Endogenous type I IFN pathways and disease activity in MS
Chapter 3.1

Spontaneous MxA mRNA level predicts relapses in patients with recently diagnosed MS

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Abstract

Objective: To determine if myxovirus resistance protein A (MxA) mRNA is related to clinical disease activity in multiple sclerosis (MS).

Methods: Baseline MxA mRNA levels were measured in a prospective cohort of 116 untreated patients with early MS and were related to clinical relapses and MRI at baseline and at follow-up.

Results: Low levels of MxA mRNA were associated with the occurrence of relapses (p= 0.002) and contrast-enhancing lesions (CELS) on baseline MRI (p= 0.045). In addition, high baseline MxA mRNA levels were related to a longer time to a first new relapse (hazard ratio (HR) 0.59; 95% confidence interval (CI) 0.35–1.00;p= 0.044). Adding the absence of CELs to high MxA mRNA, the predictive value increased (HR 0.35;95%,CI 0.17–0.74;p= 0.006), clearly showing a cumulative value for combining both factors.

Conclusions: MxA mRNA is related to clinical exacerbations, the number of CELs on MRI, and is indicative for the time to a subsequent relapse. If confirmed, MxA mRNA has potential as a biomarker for clinical disease activity in MS.
Introduction

Multiple sclerosis (MS) is an immune-mediated disease of the CNS that shows a wide range of clinical features and a variable clinical course\(^1\). The development of biomarkers that are meaningful to clinicians and patients is difficult, as no single measure captures the disease complexity. There is currently no laboratory measure that reliably correlates with or predicts disease activity. The list of demographic and clinical variables that may have predictive value on disease course is long; their contribution, however, is often small. In this context, MRI appears to be a somewhat more consistent predictor, at least at group level. The number and volume of cerebral T1- and T2-weighted lesions at the time of the first clinical symptoms have been related to the probability of disease recurrence and the conversion to clinically definite MS\(^2,3,4,5\). Myxovirus resistance protein A (MxA) mRNA expression is described as a biomarker for treatment response of recombinant interferon-β (INF-β) in MS and is related to the absence of relapses during treatment\(^6,7,8\). Mx protein level and MxA mRNA expression remains at a stable low level in the absence of viral infections and is rapidly upregulated in a dose-dependent manner by IFN type I in human mononuclear cells\(^9\). Type I IFN pathways play an important role in other chronic inflammatory diseases, such as systemic lupus erythematosus and Sjögren syndrome\(^10,11,12\). Furthermore, Mx proteins are found in the plaques of treatment-naïve patients with MS\(^13\) and are demonstrated by immunohistochemistry in viral encephalitis, confirming their role in neuroinflammatory processes. In this study, we investigated whether spontaneous (therapy-naïve) MxA mRNA expression levels are related to clinical and radiologic measures of disease activity.

Methods

Patients and study design

A prospective cohort of patients presenting with a clinically isolated syndrome (CIS), suggestive of MS, or diagnosed with relapsing-remitting MS (RRMS) in the previous 6 months, was recruited at the VU Medical Center, Amsterdam. Diagnosis was made according to standard diagnostic criteria when applicable\(^14,15\). Appropriate investigations were conducted to exclude alternative diagnoses. From this prospective cohort, unrelated Caucasian patients were selected with a follow-up of at least 1 year. Patients with progressive disease onset and patients already receiving disease modifying treatment (DMT) were excluded. Samples from 48 anonymous, untreated individuals visiting the local blood bank served as controls. Clinical assessment was performed at least annually and included the Expanded Disability Status Scale (EDSS) and the notification of relapses and treatment changes. This study was approved by the Medical Ethical Committee of the VU Medical Center and written informed consent was obtained from all participants.
MRI

Per protocol MRI scans were performed at baseline, at year 1, and at year 2. Scans were acquired either on 1.0 Tesla or 1.5 Tesla (Siemens AG, Erlangen, Germany) scanners with standard head coils, using standard 2D conventional or fast spinecho proton density (PD)– and T2-weighted images (repetition time 2,200 –3,000 msec, echo time 20–30 and 80–100 msec) with slice thicknesses of 3–5 mm, a maximum gap between slices of 0.5 mm, and an in-plane resolution of 1 1 mm2. The development of new T2-weighted lesions and gadolinium enhancing T1-weighted lesions (CEls) on brain MRI compared to baseline was identified by an independent rater, blinded for MxA mRNA expression results and clinical data. Baseline scans were performed on the day of blood sampling.

