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Original article

Serum interferon-α2 measured by single-molecule array associates with systemic disease manifestations in Sjögren's syndrome

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Abstract

Objectives. Type I IFN (IFN-I) activation is a prominent feature of primary SS (pSS), SLE and SSc. Ultrasensitive single-molecule array (Simoa) technology has facilitated the measurement of subfemtomolar concentrations of IFNs. Here we aimed to measure IFN-α2 in serum from pSS, SLE and SSc using a Simoa immunoassay and correlate these levels to blood IFN-stimulated gene (ISG) expression and disease activity.

Methods. Serum IFN-α2 was measured in patients with pSS ($n = 85$ and $n = 110$), SLE ($n = 24$) and SSc ($n = 23$) and healthy controls (HCs; $n = 68$) using an IFN-α Simoa assay on an HD-X analyser. IFN-I pathway activation was additionally determined from serum by an IFN-I reporter assay and paired samples of whole blood ISG expression of *IFI44*, *IFI44L*, *IFIT1*, *IFIT3 and MxA* by RT-PCR or myxovirus resistance protein 1 (MxA) protein ELISA.

Results. Serum IFN-α2 levels were elevated in pSS (median 61.3 fg/ml) compared with HCs (median ≤5 fg/ml, $P < 0.001$) and SSc (median 11.6 fg/ml, $P = 0.043$), lower compared with SLE (median 313.5 fg/ml, $P = 0.068$) and positively correlated with blood ISG expression ($r = 0.66$–0.94, $P < 0.001$). Comparable to MxA ELISA [area under the curve (AUC) 0.93], IFN-α2 measurement using Simoa identified pSS with high ISG expression (AUC 0.90) with 80–93% specificity and 71–84% sensitivity. Blinded validation in an independent pSS cohort yielded a comparable accuracy. Multiple regression indicated independent associations of autoantibodies, IgG, HCQ treatment, cutaneous disease and a history of extraglandular manifestations with serum IFN-α2 concentrations in pSS.

Conclusion. Simoa serum IFN-α2 reflects blood ISG expression in pSS, SLE and SSc. In light of IFN-targeting treatments, Simoa could potentially be applied for patient stratification or retrospective analysis of historical cohorts.

Key words: type I IFN, primary SS, Simoa, SLE, SSc

BASIC SCIENCE

Rheumatology key messages

- Serum IFN-α2 measured by Simoa immunoassay is elevated in primary SS, SSc and SLE.
- IFN-α2 positively correlates with blood IFN-stimulated gene and protein expression.
- IFN-α2-Simoa demonstrates high replicability, but the IFN-stimulated genes score has a greater ability to distinguish pSS from controls.

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Introduction

Sustained systemic activation of the type I IFN (IFN-I) pathway is a well-known pathophysiological feature of several systemic autoimmune diseases (SADs), including SLE, primary SS (pSS) and SSc [1]. Therapeutic strategies targeting IFN-I are under clinical evaluation. Anifrolumab, a blocking antibody against the IFN-I receptor (IFNAR), has recently been found to induce clinically relevant responses in patients with active SLE [2–4]. Measurement of IFN-I will likely assist in the selection of candidates for these targeted treatments, urging the development and validation of robust and easy-to-perform assays.

The IFN-I family comprises 16 protein subtypes that have important antiviral and immunomodulatory properties [5–7]. These functions are largely effectuated by signalling through the IFNAR complex. The diversity of subtypes and the low circulating levels complicate quantification of IFN-I protein in biological samples by routinely used laboratory techniques such as ELISA.

Various methods measuring different elements of the IFN pathway are being used to evaluate IFN-I pathway activation. The majority of these assays exploit downstream cellular responses to IFN by quantification of expression of either IFN-stimulated genes (ISGs) or proteins. The development of ultrasensitive single-molecule array (Simoa) digital ELISA technology has facilitated the direct measurement of IFN-α protein levels [8, 9].

At present, a handful of studies have been published employing this ultrasensitive technique in patients with monogenic interferonopathies or SLE, while application in other SADs has been limited [8, 10–13]. Most of these studies use custom homebrew assays developed with autoantibodies specific for all IFN-α subtypes cloned from patients with autoimmune polyglandular syndrome type 1 (APS1) [8, 10, 13].

Here we tested the performance of a commercially available ready-to-use Simoa immunoassay kit for quantification of IFN-α2 protein concentrations in serum from two independent and clinically well-characterized pSS cohorts and compared the IFN-α2 protein levels to those in SLE and SSc patients. Additionally, a side-by-side comparison of serum IFN-α2 with whole blood ISG expression, intracellular myxovirus resistance protein 1 (MxA) protein levels and serum IFN-I bioactivity was performed in these cohorts.

