EXTENDED REPORT

MxA as a clinically applicable biomarker for identifying systemic interferon type I in primary Sjögren’s syndrome

Naomi I Maria,1 Zana Brkic,1 Matti Waris,2 Cornelia G van Helden-Meeuwesen,1 Kim Heezen,1 Joop P van de Merwe,1 Paul L van Daele,1 Virgil A S H Dalm,1 Hemmo A Drexhage,1 Marjan A Versnel1

ABSTRACT

Objective To establish an easy and practical assay for identifying systemic interferon (IFN) type I bioactivity in patients with primary Sjögren’s syndrome (pSS). The IFN type I signature is present in over half of the pSS patients and identifies a subgroup with a higher disease activity. This signature is currently assessed via laborious expression profiles of multiple IFN type I-inducible genes.

Methods In a cohort of 35 pSS patients, myxovirus-resistance protein A (MxA) was assessed as a potential biomarker for type I IFN activity, using an enzyme immunoassay (EIA) on whole-blood and flow cytometric analyses (fluorescence-activated cell sorting, FACS) of isolated CD14 monocytes. In addition, potential biomarkers such as CD64, CD169 and B cell-activating factor (BAFF) were simultaneously analysed in CD14 monocytes using FACS. The IFNscore, a measure for total type I IFN bioactivity, was calculated using expression values of the IFN type I signature genes—IFI44, IFI44L, IFIT3, LY6E and MX1—in CD14 monocytes, determined by real-time quantitative PCR.

Results IFNscores correlated the strongest with monocyte MxA protein (r=0.741, p<0.001) and whole-blood MxA levels (r=0.764, p<0.001), weaker with CD169 (r=0.495, p<0.001) and CD64 (r=0.436, p=0.007), and not at all with BAFF protein. In particular, whole blood MxA levels correlated with EULAR Sjögren’s Syndrome Disease Activity Index scores and numerous clinical pSS parameters. Interestingly, patients on hydroxychloroquine showed reduced MxA levels (EIA, p=0.04; FACS p=0.001).

Conclusions The MxA assays were excellent tools to assess IFN type I activity in pSS, MxA-EIA being the most practical. MxA levels associate with features of active disease and are reduced in hydroxychloroquine-treated patients, suggesting the clinical applicability of MxA in stratifying patients according to IFN positivity.

INTRODUCTION

Primary Sjögren’s syndrome (pSS), the second most common systemic autoimmune disease, characterised by lymphocytic infiltrates in the salivary and lacrimal glands. Clinical manifestations range among others from ocular and oral dryness to vasculitis and severe fatigue. To date, effective therapy is not available and treatment has been mainly symptomatic. The aetiology of pSS is largely unknown, but evidence for a role of interferon (IFN) type I in the pathogenesis of pSS has been emerging.1–12 Further unravelling the complex pathophysiology of pSS is essential for finding disease-related biomarkers and identifying new treatment targets.

Previously, we described the systemic upregulation of IFN type I-inducible genes (IFIGs) in CD14 monocytes of pSS patients, the so-called IFN type I signature.1–7 The signature is not restricted to monocytes, as a similar IFN type I signature has been observed in pSS peripheral blood mononuclear cells (PBMCs).12,13 Assessment of the IFN type I signature is, however, a laborious real-time PCR technique for the expression of multiple IFIGs and at present, another reliable immunoassay for detection of type I IFNs in blood or serum is not available. The latter is mainly due to the presence of multiple subtypes of IFN type I.1–3,4,15 An easy and functional assay to determine the presence of IFN type I activity in the circulation would facilitate the identification of pSS patients with IFN type I signature and further contribute to unravelling the role of IFN type I in pSS patients.

The IFN type I signature, as defined by us, resulted from a factor analysis of multiple tested IFIGs and showed five genes—IFI44L, IFI44, IFIT3, LY6E and MX1—to predict 95% of the total variance of a large set of IFIGs. These five genes were used to calculate an IFN type I expression score (IFNscore) per subject. Recently, we showed this IFN type I signature in pSS monocytes to be associated with higher EULAR Sjögren’s Syndrome Disease Activity Index (ESSDAI) scores as well as higher autoantibody production, rheumatoid factor and serum IgG, lower C3 complement levels and higher B cell-activating factor (BAFF) gene expression.13

The IFN type I signature is also displayed by subgroups of patients with systemic sclerosis (SSc), systemic lupus erythematosus (SLE), dermatomyositis and rheumatoid arthritis. Several biomarkers for IFN type I activity have been proposed, among others are MxA, CD64 and CD169.13,24 However, these protein markers have never been tested systematically and simultaneously in a single (pSS) patient group.