Blood sampling and MxA mRNA measurement

Peripheral blood was collected in PAXgene tubes for mRNA extraction at baseline. Tubes were kept at room temperature for at least 2 hours before freezing at 80°. MxA mRNA expression was measured as previously described. Briefly, automated RNA isolation was performed on the BioRobot MDX (Qiagen) according to the manufacturer’s instructions (PAXgene Blood RNA Mdx kit). One-step real-time quantitative RT-PCR was performed with Taqman probes and MxA was normalized to the expression level of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to correct for experimental variations. Total leukocyte counts and differentiation were simultaneously determined to monitor the likelihood of active infections.

Statistical methods

Baseline characteristics were correlated to MxA mRNA levels using Spearman rank for linear variables and Mann-Whitney for dichotomous variables. Receiver operator characteristics (ROC) were used to calculate the optimal cutoff value to differentiate between patients in exacerbation and in remission at baseline. MxA equal and below cutoff value was considered low, MxA above cutoff value was considered high. The annualized relapse rates were calculated from the most recent available data by dividing the total number of relapses by the total follow-up duration. The numbers of new T2 lesions on MRI at 24 months compared to baseline were stratified into 4 categories: no new lesions, 1 or 2, 3 or 4, and more than 5 new T2 lesions. Also, the presence of 1 or more CELs was noted. Subsequently, MxA expression was correlated to the annualized relapse rate and the development of new T2 lesions with Kruskal-Wallis. For the time to conversion of CIS to clinically definite MS (CDMS), Kaplan-Meier survival curves were constructed for dichotomized levels of MxA, for MRI characteristics, and for the occurrence of clinical exacerbation at baseline. To account for DMT, Cox proportional hazards regression was performed and hazard ratios (HRs) and 95% confidence intervals (CIs) were reported. Changes in EDSS were annualized by dividing
the total change by the follow-up duration and underwent log transformation to establish a normal distribution. Linear regression analysis was performed adjusting for treatment with DMT during follow-up and baseline EDSS scores. In patients who initiated IFN treatment during follow-up and who were followed at least 24 months during treatment, the clinical responder status was determined. A clinical responder was defined as having no relapses and no progression on EDSS in the first 24 months of treatment. Levels of MxA mRNA were compared between responders and nonresponders to treatment with Mann-Whitney. The threshold for significance was set at 0.05.

Results

A total of 116 consecutive patients were included (67 CIS and 49 RRMS) and followed for a median period of 45 months (IQR 31–48 months) (Table 1). At baseline, 23 patients were in clinical exacerbation and 93 patients in remission, defined as having no clinical symptoms and no corticosteroid treatment for at least 4 weeks.

Table 1. Baseline variables.

<table>
<thead>
<tr>
<th>Clinical state</th>
<th>Remission</th>
<th>Relapse</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>93</td>
<td>23</td>
</tr>
<tr>
<td>Age, y, mean (SD)</td>
<td>33 (9)</td>
<td>33 (8)</td>
</tr>
<tr>
<td>Sex, n (%) female</td>
<td>58 (62)</td>
<td>16 (70)</td>
</tr>
<tr>
<td>Follow up, mo, median (IQR)</td>
<td>44 (24-48)</td>
<td>48 (46-49)</td>
</tr>
<tr>
<td>EDSS, median (IQR)</td>
<td>2.0 (1.5-3.0)</td>
<td>2.5 (2.0-4.0)</td>
</tr>
<tr>
<td>MxA mRNA/GAPDH, median (IQR)*</td>
<td>0.084 (0.021-0.145)</td>
<td>0.036 (0.009-0.075)</td>
</tr>
</tbody>
</table>

**MS subtype, n (%)**
- Clinically isolated syndrome: 60 (65), 7 (30)
- Relapsing-remitting: 33 (35), 16 (70)

**T2 lesions on baseline MRI**
- < 9: 48 (52), 8 (35)
- ≥ 9: 45 (48), 15 (65)

**CEls on baseline MRI**
- No: 60 (65), 9 (40)
- Yes: 33 (35), 14 (60)

EDSS, Extended Disability Status Scale, MxA, Myxovirus resistance protein A, SD, standard deviation, FU, follow-up, IQR, Interquartile Range, GAPDH, glyceraldehyde 3-phosphate dehydrogenase, MR, Magnetic Resonance Imaging, CELs, contrast enhancing lesions; * MxA mRNA expression was normalized to the expression level of ‘housekeeping gene’ GAPDH.