Methods

Patients and healthy controls (HCs)

Patients with pSS ($n = 85$), SLE ($n = 24$) and SSc ($n = 23$) and HCs ($n = 40$) recruited at the Erasmus MC, University Medical Center Rotterdam, from which at least paired samples of serum and PAXgene material were stored, were included in this study (Rotterdam cohort). An established cohort of pSS patients ($n = 110$) recruited at the Department of Rheumatology, Lund University, Malmö, Sweden [14] and additional HCs ($n = 28$) recruited at the Erasmus MC were used as a validation cohort (Malmö cohort). This study was approved by the Medical Ethics Review Committees of the Erasmus MC (MEC-2011-116, MEC-2016-202) and Lund University (2015/311, 2017/94). In accordance with the Declaration of Helsinki, written informed consent was obtained from all participants. Further details are provided in Supplementary Data S1, available at *Rheumatology* online. Demographic and clinical characteristics of the study cohorts are summarized in Table 1 and Supplementary Table S1, available at *Rheumatology* online.

Ultrasensitive IFN-α Simoa

IFN-α2 was measured in duplicates from serum samples (diluted two-fold in sample diluent) using the Simoa IFN-α Advantage Kit (no. 100860, Quanterix, Billerica, MA, USA) following the instructions of the supplied manual. Sample processing and analysis were done using an HD-X analyser (software version 1.6.1905.300; Quanterix). The lower limit of detection was 5 fg/ml.

Recombinant IFN-I subtypes

To verify the analytical specificity of the Simoa IFN-α Advantage Kit and HEK293-3C11-ISRE reporter cells, serum from an HC or special stripped serum (Valley Biomedical, Winchester, VA, USA) were spiked with recombinant human IFN-α1(D), -D(1), -A(2a), -2(2b), -4a(M1), -4b(4), -G(5), -K(6), -J1(7), -B2(8), -H2(14), -WA(16), -I(17), -F(21), IFN-β1a (all from PBL Assay Science, Tebu-bio, Heerhugowaard, The Netherlands) or IFN-γ (PeproTech, Cranbury, NJ, USA) and snap frozen or assayed directly.

Real-time PCR IFN-stimulated genes, MxA immunoassay and IFN-I reporter assay

Whole blood expression of ISGs *MxA*, *IFI44*, *IFI44L*, *IFIT1*, and *IFIT3* was quantified from PAXgene Blood RNA tubes (PreAnalytiX, Becton Dickinson, Vianen, The Netherlands) by RT-PCR and an IFN-I score was calculated as previously described [15]. The threshold value for the IFN-I score was set to the 97.5th percentile of IFN-I scores in HCs, consistent with the intercept of fitted Gaussian density components in a finite mixture model and the local minimum of a non-parametric density estimate of IFN-I score data in autoimmune patients (Supplementary Fig. S1, available at *Rheumatology* online). Intracellular MxA protein was measured by an immunoassay (lower limit of detection 10 μg/l) as previously described [16–18]. IFN-I activity in serum was measured using pGreenFire-ISRE reporter construct-transduced HEK293-3C11 cells (kindly provided by Jan Rehwinkel, University of Oxford, Oxford, UK) [19] and HC peripheral blood mononuclear cells (PBMCs). Assay details are provided in Supplementary Data S1, available at *Rheumatology* online.

Table 1 Demographic and clinical characteristics

Characteristics	Rotterdam				Malmö	*P*-value
	HCs (*n* = 68)	SLE (*n* = 24)	SSc (*n* = 23)	pSS (*n* = 85)	pSS (*n* = 110)	
Demographics						
Female, *n/N* (%)	62/68 (91.2)	22/24 (91.7)	20/23 (87.0)	77/85 (90.6)	102/110 (92.7)	0.782
Age, years, median (Q1–Q3)	51 (32.8–57)	49 (37–55)	61 (53–67.5)	62 (53–68)	64.5 (47–72)	0.737
Patient characteristics, median (Q1–Q3)						
Disease duration, years	–	15 (7.5–19.3)	11 (8–20.5)	12 (6–20.3)	12 (5–21)	0.354
Disease activity[a]	–	2 (0–4)	–	2 (0–7.25)	3 (1–7.75)	0.290
Clinical disease activity[b]	–	0 (0–1)	–	2 (0–9.25)	2 (0–8)	0.763
Laboratory parameters						
ANA, *n/N* (%)	–	24/24 (100)	18/21 (91.3)	68/85 (80)	89/110 (80.9)	1.000
Anti-SSA, *n/N* (%)	–	12/24 (50)	–	72/85 (84.7)	95/110 (86.4)	0.903
Anti-Ro52, *n/N* (%)	–	–	–	64/72 (88.9)	70/82 (85.4)	0.939
Anti-Ro60, *n/N* (%)	–	–	–	66/72 (91.7)	78/82 (95.1)	0.234
Anti-SSB, *n/N* (%)	–	7/24 (29.2)	–	48/85 (56.5)	63/110 (57.3)	1.000
Anti-dsDNA, IU/mL, median (Q1–Q3)	–	14.5 (1.63–39.5)	–	–	–	
Anti-Sm, *n/N* (%)	–	5/24 (20.8)	–	–	–	
Anti-RNP, *n/N* (%)	–	9/24 (37.5)	–	–	–	
IgG, g/L, median (Q1–Q3)	–	–	–	13.1 (10.2–16.7)	14.6 (11.3–17.3)	0.219
C3, g/L, median (Q1–Q3)	–	1.08 (0.89–1.17)	–	1.16 (1.03–1.30)	0.93 (0.81–1.07)	<0.001
C4, g/L, median (Q1–Q3)	–	0.19 (0.16–0.22)	–	0.19 (0.14–0.23)	0.17 (0.13–0.22)	0.137
Current medication, *n/N* (%)						
HCQ	–	17/24 (70.8)	2/23 (8.7)	42/85 (49.4)	35/110 (31.8)	0.019
HCQ monotherapy	–	4/17 (23.5)	1/2 (50)	35/42 (83.3)	17/35 (48.6)	0.003
HCQ + corticosteroids/DMARDs/biologics	–	13/17 (76.5)	1/2 (50)	7/42 (16.7)	18/35 (51.4)	
Corticosteroids/DMARDs	–	18/24 (75)	13/23 (56.5)	12/85 (14.1)	29/110 (26.4)	0.057
Corticosteroids + DMARDs	–	2/18 (11.1)	1/13 (7.7)	2/12 (16.7)	6/29 (20.7)	–
Corticosteroids only	–	10/18 (55.6)	5/13 (38.5)	9/12 (75)	22/29 (75.9)	–
DMARDs only	–	6/18 (33.3)	7/13 (53.8)	1/12 (8.3)	1/29 (3.5)	–
Rituximab	–	0/24 (0)	0/23 (0)	0/85 (0)	8/110 (7.3)	–
Belimumab	–	2/24 (8.3)	0/23 (0)	1/85 (1.2)	0/110 (0)	–