MxA (Myxovirus-resistance protein 1) is a key mediator of the IFN-induced antiviral response and is tightly regulated by type I IFNs.25–27 In SSc, MxA gene expression was proposed as a biomarker for type I IFN bioactivity and found to correlate

with disease activity. Several methods for detection of MxA have been previously described. Vallittu et al evaluated their enzyme immunoassay (EIA) for MxA in whole blood for monitoring IFN type I bioactivity in multiple sclerosis (MS) patients treated with IFN-β. The MxA-EIA was considered by the authors to be a faster and more reliable method compared with flow cytometric analysis of MxA in PBMCs. To our knowledge, MxA-EIA has never been used for the detection of IFN type I activity in systemic autoimmune diseases.

In this study, we tested MxA as a candidate biomarker for systemic IFN type I activity in pSS using EIA and flow cytometry in whole blood lysates and CD14 monocytes, respectively. In addition, the expression of CD64 (Fcγ RI), CD169 (Siglec-1) and BAFF proteins were simultaneously assessed in CD14 monocytes, as these markers have also been proposed as biomarkers for monitoring IFN type I bioactivity in systemic autoimmune diseases.

### PATIENTS AND METHODS

#### Patients

According to the 2002 American-European criteria, 35 patients positively diagnosed with pSS were recruited. Patients treated with high-dose prednisone (>10 mg daily), immunosuppressants or biologicals were excluded. Level of disease activity was assessed using ESSDAI. Twenty-seven healthy controls (HCs), or biologicals were excluded. Level of disease activity was previously described.32

#### Blood collection, preparation and monocyte isolation

Blood samples were collected in clotting tubes for serum preparation and in sodium heparin tubes (Greiner Bio-One, Germany). From heparinised blood, PBMCs and consecutively CD14 peripheral blood monocytes were isolated as previously described.32

#### Patients and control characteristics

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=27)</th>
<th>pSS IFNpos (n=21)</th>
<th>IFNneg (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (%)</td>
<td>24/27 (89)</td>
<td>16/21 (76)</td>
<td>12/14 (86)</td>
</tr>
<tr>
<td>Mean age (years)*</td>
<td>53±11</td>
<td>57 (30–85)</td>
<td>55 (37–76)</td>
</tr>
<tr>
<td>Disease duration (years)*</td>
<td>NA</td>
<td>13.8±6.4</td>
<td>10.9±7.7</td>
</tr>
<tr>
<td>pSS manifestations (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ocular symptoms</td>
<td>NA</td>
<td>20/21 (95)</td>
<td>14/14 (100)</td>
</tr>
<tr>
<td>Oral symptoms</td>
<td>NA</td>
<td>21/21 (100)</td>
<td>13/14 (93)</td>
</tr>
<tr>
<td>Positive ocular tests</td>
<td>NA</td>
<td>15/21 (71)</td>
<td>12/14 (86)</td>
</tr>
<tr>
<td>Positive histopathology</td>
<td>NA</td>
<td>11/21 (52)</td>
<td>7/14 (50)</td>
</tr>
<tr>
<td>Positive salivary gland tests</td>
<td>NA</td>
<td>1/21 (5)</td>
<td>1/14 (7)</td>
</tr>
<tr>
<td>Anti-SSA</td>
<td>NA</td>
<td>20/21 (95)</td>
<td>10/14 (71)</td>
</tr>
<tr>
<td>Anti-SSB</td>
<td>NA</td>
<td>17/21 (81)</td>
<td>6/14 (43)</td>
</tr>
<tr>
<td>Medication status (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>NA</td>
<td>5/21 (24)</td>
<td>4/14 (29)</td>
</tr>
<tr>
<td>Hydroxychloroquine</td>
<td>NA</td>
<td>13/21 (62)</td>
<td>13/14 (93)</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>NA</td>
<td>1/21 (5)</td>
<td>1/14 (7)</td>
</tr>
</tbody>
</table>

*Data are presented as means with corresponding range or SD. IFNpos, IFN type I signature positive; IFNneg, IFN type I signature negative; NA, not applicable; pSS, primary Sjögren’s syndrome.

### Measurement of complements, immunoglobulin levels and autoantibodies

C3 and C4 complements, immunoglobulin levels (such as IgG, IgM and IgA) and anti-Sjögren’s syndrome (SS) A/B autoantibody levels were measured as described previously.

