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ORIGINAL RESEARCH

Leflunomide/hydroxychloroquine combination therapy targets type I IFN-associated proteins in patients with Sjögren's syndrome that show potential to predict and monitor clinical response

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ABSTRACT

Objectives To assess to what extent leflunomide (LEF) and hydroxychloroquine (HCQ) therapy in patients with primary Sjögren's syndrome (RepurpSS-I) targets type I IFN-associated responses and to study the potential of several interferon associated RNA-based and protein-based biomarkers to predict and monitor treatment.

Methods In 21 patients treated with LEF/HCQ and 8 patients treated with placebo, blood was drawn at baseline, 8, 16 and 24 weeks. IFN-signatures based on RNA expression of five IFN-associated genes were quantified in circulating mononuclear cells and in whole blood. MxA protein levels were measured in whole blood, and protein levels of CXCL10 and Galectin-9 were quantified in serum. Differences between responders and non-responders were assessed and receiver operating characteristic analysis was used to determine the capacity of baseline expression and early changes (after 8 weeks of treatment) in biomarkers to predict treatment response at the clinical endpoint.

Results IFN-signatures in peripheral blood mononuclear cell and whole blood decreased after 24 weeks of LEF/HCQ treatment, however, changes in IFN signatures only poorly correlated with changes in disease activity. In contrast to baseline IFN signatures, baseline protein concentrations of galectin-9 and decreases in circulating MxA and Galectin-9 were robustly associated with clinical response. Early changes in serum Galectin-9 best predicted clinical response at 24 weeks (area under the curve 0.90).

Conclusions LEF/HCQ combination therapy targets type-I IFN-associated proteins that are associated with strongly decreased B cell hyperactivity and disease activity. IFN-associated Galectin-9 is a promising biomarker for treatment prediction and monitoring in pSS patients treated with LEF/HCQ.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Biomarkers that robustly monitor and predict systemic disease activity in Sjögren's syndrome (SS) are currently largely lacking. This study investigated whether RNA-based or protein-based type I interferon associated markers could fill up this need.

WHAT THIS STUDY ADDS

⇒ IFN-associated protein expression, but not RNA, seems to identify an inflammatory endotype of patients that allow prediction and monitoring of response to leflunomide/hydroxychloroquine therapy.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ IFN-associated proteins may help to broadly monitor treatment responses in primary SS and may allow stratification of patients.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is characterised by mononuclear infiltration of the exocrine glands, associated with dysfunction of the glands and dryness of primarily mouth and eyes.¹ The majority of pSS patients presents with an interferon (IFN)-signature, reflecting increased expression of type-I IFN-induced genes in circulating immune cells, which is associated with increased systemic disease activity in pSS.² Plasmacytoid dendritic cells (pDCs) are the premier type-I IFN-producing immune cells and as pDCs from patients with pSS produce enhanced levels of type-I IFN on activation³ they appear to be important IFN-producers in pSS. Type-I IFNs importantly drive hyperactivity of B cells, enhancing production of auto-antibodies and formation of immune-complexes, indicated to

contribute to systemic disease activity in pSS.^{4,5} As immune complexes in turn activate pDCs, pDCs and B cells form an amplification loop that is a potential therapeutic target in pSS.⁶ Finally, IFN signatures were suggested to predict clinical response to treatments targeting type I IFN activity and offer potential to stratify patients in systemic lupus erythematosus.⁷

We recently performed a double-blind randomised placebo-controlled clinical trial to test the clinical efficacy of leflunomide (LEF) and hydroxychloroquine (HCQ) combination therapy in patients with pSS (RepurpSS-I). In this study, we demonstrated a clear clinical effect in the treated patients compared with the placebo group.⁸ Seeing as both LEF and HCQ inhibit B cell-activation while HCQ also inhibits activation of pDCs via endosomal Toll-like receptors^{9,10} we hypothesised that targeting the type I IFN/pDC/B cell amplification loop is a mode of action of LEF/HCQ combination therapy. As such, we here evaluated the effects of LEF/HCQ on the type I IFN-signatures as potential biomarkers and three other IFN-associated protein biomarkers. In addition, as the IFN-signature is associated with disease activity, we evaluated whether changes in these biomarkers could be of use for prediction and monitoring treatment response to LEF/HCQ.