[a]Disease activity: ESSDAI for pSS and SLEDAI-2K for SLE. [b]Clinical disease activity: ClinESSDAI for pSS and ClinSLEDAI for SLE. Mann–Whitney *U* test or Pearson's chi-squared test were used to compare medians or frequencies of the Rotterdam and Malmö pSS cohorts. anti-Sm: anti-Smith; Q1: quartile 1; Q3: quartile 3.

Statistical analysis

Statistical analyses were performed in R (version 3.6.3; R Foundation for Statistical Computing, Vienna, Austria) [20] using the clikcorr (Censoring Data and Likelihood-Based Correlation Estimation) package (version 1.0) for correlation analysis [21], the pROC package (version 1.16.2) for receiver operating characteristics (ROC) analysis [22] and the CensReg (Censored Regression Tobit Models) package (version 0.5-30) for bivariate and multivariable regression analysis [23].

Results

Simoa IFN-α Advantage Kit primarily detects IFN-α2

To assess the analytical specificity of the Simoa IFN-α Advantage Kit, serum from an HC was spiked with 1 of 15 recombinant IFN-α subtypes, IFN-β or IFN-γ. Quantification indicated efficient detection of IFN-α2 subvariants in contrast to detection of IFN-α4a, IFN-α6 and IFN-α10 only at the highest concentration tested (>1500 fg/ml) (Supplementary Fig. S2, available at *Rheumatology* online).

Serum IFN-α2 concentrations are elevated in pSS, SLE and SSc

Detectable concentrations of IFN-α2 were present in 75.3% of serum samples from pSS (Rotterdam cohort), 75% from SLE, 56.5% from SSc and 45% from HCs. Serum IFN-α2 concentrations were elevated in pSS (Rotterdam cohort; median 61.3 fg/ml) compared with HCs (median ≤5 fg/ml, $P < 0.001$) and SSc (median 11.6 fg/ml, $P = 0.043$) (Fig. 1A). The highest IFN-α2 concentrations were observed in serum from SLE patients (median 313.5 fg/ml). SLE patients in remission (according to the Definitions of Remission in SLE classification [24]) showed significantly lower ($P = 0.015$) serum IFN-α2 concentrations compared with patients not in remission (Supplementary Fig. S3, available at *Rheumatology* online), which is in line with previous observations [8, 11, 12].

Serum IFN-α2 yields lower accuracy in discriminating pSS from HCs than ISG expression

Next we compared serum IFN-α2 with a whole blood 5 ISG–based transcript score (IFN-I score) reflecting *in vivo* IFN-I bioactivity. In the same cohort we also assessed whole blood intracellular MxA protein concentrations by an immunoassay that we previously described to be highly correlated with the IFN-I score in both pSS and SLE patients [16, 17]. The IFN-I score and intracellular MxA concentrations in pSS, SLE and SSc followed a similar pattern as serum IFN-α2 (Fig. 1B, C). The diagnostic accuracy of serum IFN-α2 to discriminate between pSS and HCs was evaluated by ROC analysis. The area under the curve (AUC) was lower for serum IFN-α2 (AUC = 0.77) compared with the IFN-I score (AUC = 0.86, $P = 0.019$) and intracellular MxA (AUC = 0.85, $P = 0.036$) (Supplementary Fig. S4A, available at *Rheumatology* online). The Youden's J index summarizes the overall diagnostic performance of a test as a value between 0 (no diagnostic value) and 1 (perfect test) by integrating sensitivity and specificity with equal weight and can be used as a criterion for selection of optimal cut-off values. A maximum Youden's J index of 0.54 was reached at a threshold of 19.3 fg/ml serum IFN-α2 (Supplementary Fig. S4B, available at *Rheumatology* online), yielding 62% sensitivity and 90% specificity (Supplementary Table S2, available at *Rheumatology* online).