### Real-time quantitative PCR

Total RNA was isolated from purified CD14 monocytes using RNaseq columns (Qiagen, Hilden, Germany), subsequently reverse-transcribed to cDNA using a high-capacity cDNA Reverse Transcription Kit and real-time PCR analysis using pre-designed primer/probe sets (Applied Biosystems, Foster City, California, USA). For calculation of relative expression, all samples were normalised to expression of the householder gene Abl. Fold change values were determined from normalised CT values (CT gene – CT housekeeping gene) using the Comparative CT (2-ΔΔCT) method (User Bulletin #2, Applied Biosystems, Foster City, California).

### Flow cytometry

Fluorescence-activated cell sorting (FACS) analysis was used to determine surface expression and intracellular cytokine content in purified CD14 monocytes. Membrane staining was performed with fluorescently labelled antibodies: anti-CD14 (APC/Cy7; BD Biosciences, California, USA), and either anti-CD64 (PE; Serotech, Toronto, Canada) or CD169 (Alexa Fluor-647; Serotech) were incubated in the dark for 20 min. Subsequently, cells were fixed (1% paraformaldehyde), permeabilised (0.5% saponin) and stained with either anti-BAFF (FITC; R&D Systems, Philadelphia, Pennsylvania, USA) or unconjugated anti-MxA (ProteinTech Group, Chicago, Illinois, USA), incubated in the dark for 30 min on ice; secondary antibody was goat-anti rabbit-FITC (Supertech). Unstained cells and appropriate isotype-matched controls (BD Biosciences) were used to confirm antibody specificity. Fluorescence was assayed using four-colour flow cytometry (FACS CaliburBeckton Dickinson & co, San Jose, California, USA) and analysed using FlowJo Software (TreeStar Inc, USA).

### MxA EIA

MxA-EIA was executed as previously described. Heparinised blood (25 μl) was lysed 1:20 and stored at −70°C until assayed. Briefly (see online supplementary figure S1 for full description), lysed whole blood samples and biotinylated detector-monoclonal antibody (MAb) were loaded onto MAb-coated micortitre strips and incubated overnight at 8°C; colour reaction was stopped, absorbance was measured and [MxA] read from a master standard curve. Detection limit was 10 μg/l and determined as three times the SD of eight negative control replicates.

### IFNscore

Monocyte IFN type I signature was defined by the relative expression of five IFIGs: IFI44L, IFI44, IFIT3, Ly6E and MxA. Mean_{ΔΔCT} and SD_{ΔΔCT} of each gene in the HC group were used to standardise the expression levels. IFNscores per subject represent the sum of these standardised scores, calculated as previously described. IFNscore distribution for the 35 patients was bimodal, with an overlap at a score of 10. Furthermore, HC only occasionally showed an IFNscore≥10; consequently, values of 10 or more represent IFN type I signature positivity.
Statistical analysis

Comparisons were analysed using the non-parametric Mann–Whitney U test to compare medians, and an independent t test was used to compare the means (for normally distributed data). For correlation studies, the Spearman’s rho (r_s) or Pearson’s correlation coefficient (r_p) were calculated. Values of p<0.05 were considered statistically significant. For multiple variable testing, values of p<0.01 (p<0.05/5) were considered statistically significant after applying a Bonferroni correction (n=5). Multiple group comparisons were analysed using the Kruskal–Wallis test; statistical analysis was performed using SPSS V17.0.

RESULTS

Stratification of pSS patients into IFN type I signature positive and negative patients

We used our previously described definition for the IFN type I signature and reconfirmed the presence of the signature in the current cohort—using the five indicator genes IFI44L, IFI44, IFIT3, LY6E and MX1 (figure 1A). We stratified the patient group as previously described in patients positive for the IFN type I signature (IFNpos; IFNscore≥10) and patients negative for the signature (IFNneg; IFNscore<10). Using this paradigm, none of the HC scored above 10, whereas 21 out of 35 pSS patients were IFNpos—a prevalence of 60% (figure 1B). In figure 1C, gene expression for the five signature genes is depicted in a heat map.

MxA correlates strongly to IFN type I bioactivity in pSS

To assess MxA protein as a potential biomarker for the IFN type I signature, intracellular MxA was measured in CD14 monocytes using flow cytometry and in whole-blood lysates using MxA-EIA.

Significant positive correlation was observed between the IFNscores and intracellular MxA levels, as assessed by flow cytometry (figure 2B). Relative mean fluorescence intensity scores of MxA were next compared between HC, IFNneg and IFNpos pSS patients. Quantification of CD14 monocyte MxA levels showed a significant increase in IFNpos patients compared with IFNneg patients and HC, whereas IFNneg patients showed MxA levels indistinguishable from HC (figure 2C,D).

