagnosed SjD patients compared to sicca controls;

Table III. ROC curve analysis of measured parameters.

	Newly diagnosed SjD vs. healthy controls		Newly diagnosed SjD vs. sicca controls	
	AUC (95% CI)	p-value	AUC (95% CI)	p-value
Soluble ICOS (pg/mL)	**0.8575 (0.7639-0.9511)**	**<0.0001**	**0.6957 (0.5624-0.8295)**	**0.0072**
ICOS on CD4+ T cells (MFI)	**0.7289 (0.5668-0.8910)**	**0.0143**	**0.8302 (0.7036-0.9568)**	**0.0005**
ICOS on CD8+ T cells (MFI)	0.6270 (0.4439-0.8101)	0.1785	0.7567 (0.6029-0.9105)	0.0065
ICOSL on B cells (MFI)	0.6767 (0.4454-0.9079)	0.1416	0.7650 (0.5769-0.9531)	0.0197
ICOSL on monocytes (MFI)	0.6467 (0.4081-0.8852)	0.2223	0.6095 (0.3902-0.8288)	0.3311

AUC: area under the curve; CI: confidence interval; ICOS: inducible T-cell co-stimulator; MFI: mean fluorescence intensity; SjD: Sjögren's disease.

however, the difference did not reach statistical significance. This lack of significance might be due to the relatively small number of patients included in the analysis (n=10). Although the importance of the ICOS-ICOSL axis in SjD is well recognised, evidence regarding ICOSL expression in this disease remains limited. To our knowledge, no previous studies have examined ICOSL expression in the peripheral blood of SjD patients. However, Nayar *et al*. (9) reported elevated ICOSL expression in the salivary glands of SjD patients with tertiary lymphoid structures, suggesting a discrepancy between tissue and peripheral expression. A similar pattern was observed in systemic sclerosis (SSc), where Hasegawa *et al*. (23) found elevated ICOSL-positive cells in the skin, while peripheral blood levels in B cells and monocytes remained unchanged. Our findings are in line with previous observations in SLE, where Hutloff *et al*. (24) and Yang *et al*. (25) reported decreased surface ICOSL expression on peripheral blood B cells. A possible explanation for decreased expression of ICOSL on B cells could lie in the ICOS-ICOSL expression regulatory mechanism, where ICOSL exhibits a reciprocal expression pattern to ICOS as a consequence of ICOS-ICOSL interactions (26). The decreased ICOSL expression on B cells in SjD patients in this study is consistent with the findings of Hutloff *et al*. and Yang *et al*. in SLE patients, while in contrast, Ding *et al*. (27) reported increased ICOSL expression in B cells and monocytes in RA patients. Taken together, these results suggest that ICOSL dysregulation is likely to be tissue-specific and varies across different systemic autoimmune diseases. We found no correlation or associations of ICOSL expression on B cells or monocytes with clinical characteristics. These results imply that ICOSL expression in B cells and monocytes have the least clinical significance out of all five tested parameters in our study.

In addition to alterations in soluble ICOS levels, ICOS expression on $CD4^+$ and $CD8^+$ T cells and ICOSL expression on B cells and monocytes in SjD patients at the point they enter the diagnostic process, we were also interested in alterations of these parameters in established SjD patients. Levels of soluble ICOS were elevated in SjD established patients only in comparison to healthy, but not to sicca controls, and expression of ICOS on CD4+ and $CD8^+$ T cells was only elevated in SjD established patients compared to sicca, but not healthy controls. These results indicate that levels of soluble ICOS and ICOS expression on $CD4^+$ and $CD8^+$ T cells are elevated not only early during disease onset but also remain high during the chronic phase. Moreover, their expression does not appear to be affected by treatment. Further longitudinal studies are required to clarify these findings.

One of the main objectives of our study was to assess the diagnostic potential of soluble ICOS levels, ICOS expression on $CD4^+$ and $CD8^+$ T cells and ICOSL expression on B cells and monocytes in SjD patients. To this end, we performed ROC curve analyses for all these parameters to evaluate their ability to distinguish SjD patients at the point they enter diagnostic process from both healthy and sicca controls. Levels of soluble ICOS and ICOS expressions on $CD4^+$ T cells were able to discriminate between SjD patients and both, sicca, and healthy controls, indicating the best diagnostic potential among tested parameters. However, the two parameters were not of equal value – soluble ICOS performed better in discrimination between SjD patients and healthy controls, while ICOS expression on $CD4^+$ T cells performed better in discriminating SjD patients from sicca controls. Given that clinicians typically need to differentiate between SjD patients and individuals with sicca symptoms due to other causes, rather than between SjD patients and healthy individuals, ICOS expression on $CD4^+$ T cells emerges as the most promising parameter for further evaluation of its diagnostic potential.

The specificity of elevated ICOS expression, both in its soluble and membrane-bound forms, for SjD remains unresolved. Elevated ICOS expression on $CD4^+$ and $CD8^+$ T cells was also observed in patients with SSc (23) and SLE (25) and elevated percentages of $ICOS^+$ $CD4^+$ and $CD8^+$ T cells were observed in patients with RA (27). In a study by Luo *et al*. (3), ICOS mRNA levels were compared across SjD, RA, and SLE patients, revealing higher ICOS expression in SjD patients compared to those with RA or SLE (3). However, the study did not provide details on the treatment status of the RA and SLE patients, which could potentially influence the results. In addition, elevated levels of soluble ICOS were detected in patients with SSc (23), pulmonary arterial hypertension (28), multiple myeloma (29), neuromyelitis optica spectrum disorder (30) and age-related macular degeneration (31). Therefore, further comparisons of soluble and membrane-bound ICOS expression among these diseases are necessary to better define the role of ICOS as a potential biomarker in SjD.

This study has limitations that should be considered when interpreting the findings. This was a single centre study, with a relatively small sample size and presence of missing data, particularly in the sicca control group, which may limit generaliability of the results. Future multicentre studies with larger cohorts, containing different disease control groups and more complete datasets are warranted to validate and further explore these findings.

In conclusion, our study shows that soluble ICOS and ICOS expressed on $CD4^+$ T cells are significantly elevated in SjD patients at the time they enter the diagnostic process. Moreover, the observed association of ICOS expressed on $CD4^+$ T cells with gamma globulin levels, anti-Ro and RF positivity, suggests that this parameter may help identify patients at higher risk of severe disease manifestations. Notably, this is the first study to report the high diagnostic potential of both markers in SjD, warranting further evaluation as potential biomarkers.

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