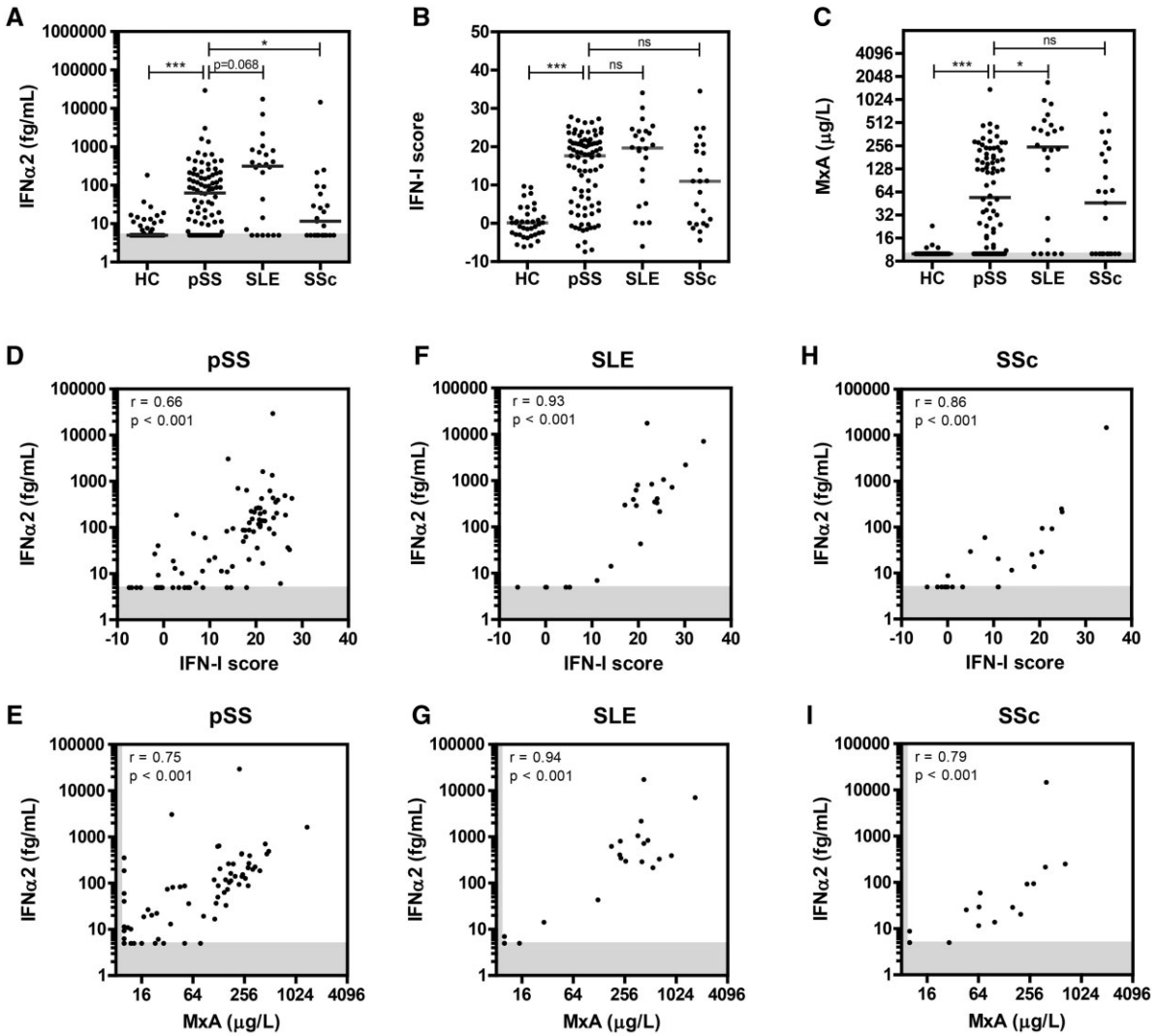
Serum IFN-α2 is positively correlated with whole blood ISG expression and serum IFN-I bioactivity

Serum IFN-α2 concentrations were positively correlated with IFN-I score ($r = 0.66$, $P < 0.001$), intracellular MxA protein levels ($r = 0.75$, $P < 0.001$) and expression of individual ISG transcripts in pSS (Fig. 1D and E; Supplementary Fig. S5A, available at *Rheumatology* online). In SLE and SSc patients, similar correlations between the IFN-I score and serum IFN-α2 were observed, with the highest correlation coefficients in SLE (Fig. 1F–I; Supplementary Fig. S5B and C, available at *Rheumatology* online). A different method to assess bioactive IFN-I in serum is an IFN-α/β luciferase reporter assay (Supplementary Fig. S6A, available at *Rheumatology* online). In this assay, ISG-inducing capacity of patients' sera was observed only for a minority of samples, mostly derived from SLE patients (Supplementary Fig. S6B, available at *Rheumatology* online). Taking an alternative approach using HC PBMCs, SLE sera induced higher levels of IFNAR-dependent ISG expression compared with pSS sera (Supplementary Fig. S6C, available at *Rheumatology* online). The IFN-I bioactivity in SLE sera positively correlated with serum IFN-α2 ($r = 0.62$, $P < 0.001$), IFN-I score ($r = 0.61$, $P < 0.001$) and MxA levels ($r = 0.51$, $P = 0.009$) (Supplementary Fig. S6D, available at *Rheumatology* online).