METHODS

Patients and study design

Twenty-nine patients fulfilling the classification criteria for pSS,¹¹ with clinically active disease (ESSDAI \geq 5) were enrolled in a randomised, double-blind and placebo-controlled trial investigating efficacy and safety of LEF/HCQ combination therapy.⁸ After randomisation, 21 patients were assigned to LEF/HCQ treatment, 8 patients to placebo treatment (table 1). Clinical assessment was performed after 8 and 16 weeks, and at the clinical endpoint (24 weeks). Patients showing a decrease of \geq 3 ESSDAI points were considered clinical responders, according to prevailing recommendations.¹² See van der Heijden *et al*⁸ for further details.

Quantification of IFN-signature biomarkers

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinised peripheral blood using Ficoll-Paque Plus (GE Healthcare) and stored at -80°C . Whole blood (WB) samples were collected in PaxGene tubes (Qiagen) and stored at -20°C . RNA was isolated using the All prep Universal Kit (Qiagen) using a Qiacube (Qiagen) according to the manufacturer's instructions. IFN-signatures were quantified as previously described^{2,3} based on the expression of *MX1*, *IFIT3*, *IFI44*, *IFI44L* and *Ly6E* as compared with GAPDH, using material from 13 (PBMC) and 23 (WB) healthy donors for comparison. IFN-signature positivity was defined as an IFN-score above the mean+2SD of scores in the healthy donors.

For MxA quantification, heparinised blood (25 μL) was lysed 1:20 and stored at -80°C until analysis. Myxovirus

Table 1 Baseline characteristics

	LEF/HCQ (n=21)	Placebo (n=8)
Age, mean (SD), years	54.7 (12.4)	53.5 (15.2)
Female, no (%)	20 (95)	8 (100)
Disease duration in years, mean (SD)	7.7 (9.8)	8.9 (6.8)
ESSDAI score, mean (SD)	10.4 (3.9)	9.1 (3.4)
ESSPRI score	6.7 (5.3–7.7)	6.7 (5.9–7.9)
Serum IgG, mean (SD), g/L*	19.4 (6.9)	13.8 (5.0)
Unstimulated saliva flow, $\mu\text{L}/5$ min	300 (10–500)	300 (75–1275)
Stimulated saliva flow, $\mu\text{L}/5$ min	400 (200–1075)	700 (150–2250)
IFN-score PBMCs	4.8 (2.4–5.5)	5.0 (2.2–5.6)
IFN-score whole blood	2.4 (1.5–2.9)	2.4 (1.2–2.9)
MxA protein expression whole blood	277 (104–442)	226 (32.8–292)
Serum CXCL10, pg/mL*	620 (335–1181)	360 (251–562)
Serum Galectin-9, ng/mL*	12.9 (7.7–17.2)	10.1 (8.8–17.5)

Data are depicted as median (IQR) unless stated otherwise.
*Indicates significant difference in baseline values between the two treatment arms at $p < 0.05$.
ESSDAI, European Sjogren Syndrome Disease Activity index; ESSPRI, European Sjogren Syndrome Patient Reported Index; LEF/HCQ, leflunomide/hydroxychloroquine; PBMCs, peripheral blood mononuclear cells.

resistance protein A (MxA) enzyme immunoassay was done as previously described.¹³ Briefly, samples and biotinylated detector-mono-clonal antibody (MAb) were loaded onto MAb-coated microtiter strips. After overnight incubation at 8°C , colour reaction was stopped, absorbance at 450 nm was measured, and MxA concentration was read from a standard curve. The detection limit was determined as three times the SD of eight negative control replicates (10 $\mu\text{g}/\text{L}$).¹⁴

Serum was collected in SST-II vacutainers (BD) according to manufacturer's instructions and stored at -80°C until analysis. Measurement of CXCL10 and Galectin-9 levels was performed using multiplex immunoassay as previously described.¹⁵

Statistical analysis

To evaluate changes in parameters between baseline and clinical endpoint within each group and the differences between LEF/HCQ and placebo arms over time, a random intercept model was applied to account for repeated measures within patients, controlling for time

and baseline values. To assess differences in changes in parameters between responders and non-responders, Mann-Whitney U test was used. Differences in the fraction of responders and non-responders between groups were evaluated using Fisher's exact test. Receiver operating characteristic (ROC) curves were used to determine the capacity of changes in biomarkers after 8 weeks of treatment to predict treatment response at the clinical endpoint. Spearman's correlation coefficient was used to assess correlations. Differences were considered to be statistically significant at $p < 0.05$. The analyses were not adjusted for multiple comparisons because of the exploratory nature of our analyses and the relative small sample size. Analyses were performed using SPSS V.25 and GraphPad Prism V.8.