Using the MxA-EIA, MxA protein levels (µg/l) were assessed in lysed whole blood of the same study subjects. Correlating whole blood MxA levels to IFNscores showed a significant positive correlation (figure 2E). IFNpos patients showed significantly elevated MxA protein levels, compared with IFNneg patients and HC. An increase was not observed in IFNneg patients, again showing MxA levels equal to HC (figure 2F).

CD64 and CD169 correlate weakly to IFN type I bioactivity in pSS

We also assessed surface protein levels of CD64 and CD169 (other proposed biomarkers of IFN activity) on CD14 monocytes using flow cytometry (see online supplementary figure S2). A significant positive correlation was observed between the IFNscore and surface expression of CD64 (figure 3A) and CD169 (figure 3B), respectively, though with considerably lower correlation coefficients as found for MxA (figure 2).

Figure 3D shows CD64 upregulation on monocytes of pSS patients irrespective of their IFN positivity. For CD64 surface expression, this was not the case as the marker was solely upregulated in IFNpos pSS patients (figure 3E).

BAFF protein does not correlate to IFN type I bioactivity in pSS

Previously, we established a positive correlation for the presence of the IFN type I signature and BAFF mRNA gene expression in
CD14 monocytes. This observation was confirmed in our current cohort (see online supplementary figure S3). We therefore assessed intracellular BAFF protein in CD14 monocytes as a candidate biomarker for IFN type I, using flow cytometry. Intracellular BAFF levels showed no correlation with the IFNscore (figure 3C; see online supplementary figure S2). Moreover, BAFF protein levels were not significantly elevated in pSS patients compared with HC, neither in IFNpos nor IFNneg patients (figure 3F).

MxA correlates with disease manifestations of pSS

Previously observed association of the IFN type I signature with high ESSDAI disease activity scores (reconfirmed in this study; figure 4A) gave rise to the question whether this also holds true for the here described potential biomarkers MxA, CD64 and CD169. We were able to assess ESSDAI disease activity scores in 23 pSS patients of this cohort. Significant correlation was observed between ESSDAI scores and MxA levels measured by either assays, even though the correlation was the strongest when the EIA was used (table 2 and figure 4B,C). For the other biomarkers, no correlation was found (see online supplementary table S1).

We subsequently determined whether the potential biomarkers were associated with classical aberrant immune parameters of pSS such as anti-SSA and anti-SSB autoantibodies, rheumatoid factor, immunoglobulin levels and neutrophil counts (table 2). In particular, whole blood MxA levels showed the strongest and the most significant correlations with many of these parameters; patients with autoantibodies showed higher levels of whole blood MxA compared with patients free of autoantibodies (figure 4D). Significant positive correlations were also found between whole-blood MxA and rheumatoid factor, and immunoglobulin levels (IgG, IgA and IgM) (table 2, figure 4E). Significant negative correlations were found between whole-blood MxA, haemoglobin levels and neutrophil counts. Monocyte MxA(Facs) and CD169 levels performed less well in these comparisons (table 2; see online supplementary table S1).

Patients on hydroxychloroquine (HCQ, Plaquenil) treatment had significantly reduced biomarkers of IFN activity (figure 4F, G); table 2 and figure 4G show patients on HCQ treatment to have reduced levels of MxA, measured by EIA and FACS. HCQ was, however, found to have no significant effect on ESSDAI levels (figure 4H).
Figure 3  Flow cytometric analysis of CD14 monocytes from healthy controls (HCs) and primary Sjögren’s syndrome (pSS) patients. Correlation plots shown between interferon (IFN) scores for all study subjects and (A) CD64, (B) CD169 and (C) B cell-activating factor (BAFF) protein levels, respectively. Relative mean fluorescent intensity (MFI) for (D) CD64 (E) CD169 and (F) BAFF protein levels are shown for CD14 monocytes from HC (n=27) and pSS patients, stratified in IFN type I signature negative (IFNneg) (n=14) and IFN type I signature positive (IFNpos) (n=21) patients. Each symbol represents an individual sample; horizontal lines represent the median. Relative expression was calculated as protein-specific staining (MFI)/isotype control (MFI). The correlation coefficients (r) and p values are shown. For correlations on CD64 and CD169, Spearman’s rho correlation test was used and to compare medians, the Mann–Whitney U test was used. For BAFF, Pearson’s correlation test was used and to compare means, the independent t test was used.