Serum IFN-α2 identifies pSS with high ISG expression

The IFN-I scores in pSS patients follow a bimodal distribution on the basis of which patients were classified as IFN-low or IFN-high. As expected, IFN-α2 concentrations were significantly higher in IFN-high compared with IFN-low pSS (Fig. 2A). ROC analysis indicated comparable discriminative ability of serum IFN-α2 (AUC = 0.9) and intracellular MxA (AUC = 0.93) to identify IFN-high pSS patients (Fig. 2B). The maximum accuracy of 83.7% and maximum Youden's J index of 0.67 were obtained for serum IFN-α2 at a threshold of 19.8 fg/ml and only changed marginally when increasing the threshold to 28 or 66 fg/ml (Supplementary Fig. S7, available at *Rheumatology* online; Table 2). Using these thresholds, sensitivity ranged from 71 to 84% and specificity from 80 to 93%. Blinded validation in the second, independently collected pSS cohort (Malmö cohort) expanded with 28 additional HCs confirmed the measures of discriminative property and predictive ability of

FIG. 1 Serum IFN-α2 concentrations positively correlated with blood ISG expression in pSS, SLE and SSc



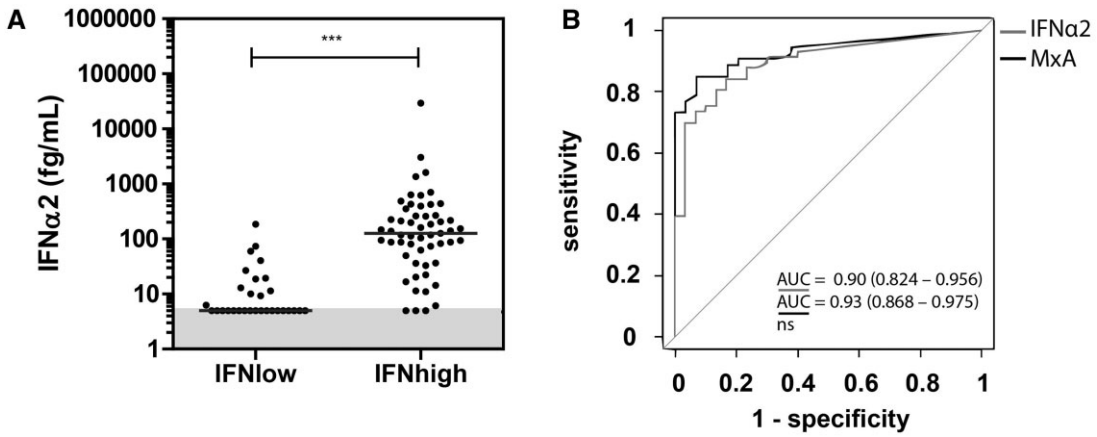
(**A**) Serum IFN-α2, (**B**) blood IFN-I score and (**C**) blood intracellular MxA protein in HCs ($n = 40$) and patients with pSS ($n = 85$), SLE ($n = 24$) and SSc ($n = 23$). Correlation between serum IFN-α2 and (**D**, **F**, **H**) IFN-I score or (**E**, **G**, **I**) intracellular MxA protein concentrations in (**D**, **E**) pSS, (**F**, **G**) SLE and (**H**, **I**) SSc. Horizontal lines represent medians and shaded regions indicate values below the lower limit of detection. Statistics: (A, C) censored regression analysis, (B) Kruskal–Wallis H test, (D–I) likelihood-based correlation coefficient estimation. $^{*}P < 0.05$, $^{***}P < 0.001$; ns: not significant.

serum IFN-α2 (Table 2; Supplementary Table S2, available at *Rheumatology* online). Notably, serum IFN-α2 data, IFN-I scores and the correlation between them were replicable in two sets of HCs and pSS patients from both cohorts despite independent sample collection, processing and measurement (Supplementary Fig. S8, available at *Rheumatology* online). A total of 15 pSS patients from both cohorts that were classified as IFN-high based on their IFN-I score exhibited serum IFN-α2 concentrations <19 fg/ml. In comparison to IFN-high pSS with IFN-α2 >19 fg/ml, these patients had lower IFN-I scores, intracellular MxA and total IgG and were more frequently negative for ANA and anti-SSB autoantibodies (Supplementary Table S3, available at *Rheumatology* online). No differences between these groups were observed in the use of medication, complement factors, disease activity and age.