RESULTS

LEF/HCQ treatment decreases type I IFN-signatures and IFN-associated protein biomarkers

At each time point during the trial (baseline, 8 weeks, 16 weeks, 24 weeks), we measured five different biomarkers that represent the IFN activity and are increased in patients with pSS: the RNA type I IFN signatures quantified in PBMCs and WB,² WB MxA protein,¹³ serum CXCL10,¹⁶ and Galectin-9 protein levels.¹⁵ In addition, we included serum IgG in our analyses as a conventional laboratory parameter for B cell hyperactivity as comparison. At baseline, the biomarkers generally correlated with each other and the ESSDAI (online supplemental table 1), consistent with literature. After 24 weeks of LEF/HCQ treatment, all biomarkers except the PBMC IFN-scores a significantly

decreased compared with baseline (figure 1). Similarly, when assessed over time compared with placebo and corrected for baseline, all but the PBMC IFN-scores significantly decreased in the LEF/HCQ treatment group (online supplemental figure 1). Interestingly, changes (Δ) in ESSDAI (Δ ESSDAI) at 24 weeks only correlated with changes in the IFN-induced proteins MxA, Galectin-9, and CXCL10; but not with changes in the type I IFN-signatures or serum IgG. An univariate linear regression model evaluating Δ Galectin-9 and the change in clinical response (Δ ESSDAI) showed an adjusted R^2 of 0.2648. Adding delta MxA in a multivariate linear regression model only slightly improved the adjusted R^2 (adj. $R^2=0.3354$). A multivariate analysis with the three IFN-associated proteins resulted in adjusted $R^2=0.2964$.

Changes in CXCL10 only significantly correlated with changes in ESSPRI (online supplemental table 2). Analysis of the ESSPRI subscores showed significant correlation of ESSPRI fatigue and pain, but not ESSPRI dryness with changes in CXCL10 ($r=0.602$, $p=0.003$; $r=0.563$, $p=0.007$ and $r=0.349$, $p=0.120$, respectively).

In line with previous data, we found only modest increased type II IFN signatures in WB ($p=0.0009$) but not in PBMC ($p=0.1780$) of HCs as compared with pSS patients.¹⁷ The type II IFN signature in WB and also PBMC was not affected by LEF/HCQ treatment ($p=0.3838$ and $p=0.5833$, respectively). Given these findings for the further analyses we only focused on type I IFN signatures and IFN-associated proteins (online supplemental figure 4).