Figure 4  EULAR Sjögren’s Syndrome Disease Activity Index (ESSDAI) scores stratified in (A) interferon (IFN) type I signature negative (IFNneg) and IFN type I signature positive (IFNpos) primary Sjögren’s syndrome (pSS) patients (B) MxA (μg/l) levels as measured by the MxA-EIA; MxA<100 μg/l (low) and MxA>100 μg/l (high). Cutoff values were determined as meanHC + 2×SDHC (C) Correlation plot is shown between EULAR Sjögren’s Syndrome Disease Activity Index (ESSDAI) scores and MxA (μg/l) levels. (D) MxA (μg/l) levels in pSS patients in presence (+) or absence (−) of autoantibodies; anti-SSA (divided in Ro52 and Ro60) and anti-SSB (E) immunoglobulin levels (g/l) such as IgG, IgA and IgM in pSS patients stratified in MxA<100 μg/l and MxA>100 μg/l. Comparisons for pSS patients in presence (+) or absence (−) of hydroxychloroquine (Plaquenil) treatment plotted for (F) the IFNscore; line represents IFNscore cutoff ≥10, (G) MxA (μg/l), as measured by the MxA-EIA and (H) ESSDAI scores. Each symbol represents an individual sample; horizontal lines represent the median. The correlation coefficient (r) and p values (*p>0.05; **p>0.01; ***p>0.001) are shown, Spearman’s rho correlation test was used and to compare medians, the Mann–Whitney U test was used.
DISCUSSION

Our study is the first to simultaneously assess protein levels of MxA, CD64, CD169 and BAFF as biomarkers for IFN type I detection. Herein, MxA was distinguished as the best functional biomarker for systemic IFN type I activity in pSS.

MxA protein is considered as an important mediator of early innate immune defence and its expression has been used as a marker for IFN bioactivity in experimental as well as clinical settings. The MxA gene, in contrast to other IFIGs, is not directly induced by viruses and strictly depends on IFN signalling. Furthermore, the stability of MxA protein in cells was observed to be higher than the corresponding mRNA levels. We assessed intracellular MxA protein using an EIA method on whole blood and flow cytometric analyses on circulating CD14 monocytes. In our patients, flow cytometric detection of MxA in monocytes showed a slightly better distinction between IFNpos and IFNNeg patients within this pSS group, relative to the EIA in whole blood. Nevertheless, the MxA-EIA was a more practical assay as it requires only 25 μl of whole blood—obtained during routine blood collection and easily stored at −70°C to the time of assessment—without necessity of laborious PBMC isolation and subsequent monocyte isolation. Vallittu et al., who previously validated the MxA-EIA for monitoring MS patients treated with IFN-β, concluded the EIA favourable for large-scale clinical application due to its high level of standardisation and reproducibility. We also experienced the EIA to be less variable than FACS (data not shown). This report shows whole-blood MxA levels measured by EIA to have the strongest correlation with features of active pSS, that is, with higher ESSDAI scores, anti-SSA/SSB antibodies, rheumatoid factor, immunoglobulin levels, lower haemoglobin levels and neutrophil counts. We found no association with complement consumption as previously found for the IFNscore. On the basis of these observations, we propose to use the MxA-EIA for identification of IFN type I bioactivity in pSS.

Alongside MxA, we also considered CD64, CD169 and BAFF as candidates, observing a significant correlation between CD64 and CD169 and the IFNscore, be it with a relatively low correlation coefficient.

CD64, the high-affinity Fc-gamma receptor I (FcγRI) recognising Fc portion of IgG, is exclusively expressed on myeloid cells and found to correlate with IFIG expression in SLE. Levels of CD64 on monocytes did neither correlate with IFN type I activity, nor with the clinical and laboratory parameters. In contrast, the surface marker was overexpressed in all pSS patients. We therefore consider it as a marker of systemic autoimmunity in general.

CD169, also termed sialoadhesin (Siglec-1), previously identified as a biomarker for IFN type I activation in SLE, was found to correlate with disease severity. Also in SSc, elevated CD169 levels were found on monocytes. In our hands, CD169 was elevated in IFNpos pSS patients and correlated to several clinical and laboratory parameters, even though not as strongly as the MxA assays.

BAFF overexpression has been observed in serum, salivary glands and monocytes in pSS patients. We previously...
observed a strong correlation between the IFN type I signature and BAFF mRNA in pSS monocytes.11 Moreover, we found BAFF serum protein level increased in pSS compared with HC, but did not find a correlation with the IFNscore; additionally, BAFF serum protein did not correlate with BAFF mRNA. We report here that BAFF protein levels showed a slight but not significant increase in IFNpos patients, whereas BAFF mRNA was indeed significantly increased in IFNpos patients. This again indicates the lack of correlation between BAFF mRNA and BAFF protein.