Serum IFN-α2 is associated with serological disease and HCQ treatment in pSS

Bivariate analyses indicated higher serum IFN-α2 concentrations in pSS patients with autoantibodies and lower IFN-α2 in patients currently treated with HCQ (Table 3). Serum IFN-α2 was positively correlated with total IgG levels in both cohorts and negatively correlated

Fig. 2 Serum IFN-α2 identified pSS with high ISG expression



(**A**) Serum IFN-α2 in pSS patients stratified according to IFN-I score. IFN-I score threshold: 97.5 percentile of HCs. Horizontal lines represent medians and shaded region indicates values below the lower limit of detection. (**B**) ROC curves of serum IFN-α2 and MxA for discrimination of IFN-low and IFN-high pSS ($n = 85$). Statistics: (A) censored regression analysis and (B) bootstrap test for two correlated ROC curves to compare AUCs. ***$P < 0.001$; ns: not significant.

Table 2 Measures of accuracy of serum IFN-α2 to identify IFN-high pSS

Measure	Rotterdam			Validation in Malmö cohort		
	19 fg/mL	28 fg/mL	66 fg/mL	19 fg/mL	28 fg/mL	66 fg/mL
Accuracy	0.83 (0.74, 0.90)	0.83 (0.74, 0.91)	0.79 (0.70, 0.87)	0.89	0.89	0.76
Sensitivity	0.84 (0.75, 0.93)	0.80 (0.70, 0.91)	0.71 (0.59, 0.82)	0.92	0.91	0.73
Specificity	0.80 (0.63, 0.93)	0.87 (0.73, 0.97)	0.93 (0.83, 1)	0.81	0.85	0.89
NPV	0.73 (0.61, 0.86)	0.70 (0.60, 0.83)	0.63 (0.54, 0.74)	0.75	0.73	0.50
PPV	0.89 (0.81, 0.96)	0.92 (0.85, 0.98)	0.95 (0.88, 1)	0.94	0.95	0.95

Data represent measures of accuracy (95% CI) calculated from the ROC analysis of the Rotterdam cohort (pSS, $n = 85$; HCs, $n = 40$) and blinded validation of the presented threshold values in the Malmö cohort (pSS, $n = 110$) extended with 28 additional HCs. NPV: negative predictive value; PPV: positive predictive value.

with complement components C3 and C4 in the Rotterdam cohort (Table 3). Neither total EULAR SS Disease Activity Index (ESSDAI) score [25] nor the number of active ESSDAI domains were significantly correlated with serum IFN-α2 (Table 3; Supplementary Table S4, available at *Rheumatology* online). Considering each ESSDAI domain separately, active involvement of haematological or cutaneous domains was associated with higher IFN-α2 (Supplementary Table S4, available at *Rheumatology* online). No differences in serum IFN-α2 were observed between patients with active cutaneous vasculitis/purpura ($n = 4$) and patients with subacute cutaneous lupus erythematosus ($n = 5$) (data not shown). In contrast, patients with active articular involvement had lower IFN-α2 levels (Supplementary Table S4, available at *Rheumatology* online). Multivariable regression analysis was performed on the pooled data from both cohorts to increase statistical power. Multiple regression revealed independent associations of total IgG levels, autoantibodies, use of HCQ and activity in articular and cutaneous ESSDAI domains, but not total ESSDAI score or the number of active ESSDAI domains with serum IFN-α2 in pSS (Table 3; Supplementary Table S4, available at *Rheumatology* online).

Higher serum IFN-α2 in pSS with extra-epithelial extraglandular manifestations (EGMs)

Patients with EGMs are at higher risk for morbidity and mortality [26–28]. We evaluated the association between serum IFN-α2 and EGMs defined as current or past pSS-related systemic involvement in any of the organ-specific ESSDAI domains. Although a trend for higher IFN-α2 was observed in pSS with EGMs compared with patients with exclusive glandular disease, this difference did not reach statistical significance (Supplementary Table S5, available at *Rheumatology* online). Based on the underlying pathophysiological mechanisms, EGMs