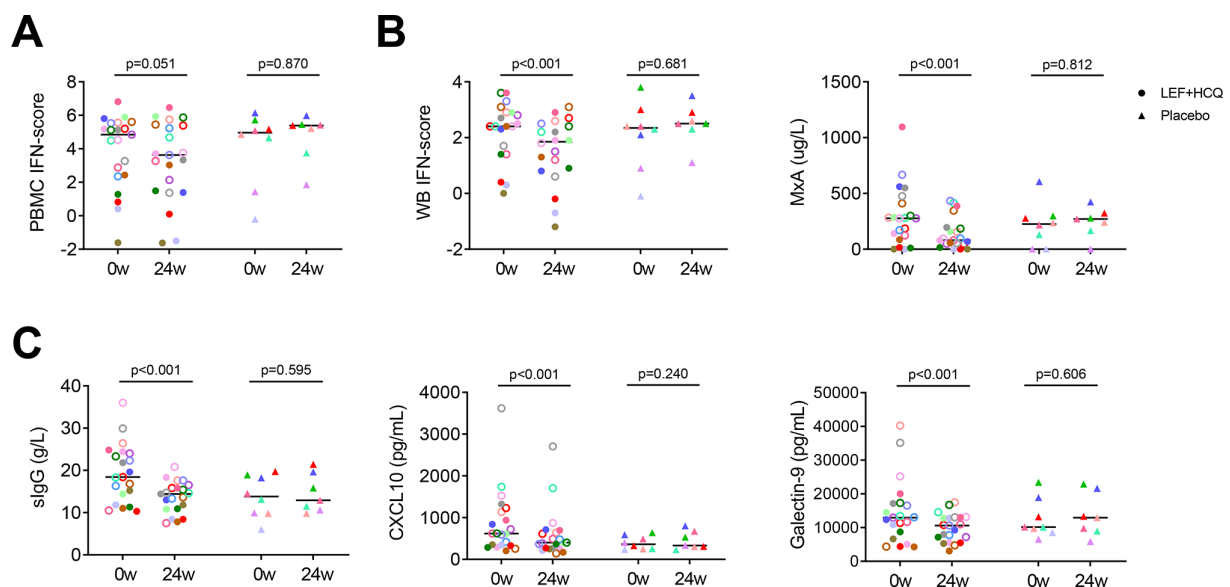


Figure 1 IFN-associated biomarkers decrease on LEF/HCQ combination treatment. Blood was drawn from patients from the LEF/HCQ and placebo groups at baseline and 24 weeks. Five biomarkers associated with the IFN activity were quantified as compared with serum IgG concentrations. In peripheral blood mononuclear cells (PBMCs) (A), whole blood (WB, left panel) (B) type I IFN signatures were measured based on gene expression. In whole blood protein concentrations of MxA (B, right panel) and in serum IgG, CXCL10 and Galectin-9 were measured (C). Differences between 24 weeks and baseline within the LEF/HCQ and Placebo groups were evaluated. Median values are shown. LEF/HCQ, leflunomide/hydroxychloroquine.

Baseline protein levels of Galectin-9, in contrast to IFN-signatures, are associated with clinical response

Of the 21 LEF/HCQ treated patients, 11 were responders at the clinical endpoint. There was a clear difference in Δ ESSDAI between the responders (mean -7.2) and non-responders (mean -0.2) after 24 weeks of treatment (online supplemental figure 2). As such, finding molecular differences between these two groups could help in prediction of clinical response and elucidate the mode-of-action of LEF/HCQ and could be of use for biomarker discovery. We first assessed whether patients with a baseline type I IFN-signature (based on either IFN-score) would be more likely to respond to treatment. However, there were no differences in Δ ESSDAI or in the fraction of responders between IFN-signature negative and positive patients using either method (online supplemental figure 3). Interestingly, a better association was found for

IFN-associated protein Galectin-9, which was significantly different between responders and non-responders at baseline (figure 2). In addition to Galectin-9 at baseline also CXCL10 significantly differed between responders and non-responders. This was not observed for MxA protein or IFN signatures. In line with these differential expression patterns, ROC analyses indicated good prediction for Galectin-9 and CXCL10 (area under the curve, AUC of 0.809 and 0.800, respectively, figure 2B) but moderate to poor prediction for MxA, sIgG and IFN signatures of PBMCs and WB (AUC 0.700, 0.745, 0.700 and 0.590, respectively). Combining baseline CXCL-10 and Galectin-9 in the ROC analysis resulted only in a modest insignificant increased AUC values (AUC of 0.83). Also when analysing continuous data, significant correlation was found between baseline Galectin-9 protein concentrations and ESSDAI response ($r=-0.4671$, $p=0.0328$,