Some limitations of our study are for one, the relatively small sample size for CD64. Assessing this surface marker in a large set of patients is warranted to better understand its role in systemic autoimmunity. Second, MxA-EIA levels were not assessed for all subjects due to whole-blood necessity. Furthermore, absence of viral infections was defined as ‘no symptoms of underlying viral infections’ at inclusion, whereas no viral screening was done to confirm. In HC with relatively high MxA levels, however, ENA profiles were run to rule out the presence of autoantibody. Third, the ESSDAI as well as some clinical and laboratory parameters were not assessed in all pSS patients.

In this study, we reconfirmed a monocyte IFN type I signature prevalence in over half of the pSS patients, a subgroup with higher disease activity.11 We proposed the identification of these subgroups useful in establishing a patient’s eligibility for specific treatments. Indeed, an interesting finding here was that patients on HCQ treatment had considerably reduced MxA levels, indicating HCQ to interfere with IFN production. A recent report shows HCQ to impair systemic IFNα production.19 Our data suggest that identifying the IFN type I signature by performing MxA assays might prove useful in predicting HCQ treatment responsiveness. In this cohort, there are more HCQ users in the IFNneg group compared with IFNpos. However, it is not likely that the increased ESSDAI levels in the IFNpos group are an effect of less HCQ use in this group, because in the IFNpos group, 62% of the patients use HCQ and are nevertheless IFNpos. Moreover, we previously showed 61% (23/38) of the IFNpos patients to use HCQ compared with 55% (17/31) of IFNneg patients in addition. HCQ use did not significantly affect ESSDAI scores (figure 4H). Larger studies are, however, warranted to fully elucidate the effects of HCQ on the IFN type I system, in particular, longitudinal studies monitoring MxA levels in HCQ-naïve patients starting HCQ treatment.

The association between the IFN type I signature, MxA protein and disease activity levels in pSS needs to be further assessed, by for one looking longitudinally, in order to validate MxA as a disease activity marker. The same holds true for association between IFN type I polymorphisms and MxA, as these polymorphisms have been linked to increased IFNα sensitivity.40–41 To determine the applicability of the MxA-EIA in other IFN type I-related diseases, MxA levels were assessed in a small SLE cohort (see online supplemental figure S4) and showed results similar to pSS—a significant correlation between IFNscores and MxA levels (r=0.885, p<0.0001)—suggesting a broad applicability of MxA-EIA in systemic autoimmunity.

In conclusion, we identify MxA protein expression by EIA and FACs analysis as practical and rapid approaches for assessment of IFN type I bioactivity in pSS. MxA-EIA is the simplest and most reliable of these assays and indicates clinical applicability in stratifying pSS patients according to IFN positivity. The use of MxA as a biomarker for identifying patients eligible for therapy in pSS clearly requires further research.

ACKNOWLEDGEMENTS

The authors acknowledge Ms M Kivivirta (University of Turku, Finland) for her technical assistance in running the MxA-EIAs, Dr M Schreurs (Erasmus MC Rotterdam, The Netherlands) for measurement of complement, immunoglobulin levels and autoantibodies and MSc, W Beumer (Erasmus MC Rotterdam, The Netherlands) for his scientific perspective as well as aid in figure design.

Contributors NIM and ZB (guarantors) were involved in conception and study design, overall data acquisition and monitoring, analysis and interpretation of data, drafting the article. MW was involved in laboratory data acquisition, analysis and interpretation and revising the article. CGVH-M and KH were involved in laboratory data acquisition and analysis, and revising the article. IPvDM, PlVd and VASHD were involved in clinical data acquisition and interpretation, and revising the article. HAD was involved in conception and study design, interpretation of data and revising the article. MAV (guarantor) was involved in conception and study design, data monitoring and interpretation and revising the article. All authors approved the final version.

Funding This study was supported by grants from the Dutch Arthritis Foundation (Reumafonds); 10-1-401 and the Netherlands Organisation for Scientific Research (NWO; 017.007.095).

Competing interests None.

Patient consent Obtained.

Ethics approval The Medical Ethical Review Board of the Erasmus MC Rotterdam.

Provenance and peer review Not commissioned; externally peer reviewed.