Table 3 Clinical characteristics associated with serum IFN-α2 in primary SS

Variables	Bivariate analysis				Multivariable analysis		
	Rotterdam		Malmö				
	Serum IFN-α2	*P*-value	Serum IFN-α2	*P*-value	Estimate	Standard error	*P*-value
Intercept					1.647	0.909	0.070
ESSDAI score	0.166[a]	0.139	0.014[a]	0.889	−0.017	0.024	0.482
Age	−0.158[a]	0.156	−0.122[a]	0.199	−0.015	0.009	0.116
Gender		0.349		0.933	0.332	0.469	0.479
Female	49.9 (≤5–185.8)[b]		80.9 (15.3–308.3)[b]				
Male	88.9 (49.3–188.9)[b]		102.3 (28.2–265.5)[b]				
IgG	0.552[a]	**<0.001**	0.367[a]	**<0.001**	0.092	0.027	**<0.001**
C3	−0.249[a]	**0.026**	−0.046[a]	0.629	−0.050	0.656	0.939
C4	−0.317[a]	**0.004**	−0.089[a]	0.351	−0.887	2.434	0.716
HCQ		0.056		**0.038**	−1.068	0.270	**<0.001**
No	88 (11.4–243.5)[b]		90.5 (37.8–330.5)[b]				
Yes	30 (≤5–124)[b]		47 (7–110.4)[b]				
Corticosteroids		0.777		0.676	0.355	0.350	0.311
No	61.3 (5.1–179.9)[b]		84.5 (13.2–259.3)[b]				
Yes	95.2 (9.5–192)[b]		64.9 (41.7–506.7)[b]				
DMARDs		0.722		0.485	0.444	0.636	0.486
No	67.9 (6.2–185.9)[b]		79.5 (13.3–286.1)[b]				
Yes	19.3 (12–221.2)[b]		118.8 (64.9–467.8)[b]				
ANA		**<0.001**		**<0.001**	1.040	0.407	**0.011**
Negative	≤5 (≤5–11.4)[b]		7.2 (≤5–73.9)[b]				
Positive	91 (16.1–218.9)[b]		95.5 (42.7–405.5)[b]				
SSA		**<0.001**		**<0.001**	1.412	0.450	**0.002**
Negative	≤5 (≤5–10.1)[b]		≤5 (≤5–10.5)[b]				
Positive	87.9 (12.6–214.5)[b]		86.5 (40.6–330.5)[b]				
SSB		**<0.001**		**<0.001**	0.765	0.295	**0.010**
Negative	6.1 (≤5–26.8)[b]		37.5 (6.2–148.5)[b]				
Positive	122 (57.6–259.7)[b]		90.5 (51.1–427.4)[b]				
Storage years	−0.188[a]	0.283	–	–	−0.145	0.108	0.179

Bivariate analysis: data represent [a]correlation coefficients or [b]medians (Q1–Q3) of IFN-α2 concentrations (fg/mL) in pSS patients from the Rotterdam and Malmö cohorts. Correlations between log(IFN-α2) and indicated variables were assessed by likelihood-based correlation coefficient estimation. Multivariable analysis: independent associations of indicated variables with log(IFN-α2) in the pooled cohort were evaluated in a censored regression (tobit) model. For both cohorts, absolute C3 levels were centralized before inclusion in multivariable analysis. Bold values denote statistical significance at the $p < 0.05$ level.

were further classified into peri-epithelial (pulmonary, central nervous system, muscular, renal: tubulointerstitial nephritis), extra-epithelial (cutaneous, peripheral nervous system, lymphadenopathy, haematological, renal: glomerulonephritis) and non-specific (constitutional, articular) [29, 30]. Current or past extra-epithelial EGMs, but not peri-epithelial or non-specific EGMs, were associated with higher IFN-α2 concentrations and a positive correlation was observed between the number of extra-epithelial EGM domains and serum IFN-α2 (Supplementary Table S5, available at *Rheumatology* online). This association remained significant in a multivariable regression analysis adjusting for peri-epithelial and non-specific EGMs, current use of medication, age, gender and serological parameters (Supplementary Table S6, available at *Rheumatology* online). Considering the individual extra-epithelial EGM domains, median IFN-α2 levels were higher in patients with a history of one or a combination of cutaneous (69% cutaneous vasculitis), haematological or lymphadenopathy (including lymphoma) domains, but not the peripheral nervous system domain, compared with patients without a history in any of the extra-epithelial domains (Supplementary Fig. S9, available at *Rheumatology* online).

Discussion

IFN-I pathway activation is a well-known feature in multiple SADs, but direct ultrasensitive measurement of IFN-α protein has been very limited in patients with SADs other than SLE. Using a commercially available Simoa IFN-α immunoassay kit, we found elevated levels of circulating IFN-α2 protein in two independent cohorts of pSS patients and patients with SSc and SLE. In pSS, SLE and SSc, these IFN-α2 protein levels were positively correlated with blood ISG transcript and MxA protein expression, commonly used as surrogate markers for IFN-I pathway activation. These correlations were strongest in SLE patients. In SLE, we found higher IFN-α2 levels in patients not in remission and additionally observed high IFN-α2 levels in a proportion of patients in remission. These data confirm previous observations that linked high levels of serum IFN-α to active disease and risk of relapse [8, 11, 12]. The results obtained from two independent pSS cohorts were highly comparable, indicating good replicability of IFN-α2 measurements between cohorts. Regression analysis did not show a significant negative association between IFN-α2 and the duration of serum storage in this cross-sectional cohort. This indicates that IFN-α2 protein levels in serum were relatively stable for up to 6 years (−80°C) as occurred in our study. Hence this technique is applicable for retrospective analysis of historical cohorts from which only serum is stored.