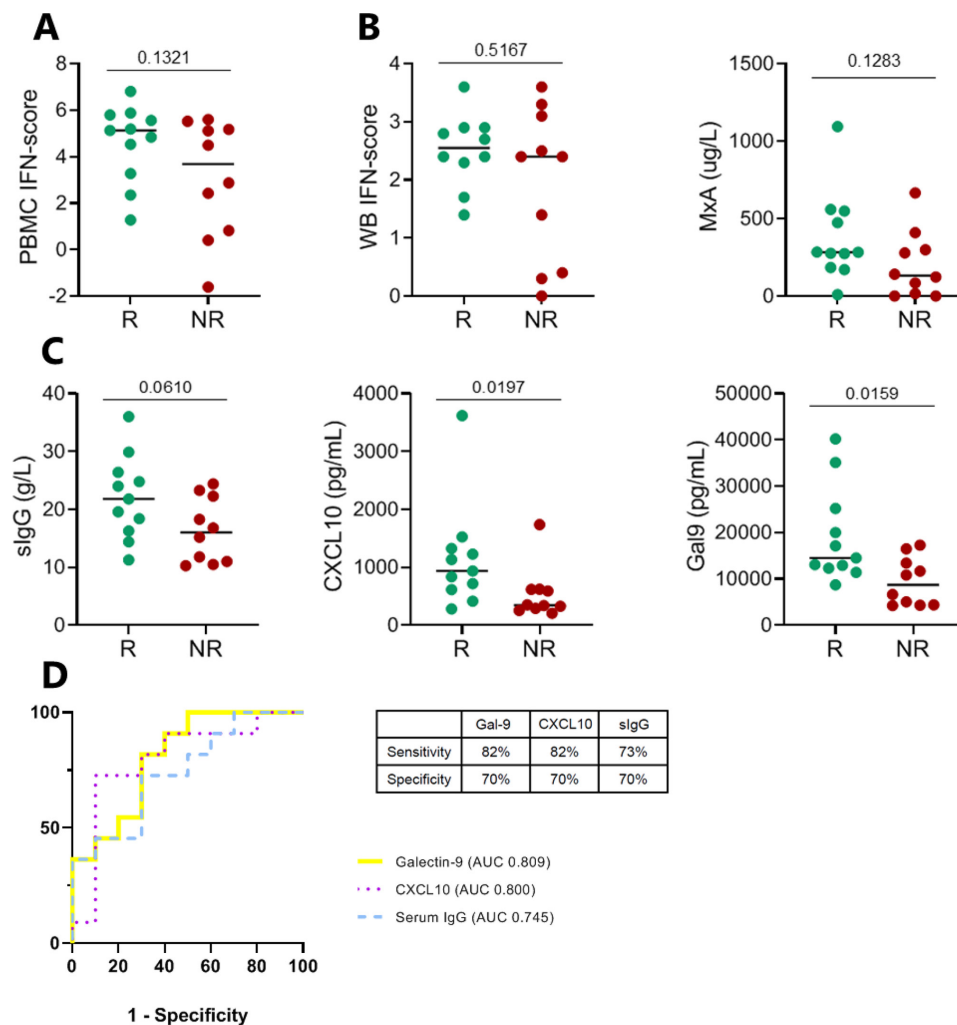


Figure 2 Baseline Galectin-9 and CXCL10 might be used to predict clinical response after 24 weeks (endpoint). IFN-associated biomarkers quantified in PBMCs (A), WB (B), and serum (C) at baseline. Receiver operator characteristic analysis was used to assess whether baseline values of the quantified biomarkers could be used to predict clinical response at 24 weeks. Area under the curve (AUC), sensitivity and specificity are given for serum IgG and the IFN-associated markers (D) (Cut-off values for the sensitivity and specificity were: 12013 pg/L (Gal9), 604.4 pg/L (CXCL10) and 18.35 g/L (sIgG)). LEF/HCQ-treated patients who showed a decrease in ESSDAI of ≥ 3 points at 24 weeks (responder, green diamonds) or not (NR, red circles) are indicated. Individual data (dots) and median values (dash) are shown and p values are indicated. LEF/HCQ, leflunomide/hydroxychloroquine; NR, not reported; PBMCs, peripheral blood mononuclear cells; WB, whole blood.