Open Access This is an Open Access article distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 3.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/3.0/

REFERENCES


Table S1

<table>
<thead>
<tr>
<th>Variable</th>
<th>CD169</th>
<th></th>
<th>CD64</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>r</td>
<td>p value</td>
<td>N</td>
</tr>
<tr>
<td>ESSDAI score</td>
<td>28</td>
<td>0.28</td>
<td>n.s.</td>
<td>18</td>
</tr>
<tr>
<td><strong>Laboratory parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rf (IE/ml)</td>
<td>26</td>
<td>0.19</td>
<td>n.s.</td>
<td>18</td>
</tr>
<tr>
<td>C3 (g/l)</td>
<td>33</td>
<td>0.13</td>
<td>n.s.</td>
<td>20</td>
</tr>
<tr>
<td>C4 (g/l)</td>
<td>33</td>
<td>0.07</td>
<td>n.s.</td>
<td>20</td>
</tr>
<tr>
<td>IgG (g/l)</td>
<td>34</td>
<td>0.40</td>
<td>0.02</td>
<td>20</td>
</tr>
<tr>
<td>IgA (g/l)</td>
<td>33</td>
<td>0.10</td>
<td>n.s.</td>
<td>20</td>
</tr>
<tr>
<td>IgM (g/l)</td>
<td>33</td>
<td>0.36</td>
<td>0.04</td>
<td>20</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>31</td>
<td>0.14</td>
<td>n.s.</td>
<td>19</td>
</tr>
<tr>
<td>Hb (mmol/l)</td>
<td>34</td>
<td>-0.29</td>
<td>n.s.</td>
<td>20</td>
</tr>
<tr>
<td>Thrombocytes (*10E9/l)</td>
<td>33</td>
<td>-0.01</td>
<td>n.s.</td>
<td>19</td>
</tr>
<tr>
<td>Lymphocytes (*10E9/l)</td>
<td>31</td>
<td>0.00</td>
<td>n.s.</td>
<td>19</td>
</tr>
<tr>
<td>Neutrophiles (*10E9/l)</td>
<td>31</td>
<td>-0.24</td>
<td>n.s.</td>
<td>19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Variable</th>
<th>N (%)</th>
<th>Z</th>
<th>p value</th>
<th>N (%)</th>
<th>Z</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Auto-antibodies</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-SSA</td>
<td>29/34 (85)</td>
<td>-2.16</td>
<td>0.03</td>
<td>16/20 (80)</td>
<td>-0.95</td>
<td>n.s.</td>
</tr>
<tr>
<td>Anti-Ro52</td>
<td>21/27 (78)</td>
<td>-0.99</td>
<td>n.s.</td>
<td>15/19 (79)</td>
<td>-1.20</td>
<td>n.s.</td>
</tr>
<tr>
<td>Anti-Ro60</td>
<td>19/27 (70)</td>
<td>-1.97</td>
<td>0.05</td>
<td>14/19 (74)</td>
<td>-1.94</td>
<td>n.s.</td>
</tr>
<tr>
<td>Anti-SSB</td>
<td>22/34 (65)</td>
<td>-2.05</td>
<td>0.04</td>
<td>13/20 (65)</td>
<td>-0.83</td>
<td>n.s.</td>
</tr>
<tr>
<td><strong>Medical therapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>9/34 (26)</td>
<td>-2.25</td>
<td>0.03</td>
<td>6/20 (30)</td>
<td>-0.17</td>
<td>n.s.</td>
</tr>
<tr>
<td>Plaquenil</td>
<td>25/34 (74)</td>
<td>-1.93</td>
<td>n.s.</td>
<td>13/20 (65)</td>
<td>-0.20</td>
<td>n.s.</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>3/34 (9)</td>
<td>-1.12</td>
<td>n.s.</td>
<td>2/20 (10)</td>
<td>-0.25</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Data are presented as Spearman’s rho (r) or according to \( \chi^2 \) test (Z), unless otherwise mentioned.

Data normally distributed are presented as Pearson’s rho (r).