Ultrasensitive IFN-α protein measurement has well-defined specificity. In this study we used the sole commercially available IFN-α Simoa kit, which we verified to primarily detect IFN-α2, one of the most potent inducers of ISG expression of the IFN-α subtypes [31]. Others have used homebrew Simoa assays with high-affinity antibodies, cloned from APS1 patients, that recognize all IFN-α subtypes [8, 10, 13]. However, these antibodies are currently not commercially available. Using these antibodies, the authors reported a positive correlation between serum IFN-α concentrations and ISG expression score in SLE patients [8]. Here we show similar correlations between blood ISG score and serum IFN-α2. Although the timing and magnitude of expression may vary between the different human *IFN-A* genes, IFN-α2 seems to be expressed exclusively in parallel with other IFN-α subtypes [32, 33]. The analogous results obtained by two Simoa assays with distinct specificities and the current knowledge on human IFN-α biology suggest co-expression of multiple IFN-α subtypes contributing to ISG expression in patients with SADs. A gold standard capturing the entirety of the concept IFN-I pathway activation does not exist. Measurement of ISGs is the most frequently used method, but interpretation of this method for IFN-I specificity has been complicated by the significant overlap between gene expression modules that are activated by distinct IFN receptor complexes and even IFN-independent signalling [34–38]. Nevertheless, attempts have been made to define gene modules preferentially regulated by IFN-I or IFN-II [39, 40]. The five genes of the composite ISG score that we used here belong to an IFN module induced *in vivo* upon treatment with recombinant IFN-α or IFN-β and upregulated *in vitro* preferentially by IFN-I over IFN-II [39]. This ISG score showed an excellent correlation with circulating IFN-α2 protein.

A small subset of pSS patients, mainly characterized by overall milder serological disease activity, exhibited a discrepancy between ISG expression score and IFN-α2 levels. Therefore, in accordance with studies in SLE, whole blood ISG expression seems to be a more sensitive method for IFN-I activation in patients with low levels of IFN-α [8, 11]. It should be noted, however, that altered distribution of leucocyte subsets, a known phenomenon in autoimmune diseases, can cause variation in whole blood ISG expression [41]. The absence of elevated IFN-α2 in serum while ISG expression was positive might be explained by high-affinity binding of low levels of IFN-α to the membrane-bound IFNAR inducing transcriptional responses *in vivo* [42]. Alternatively, these patients may have increased concentrations of IFN-α subtypes or other type I IFNs not quantified by this Simoa assay. Conversely, apparent discordant measurements of high IFN-α2 and a low IFN-I score were found in only a few samples with similar frequency in pSS and HCs. As this might be caused by autoantibodies against IFN-α that could potentially have neutralizing capacity, we analysed sera for the presence of anti-cytokine antibodies using a bead-based method described previously [43, 44]. No autoantibodies against IFN-α2 could be detected in these samples, while these were readily detected in serum from an APS1 patient (Supplementary Fig. S10, available at *Rheumatology* online).

Compared with both ISG expression and direct IFN-α2 measurement, the IFN-I reporter assay was far less sensitive in our cohort, as was also reported by others [11]. Accounting for potential modulating factors in serum upstream of the IFNAR affecting cellular responses to IFN-I, reporter assays measure the bioactivity of IFN-I present in samples rather than the concentration of protein. These assays are limited by a poor sensitivity to detect low levels of IFN-I in patient samples and are hard to standardize for use in clinical practice.

In the two pSS cohorts, serum IFN-α2 concentrations were mainly associated with disease-relevant serological parameters IgG, autoantibodies and complement components. These results are consistent with previous literature describing associations between IFN-I pathway activation and haematological or serological parameters in pSS and other SADs [10, 15, 45–47]. In line with our findings from a placebo-controlled trial of HCQ in pSS and several cross-sectional cohorts [15, 17, 48, 49], we found an independent negative association of HCQ treatment with serum IFN-α2 protein concentrations in pSS. This indicates a persistent effect of HCQ on IFN-I pathway activation after long-term treatment. In addition, we found higher IFN-α2 levels in patients with active cutaneous disease and patients with a history of extra-epithelial manifestations. Extra-epithelial manifestations, in particular cutaneous vasculitis, and several specific serological parameters are well established risk factors for lymphoma in pSS [50]. A limitation of our study is that we were unable to study IFN-I in salivary gland biopsies and correlate these to peripheral IFN-I pathway activation and clinical manifestations in our patient cohorts. Considering our results and the link between IFN-I pathway activation and biological markers of disease activity in pSS, future studies should evaluate potential additional prognostic value of IFN-I measurement for risk stratification.

The current development and clinical evaluation of treatments directed against the IFN-I pathway and related signalling components urges identification of biomarkers for patient stratification. These markers for selection of treatment candidates should accurately reflect the pathway of interest, be useful for prediction of treatment responses and be feasible for application in clinical practice using robust methods that can be standardized across laboratories. A hypothetical marker suitable for monitoring treatment responses or clinical disease activity should additionally be susceptible to change over time. Direct quantification of serum IFN-α by Simoa has a well-defined specificity for the pathway of interest and can be easily standardized considering the automated analytical procedure. The potential clinical relevance of serum IFN-α for use in risk stratification, prediction of treatment responses or monitoring in individual patients of distinct SAD entities remains to be elucidated.

In conclusion, the whole blood ISG expression in pSS, SLE and SSc is closely linked to elevated concentrations of circulating IFN-α2. Direct measurement of IFN-α protein at low concentrations in biological samples has been a considerable step forward in understanding IFN-I pathway activation in SADs and may be relevant in light of patient stratification and targeted treatment.

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Data availability statement

The data underlying this article are available in the article and in its online supplementary material, available at *Rheumatology* online.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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