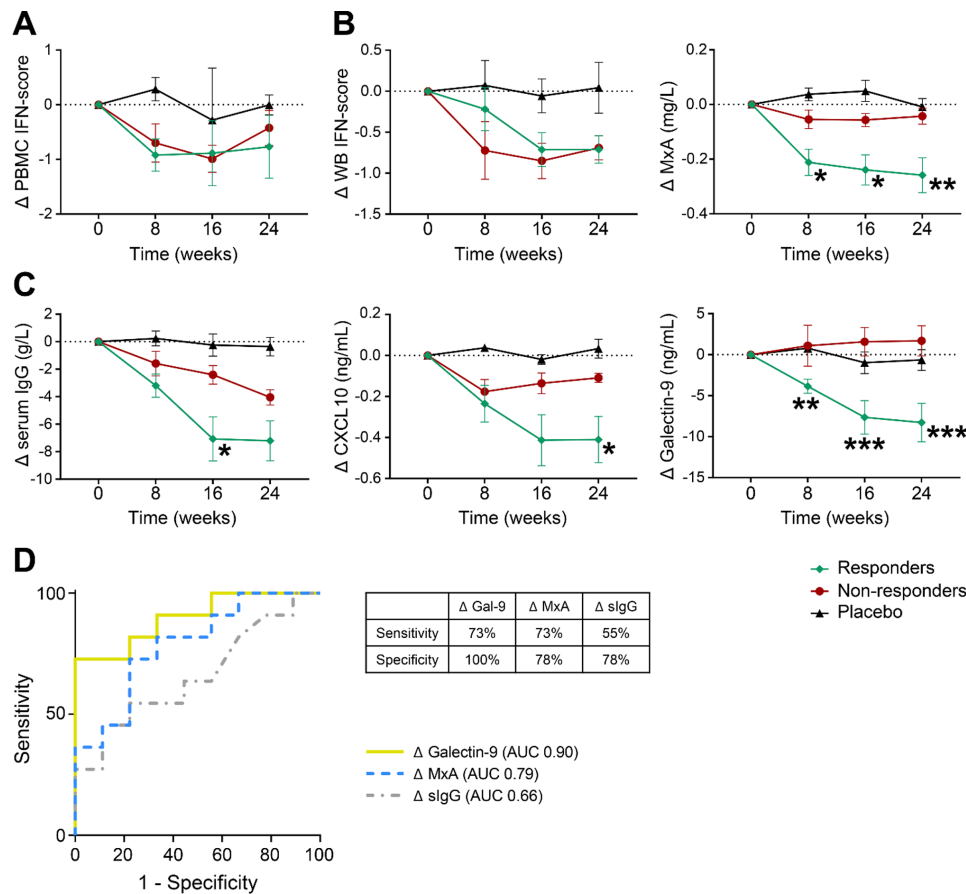


Figure 3 Early changes in Galectin-9 can be used to predict clinical response after 24 weeks (endpoint). Changes over time in the biomarkers quantified in PBMCs (A), WB (B), and serum (C). Receiver operator characteristic analysis was used to assess whether changes (Δ) in quantified biomarkers between 8 weeks and baseline could be used to predict clinical response at 24 weeks. Area under the curve (AUC), sensitivity, and specificity are given for serum IgG and the markers that outperformed serum IgG (D) (cut-off Δ values for the sensitivity and specificity were: -2300 pg/L (Gal-9), -116.5 pg/L (MxA), -2.75 g/L (sIgG)). LEF/HCQ treated patients who showed a decrease in ESSDAI of ≥ 3 points at 24 weeks (responders, green diamonds) or not (NR, red circles) and the placebo-treated group are indicated (black triangles). Mean \pm SEM are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, respectively. LEF/HCQ, leflunomide/hydroxychloroquine; NR, not reported; PBMCs, peripheral blood mononuclear cells; WB, whole blood.

(online supplemental table 3). This was not statistically significant for CXCL10 ($r = 0.3862$, $p = 0.0838$), in contrast to dichotomised clinical data. IFN signatures and all IFN associated proteins at baseline correlated with the change in serum IgG levels after 24 weeks. Interestingly, on further analyses of ESSPRI subscores, baseline Galectin-9, CXCL10 and sIgG levels significantly correlated with the ESSPRI pain scores ($r = -0.51$ $p = 0.018$, $r = -0.71$ $p = 0.0003$, $r = -0.44$ $p = 0.047$, respectively), but not with ESSPRI dryness or fatigue scores (online supplementary table 3). No other correlations with clinical outcome measures were observed for baseline levels of MxA or sIgG protein levels or IFN signatures of PBMCs and WB.

MxA and Galectin-9 are promising biomarkers for monitoring and prediction of treatment response

We next evaluated the differences between responders and non-responders in the decrease of IFN-signature biomarkers over time. Consistent with the lack of correlation between Δ ESSDAI and Δ IFN-signatures, there were no differences in Δ IFN-signatures between responders

and non-responders at any timepoint (figure 3A,B). Levels of serum CXCL10 and serum IgG decreased strongly in the responders but also went down in the non-responders, leading to significant differences only at single timepoints (figure 3C). Strikingly, after only 8 weeks of treatment responders showed clear changes in both MxA and Galectin-9 levels, while non-responders showed changes comparable to those in the placebo group (figure 3B,C). As such, early changes in MxA and Galectin-9 between baseline and 8 weeks could potentially be used to predict treatment response.