Rf, Rheumatoid factor; CRP, C-reactive protein; Hb, Hemoglobin
SUPPLEMENTAL FIGURES LEGENDS

Figure S1. MxA Enzyme immunoassay (EIA)

MxA-EIA was executed as previously described with new monoclonal antibodies and recombinant MxA as standard, subsequent to validation for corresponding results[19]. Mouse MAbs AFI-7B3 and AFI-10G10 (Institute of Clinical Medicine, University of Eastern Finland) were used as capture and biotinylated detector-antibody, respectively. MAbs were used as IgG, purified from cell culture supernatants by using FPLC with CIM protein G monolithic column (BIA separations, Ljubljana, Slovenia). Detector antibody was biotinylated with NHS-PEO₄-Biotin reagent (Pierce, Rockford, IL) according to manufacturer’s instructions. Recombinant MxA, produced with baculovirus expression system, was kindly provided by Ilkka Julkunen and Krister Melen (National Institute of Health and Welfare, Helsinki, Finland)[26]. The heparinized whole blood samples (25μl) were lysed by diluting 1:20 in hypotonic buffer containing 1.5% bovine serum albumin (BSA), 1% ascorbic acid, 0.5% NaHCO₃ and 0.05% NaN3 and stored at -70°C until assayed. Standard dilutions for the assay were prepared in hypotonic buffer. Microtitre strip wells were coated by overnight incubation with 0.4 μg per well of AFI-7B3 IgG in 0.1 M carbonate, pH 9.6. The strips were washed twice with 5 mm Tris, 0.15 M NaCl, 0.05% Tween 20, pH 7.75 and saturated for 1 h with the assay buffer containing 0.5% BSA, 0.05% Tween 20 and 0.1 mM merthiolate in PBS. Strips were washed four times, and 50 μl each of the sample and 0.8 μg/ml biotinylated AFI-10G10 IgG, in the assay buffer, were added into the duplicate wells. After overnight incubation with shaking at 8°C, the strips were washed six times, and 100 μl of streptavidin-peroxidise-HRP (Pierce, #21127) diluted 1:15.000 in assay buffer was added into the wells. Strips were shaken for 75±15 min, washed eight times and incubated with 100 μl of tetramethylbenzidine (TMB) peroxidise substrate solution (Ani Labsystems, Vantaa, Finland) for 10–30 min in the dark. Color development was stopped by adding 100μl of 0.5 N H₂SO₄ and absorbance values were measured at 450 nM using Victor² multilabel counter (PerkinElmer, Turku, Finland). MxA concentrations (μg/l) of the specimens were read from a master curve (50-800μg/l) plotted with the standard values using Mutlicalc software with spline smoothed fitting algorithm. Hypotonic buffer was used as the negative control. The limit of detection of the MxA EIA
assay was 10μg/l, which was determined as three times the standard deviations (SD) of eight negative control replicates. One negative and one positive control sample were also included in each assay.

**Figure S2.** Flow cytometric analysis was performed on CD14+ cells isolated from HC and pSS patients (stratified in IFNneg and IFNpos). (A) Representative dot plots show CD14 and consecutive CD64, CD169 and BAFF protein expression (top to bottom) on monocytes from study subjects. Appropriate isotype-matched controls were used to confirm antibody-specificity. (B) CD64 (C) CD169 and (D) BAFF protein expression for IFNneg (left) and IFNpos (right) pSS patients are shown. Representative histograms are depicted; Shaded histogram represents the isotype control, dotted line represents the HC and the solid line the pSS patients.

**Figure S3.** For relative BAFF mRNA gene expression (A) a correlation plot shown with IFNscores for all study subjects and (B) BAFF mRNA gene expression levels stratified in HC (n=27), IFNneg (n=14) and IFNpos (n=21) pSS patients. Each symbol represents an individual sample; horizontal lines represent the mean. The correlation coefficient (r) and P values are shown. For BAFF a Pearson correlation was used and to compare means the independent T-test was used.

**Figure S4.** In a small cohort of systemic lupus erythemathosis (SLE) patients (A) a correlation plot is shown between IFNscores (for all age and gender matched study subjects) and intracellular MxA (µg/l) levels, as assessed by the MxA-EIA. (B) IFNscores shown for HC (n=12) and SLE patients (n=12), stratified in IFNneg and IFNpos by red dotted line at 10; Red dots represent IFNpositivity (IFNscore≥10). (C) MxA-EIA (µg/l) levels shown for HC (n=12) and SLE patients (n=12). Each symbol represents an individual sample; horizontal lines represent the median. The correlation coefficients (r) and P values are shown. For correlations Spearman's rho correlation test was used and to compare medians the Mann-Whitney U test was used.
Whole blood samples lysed in hypotonic buffer (1:20)

50μl of standard or lysate added per MAb coated microtiter well

50μl of Biotinylated detector MAb added

Incubated overnight on shaker at 8°C

Incubated with Streptavidin- HRP for 75±15min RT

Peroxidase reaction developed

Color reaction with TMB-substrate solution

Reaction stopped with H₂SO₄

A₄₅₀ nm measured
MxA concentrations (μg/l) read from standard curve