MxA mRNA expression levels at baseline

Compared to controls, MxA mRNA levels were lower in patients with MS, both in exacerbation (p < 0.001) and in remission (p < 0.001). Furthermore, MxA mRNA expression
was lower in patients sampled during clinical exacerbation compared to those in remission (p= 0.002; Figure 1). To exclude a possible confounding effect of IV steroids in patients with exacerbation, the analysis was repeated after exclusion of those 4 patients who had received IV steroids within 1 month before blood sampling; again, lower MxA mRNA expression was found in patients sampled during a clinical exacerbation (p= 0.004). In line with this, patients with low MxA mRNA values had a higher number of CELs on baseline MRI (p=0.045; mean [SD] number of CELs for low MxA 1.7 [2.6] and for high MxA 0.9 [1.9]). No significant correlation was found between baseline MxA and T2 lesion load. With ROC analysis the optimal cutoff was calculated (MxA= 0.0750) to discriminate between relapse and remission at baseline (data not shown, area under the curve = 0.705). MxA levels below this cutoff level were considered low; MxA levels above this cutoff level were considered high. Dichotomized levels of MxA were subsequently correlated to the annualized relapse rates at follow-up and the time to first new relapse.

**Figure 1.** MxA mRNA expression levels in patients and controls. MxA mRNA expression levels relative to GAPDH expression levels. Box and whiskers representing 25-75% expression level and bar representing 95% confidence interval. MxA mRNA expression is higher in controls compared to relapsing-remitting MS patients (both in remission and in exacerbation) and expression is higher in MS patients in remission compared to patients in a clinical exacerbation. MxA, Myovirus resistance protein A; GAPDH, glyceraldehyde 3- Phosphate dehydrogenase.

**Predictive value of MxA mRNA expression for measures of disease activity at follow-up**

During followup, the mean relapse rate was lower in patients with high MxA levels at baseline (mean relapse rate 0.22 vs 0.36; p= 0.010). In addition, patients with high MxA mRNA experienced a new relapse at a later time than patients with low MxA mRNA when adjusting for the use of DMT during follow-up (HR 0.59; 95% CI 0.35–1.00; p= 0.044; Figure
2). The absence of CELs on baseline MRI was also related to a longer time to a subsequent relapse (HR 0.54; 95% CI 0.31–0.93; p = 0.019). Combined analysis of high MxA and the absence of enhancement on MRI provided an additional predictive value for the time to a new relapse (HR 0.35; 95% CI 0.17–0.74; p = 0.006; Figure 3), compared to either MxA expression or CELs alone. Baseline T2 lesion load did not show predictive value for relapses in this study. The absence of a clinical exacerbation at baseline, however, also predicted the time to a subsequent relapse (HR 0.35; 95% CI 0.20–0.61; p < 0.001). MxA mRNA expression did not significantly correlate to the time of conversion to CDMS for the subgroup of patients with CIS. Overall, the changes in EDSS during the scope of this study were small and no statistically significant differences were found between groups (data not shown). Finally, although the proportion of patients with no or low numbers of new T2 lesions was higher in patients with high baseline MxA mRNA at year 1, overall no significant correlation was found for baseline MxA mRNA expression with the annualized number of new T2 lesions (Figure 4) and the occurrence of CELs during follow-up.

![Figure 2](image-url)  
*Figure 2. Cox proportional hazard analysis for the prediction value of baseline MxA for the time to a new relapse.*  
Patients with low MxA mRNA expression have a shorter time to a new relapse (p = 0.044). Calculated with Cox proportional Hazard analysis with disease modifying treatment as a confounder. MxA, Myxovirus resistance protein A.
Figure 3. Cox proportional hazard analysis for the prediction value of contrast enhancing lesions (CELs) on baseline MRI and MxA for the time to a new relapse. Patients with no CELs and a high MxA have the longest time to a new relapse. If MxA is low or baseline MRI is positive for CELs, the time to a relapse is shorter, which is comparable with either factor. The combination of CELs and a low MxA mRNA expression level results in an even shorter time to a new relapse ($p = 0.006$). CELs, contrast enhancing lesion; MxA, Myxovirus resistance protein A. Calculated with Cox proportional Hazard analysis with disease modifying treatment as a confounder.