To evaluate this, the changes in MxA and Galectin-9 between baseline and 8 weeks were used to discriminate responders from non-responders using ROC analysis. For comparison, changes in serum IgG at 8 weeks showed mediocre potential for predicting clinical response at 24 weeks (AUC 0.66). Strikingly, both Δ MxA (AUC 0.79) and Δ Galectin-9 (0.90) substantially outperformed Δ serum IgG. Δ Galectin-9 in particular showed promise as a biomarker with a specificity of 100% and a sensitivity

of 73% for predicting clinical response at 24 weeks (figure 3D). Δ PBMC IFN-score (AUC 0.55), Δ WB IFN-score (AUC 0.64) and Δ CXCL10 (AUC 0.52) performed poorly and did not outperform Δ serum IgG. The AUC did not improve when combining the best performing proteins (Δ MxA and Δ Galectin-9, resulting in an AUC of 0.88).

DISCUSSION

The present study demonstrates that LEF/HCQ combination therapy more strongly downregulates systemic IFN-associated protein levels than systemic type I IFN signatures. Surprisingly, changes in ESSDAI scores were robustly associated with changes in IFN-induced proteins MxA and Galectin-9, but not with changes in either IFN-score. Finally, baseline concentrations and early changes in IFN-associated proteins are associated with clinical endpoint responses.

Supporting previous data, we demonstrated modestly upregulated type II IFN signatures in the blood of pSS patients, which were not changed by the treatment.¹⁷ LEF/HCQ treatment did decrease systemic type-I IFN activity in patients with pSS, which might be induced by HCQ, as this was recently demonstrated on HCQ monotherapy in pSS patients.¹⁸ However, recently it was also demonstrated that LEF in vitro, in concentrations clinically relevant, significantly and robustly inhibits type I IFN activity by mononuclear cells from pSS patients.¹⁹ Previously, it was demonstrated that the clinical response to LEF was also associated with downregulation of IFN γ production, a type II IFN.^{20–21} Increased type II IFN activity on the RNA and protein level have been demonstrated in pSS salivary glands.^{17–22} LEF in vitro was shown to also inhibit IFN γ production. The here recorded inhibition of IFN activity by LEF/HCQ treatment may, therefore, be partly derived from IFN γ inhibition at the site of inflammation. Hence, both LEF and HCQ may contribute to the observed changes in IFN signatures and IFN-associated proteins in the present study.

Overall, IFN-associated proteins (MxA, CXCL10 and Galectin-9) performed well in monitoring and predicting disease activity in contrast to RNA-based IFN signatures and serum IgG levels. This discrepancy between the lack of correlation of changes in disease activity with changes in IFN signatures and positive correlation with IFN-associated proteins, may be explained by a difference in compartments. Circulating type I IFNs and IFN-associated proteins are likely abundantly produced at the site of inflammation, in-line with production of Galectin-9 in skin and muscle tissue from patients with juvenile dermatomyositis,²³ while the IFN-signatures are quantified in circulating immune cells. As circulating cells are long-lived, it may take longer for changes in local inflammation to be reflected at the gene-expression level in circulating cells. Also, IFN-induced imprints may be long-lived, requiring longer time to normalisation or more maximal inhibition of interferons. Alternatively,

differences in regulation between protein and RNA expression may contribute to the observed changes. In this respect, we found only a moderate correlation between Δ MxA and changes in expression of its gene *MXI*, which makes up part of the IFN-score, within WB ($r=0.44$, $p=0.044$).

Galectin-9 in the current study was the best performing cytokine reflecting disease activity when analysed cross-sectionally and allowed monitoring of disease activity over time following treatment with LEF/HCQ. Galectin-9 is a protein that can be induced by IFN α signalling and was found upregulated in pSS patients.^{15–24} Galectin-9 also is a negative regulator of B cell activity, serving as a feedback to control B cell activation in pSS. In support of this in the present study it significantly correlated with serum IgG levels. Hence, Galectin-9 levels reflects both B cell hyperactivity and IFN-induced activation in pSS. Interestingly, baseline values of Galectin-9 showed good predictive value. In addition, early (after 8 weeks) changes in serum Galectin-9 most robustly predicted which patients responded to LEF/HCQ at 24 weeks. Thus, serum Galectin-9 seems to be a promising biomarker for monitoring and prediction of treatment response to LEF/HCQ combination therapy in patients with pSS.