**DMT treatment at follow-up**

None of the patients had ever been treated with DMT at study enrolment (to select patients in treatment-naïve circumstances a history of DMT use was entered as exclusion criterion). However, during follow-up 55 (47%) patients started with DMT. Twenty-one patients started with IFN-β-1b SC (18%), 20 patients with IFN-β-1a SC (17%), 9 patients with IFN-β-1a IM (8%), and 5 with glatiramer acetate (4%). The mean time to start of treatment was 23.7 months (SD 20). The proportion of patients starting with DMT was comparable between patients with a high MxA and a low MxA at baseline (45% vs 50%).

**MxA mRNA expression levels as a predictor of treatment response**

For patients treated with IFN-β for at least 24 months during follow-up of this study, we determined treatment responder status. Patients who remained relapse-free during the first 24 months of treatment and showed no progression on EDSS in this period were considered clinical responders. In 40 patients responder status could be determined, of which 18 (45%)
were clinical responders and 22 (55%) clinical nonresponders. Baseline MxA mRNA levels did not significantly differ between responder status groups.

![Figure 4. MxA mRNA expression and the development of new T2 lesions on MRI at year 1 and year 2 of follow-up.](image)

Although the proportion of patients with no and low numbers of new T2 lesions seemed to be higher in patients with high baseline MxA mRNA at year one, no significant correlations were found for baseline MxA mRNA expression and the development of new T2 lesions. MxA, Myxovirus resistance protein A; MRI, Magnetic Resonance Imaging.

**Discussion**

To our knowledge, this is the first study reporting that MxA mRNA expression has potential as a biomarker for disease activity in recently diagnosed relapsing-remitting MS. Low MxA expression was associated with the occurrence of relapses and the number of CELs at baseline, and an increased relapse rate and a shorter time to a new relapse during follow-up. Its potential value as a biomarker for disease activity should be confirmed in other prospective cohorts. Patients with MS had a lower MxA mRNA expression compared to controls, which suggests an overall different activity level of endogenous IFN-β pathways in patients with MS. The subsequent finding that MxA mRNA expression levels may be useful to differentiate patients with active disease from those with no or only modest disease activity is even more exciting. The role of IFN-stimulated genes in clinical MS disease activity was suggested by Feng et al\(^{18}\), and confirmed in a Danish study of 39 patients with MS in which a correlation between spontaneous MxA mRNA expression at baseline and CELs during follow-up was shown\(^{19}\). Our study confirms this correlation between MxA mRNA expression and
CELs, albeit cross-sectional. MxA mRNA expression is unequivocally associated with titers of neutralizing antibodies (NAb) in IFN-β-treated patients with MS and generally accepted as a measure of recombinant IFN-β bioactivity. In NAb-positive patients without MxA response, no differential expression was detected of any of more than 1,000 IFN-β regulated genes identified in NAb-negative patients. Lack of in vivo MxA response in patients with MS with NAb is a reliable marker of a completely blocked biologic response to IFN-β. Anti-IFN-β NAb that persist after IFN-β treatment withdrawal have been associated with a more active MS disease course. Furthermore, circulating NAb have been demonstrated to chronically suppress endogenous IFN-β. This observation and our study results might explain and may be manifestations of the same phenomenon: the involvement of functional IFN pathways in the modulation of disease activity in MS. Relapses are considered the primary outcome in clinical trials, as current treatments show their efficacy primarily with relapse rate reduction. MRI is a well-known and sensitive surrogate measure that is considered more objective than clinical outcome measures. CELs and T2 lesions on MRI, however, only moderately predict or correlate with clinical disease activity. Remarkably, in our study, the MxA levels appeared to correlate better to clinical relapses than to the development of new T2 lesions and CELs on MRI during follow-up, suggesting potential value of MxA mRNA as a predictive marker for clinical disease activity in addition to MRI. The prediction of future relapses by MxA mRNA expression at baseline was more or less comparable to the predictive value of clinical relapses at baseline and CELs. If confirmed in future studies, a comparison between CELs and MxA obviously would be in favor of the use of the blood test, since magnetic resonance monitoring includes high costs and time-consuming procedures. Combining MRI and MxA mRNA showed an important additional predictive value for the time to a subsequent relapse. This suggests that combining both measures will be a more robust predictor of clinical disease activity than the finding of CELs alone. Baseline MxA mRNA did not significantly differ between clinical responders and nonresponders in our study. However, this may be due to the small sample of patients (n = 40) who started IFN-β during follow-up. A possible confounding factor in this study could be the occurrence of viral infections at the time of blood sampling, as MxA mRNA expression levels are known to rise in response to a viral stimulus. However, none of the patients reported complaints suggesting a viral infection at the time of blood sampling, which was confirmed by normal leukocyte counts and differentiation.
References