The present data demonstrating differential expression of CXCL10 between responders and non-responders at baseline corroborate previous CXCL10 baseline measurements using a different proteomic platform.⁸ Here, we show robust decreases of CXCL10 by LEF/HCQ therapy. Changes in CXCL10 to lesser extent than Galectin-9 and MxA correlated with changes in disease activity. However, interestingly CXCL10 was the only IFN-associated biomarker that showed significant correlation with the patient reported outcome ESSPRI over time. Since the improvement of saliva output was also significantly, although modestly, correlated to ESSPRI scores, this finding was suggested to be related to dryness symptoms. However, ESSPRI dryness was not significantly correlated to CXCL10 levels, but instead correlated to ESSPRI pain and fatigue subscores. CXCL10 previously has been associated with neurological phenomena such neuropathic pain in mice and pain and fatigue in different human inflammatory conditions.^{25–27} This seems to indicate involvement of CXCL10 in pain and fatigue processes in pSS.

Effective prediction of clinical response at baseline may greatly reduce the number of patients who receive inefficient long-term treatment, adding to cost-effectiveness and preventing unnecessary side effects. Recently, we found a serum proteome that seems to indicate that an inflammatory endotype typifies patients that are inclined to respond to LEF/HCQ treatment. The current study extends these observations indicating that IFN-associated proteins such as Galectin-9, MxA and CXCL10 could help in prediction and provide a means to monitor therapy efficacy in patients. These results should, however, be validated in larger follow-up studies that are presently ongoing.

Larger follow-up studies will also need to demonstrate to what extent the here described IFN-associated biomarkers are valuable tools for monitoring or predicting disease activity in SSA patients or those with high patient reported outcomes, for example, those with an inflammatory endotype. In the present study, results were corrected for baseline values, warranting the significance of our data. Nonetheless, randomisation could not overcome differences in some biomarkers at baseline (sIgG, CXCL10) due to low n-values, in particular in the placebo group. A higher number of patients and 1:1 randomisation in future studies will most likely prevent this, allowing more optimal comparison, also at the molecular level.

The decreased type-I IFN activity and B cell hyperactivity in treated patients as well as the specific decrease in MxA and Galectin-9 observed in responders, suggest that LEF/HCQ combination therapy thwarts the pDC/B cell IFN amplification loop. The lack of differences in response between IFN-positive and IFN-negative patients at baseline may be due to the above-mentioned mechanisms leading to discordance of IFN activity and IFN signatures. Alternatively, the observed decrease in Galectin-9 levels may be in part driven via inflammatory pathways other than type-I IFN, such as IFN- γ and TNF- α which are known to induce its production.²⁸

Thus, our data indicate that the type I IFN pDC/B cell amplification loop is a target of LEF/HCQ therapy. In addition, we show that IFN-associated proteins in particular serum Galectin-9 is associated with clinical response and a promising biomarker for prediction and monitoring of clinical response to LEF/HCQ combination therapy. These data represent the first analysis of the relationship between IFN-activity and clinically relevant changes in ESSDAI within longitudinal data in patients with pSS, and provide compelling evidence for a central role of IFN-activity in pSS immunopathology.

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Contributors EvdH, AAK, TR and JAvR were involved in conception and design of the study. EvdH, SLMB, CPJB, AAK, CGVH-M and MV were involved in data acquisition. EvdH, SH, APL, MH, HL and JAvR were involved in data analysis and interpretation. EvdH, SH, MH and JAvR drafted the manuscript. All authors contributed to the article and approved the submitted version. MH and JAvR are joint last authors. JAvR is the guarantor of this study

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Competing interests None declared.

Patient consent for publication Consent obtained directly from patient(s).

Ethics approval This study involves human participants and was approved by Medical ethical committee of the University Medical Center Utrecht protocol number 15-427. Participants gave informed consent to participate in the study before taking part.

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