Chapter 2

Reduced biological activity of recombinant IFN-β and treatment response
Chapter 2.1

IFN-β bioactivity measurement in MS: feasibility for routine clinical practice

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Abstract

**Background:** Neutralizing antibodies (NAb) to interferon beta (IFN-β) are associated with a reduced bioactivity and efficacy of IFN beta in multiple sclerosis (MS). Unclear is how to apply IFN-β bioactivity measurements (quantification of Myxovirus resistance protein A (MxA) mRNA) in clinical practice.

**Objectives:** To evaluate value and feasibility of IFN-β bioactivity measurement with a single MxA mRNA measurement for screening and a second measurement before and after IFN-β administration for definite confirmation of IFN-β bioactivity status.

**Methods:** In 79 MS patients MxA mRNA expression was determined 4 hours after IFN-β administration. If inadequate, MxA mRNA expression testing was repeated 3 months afterwards, comparing post- and pre injection samples to determine whether IFN-β bioactivity was persistently lacking. MxA mRNA expression was compared to NAb titres, determined by the cytopathic effect assay (CPE).

**Results:** NAb titres correlated significantly with MxA mRNA expression and MxA mRNA induction. Of all screened patients, only one patient had adequate MxA mRNA expression and high NAb titres simultaneously. Of the biological non-responders at second measurement (21/55), 17 (81%) were high-titre NAb positive, 1 (5%) was low-titre NAb positive and 3 (14%) were NAb negative. Without considering the pre-injection measurement, two more NAb negative patients would have tested negative for IFN-β bioactivity, emphasizing the need of a pre-injection sample.

**Conclusions:** Our data suggest that for IFN-β bioactivity screening a single post-injection measurement seems reasonable. However, MxA induction measurement based on both pre- and post- IFN-β injection samples at second measurement is somewhat more precise in determining ultimate IFN-β bioactivity status.
Introduction

Neutralizing antibodies (NAb) to Interferon beta (IFN-β) therapy develop in a significant number of treated multiple sclerosis (MS) patients and are associated with a reduced bioactivity and efficacy of IFN-β\(^1\). The immunogenicity of recombinant IFN-β preparations is variable among the different products and antibody responses are influenced by several factors, such as route and frequency of administration, treatment duration and drug dose\(^2\). There is much debate on how to optimise testing for NAb and bioactivity of IFN-β in daily practice\(^3\). Different testing methods for NAb are proposed and different schedules of blood sampling are used. Existing assays use either the anti-viral effect of IFN-β (the cytopathic effect assay; CPE) or measure IFN-β bioactivity by quantification of IFN-β induced gene-products (Myxovirus resistance protein A (MxA) or its mRNA)\(^4,5,6,7\). The CPE assay is at present one of the most commonly used\(^8\), although it clearly has some disadvantages. Firstly, inter-laboratory comparison is difficult because the results vary with the use of different cell lines and viruses. Secondly, there is controversy about at which level of NAb titres IFN-β bioactivity is abolished completely\(^9,10\). MxA based assays assess the stimulation of the IFN-β receptor (IFNAR) and reflect bioactivity of IFN-β. Different approaches of measuring MxA mRNA based bioactivity of IFN-β have been described; MxA expression reflects the absolute level of MxA mRNA several hours after IFN-β injection, whereas MxA induction is based on blood samples taken before and after the administration of IFN-β, and the relative increase of MxA compared to the pre-injection level\(^11,12\). The rise in MxA mRNA expression after IFN-β injection does not occur or occurs only partially in the presence of NAb\(^13\). A disadvantage of the bioactivity measurements is that sampling of blood has to take place at a standardised point of time between 4 and 24 hours post-injection of IFN-β. Despite the fact that there is considerable individual variation in MxA responses, there still is controversy about whether a pre-injection sample is needed. Obviously, a pre-injection sample and an interval of more than 4 hours between injection and the post-injection sample make the bioactivity assays less suitable for routine clinical practice. To assess whether a bioactivity measurement protocol of blood sampling 4 hours after IFN-β injection without pre-injection sampling is feasible and valid for screening and whether pre-injection samples are necessary during measurements at a second time-point, we correlated MxA mRNA expression measured in a single post IFN-β -injection sample with CPE determined NAb titres. In addition, we measured MxA induction (pre- versus post-injection measurement) in the patients with inadequate MxA expression at first assessment.
Patients & Methods

Patients and visits
Between March 2006 and November 2007, consecutive relapsing-remitting MS patients, who were treated with IFN-β for at least 6 months at the out-patient department of the VU medical center, were invited to participate. Patients treated with corticosteroids within 4 weeks before blood sampling were excluded. Patient characteristics are summarised in Table 1. At the first visit a single blood sample was taken 4 hours after IFN-β administration. When MxA-expression was not adequate (for criteria see below) the patient was invited for a second visit at least 3 months afterwards. At this visit 2 blood samples were taken, one immediately before and one 4 hours after IFN-β injection to assess MxA induction. Blood was drawn after local ethics committee approval and patient informed consent was obtained.

Methods

Serum and PAXgene (Preanalytix) samples were collected at both visits. Serum was stored immediately at -80 degrees, before shipment to “Centro Riferimento Regionale Sclerosi Multipla” (CRESM) in Orbassano (Italy) for NAb titre measurements, using a validated CPE assay described previously4,14,15. NABs were tested against the type of IFN-β used by the individual patients. The neutralising titre of the sera was calculated according to the Kawade’s formula and expressed in ten-fold reduction units (TRU)16,17. For this study, patients with NAb titres of ≤20 TRU/mL were considered as NAb negative, NAb titres of 20 - 150 TRU/mL were considered as ‘low- titre NAb positive’ and patients with NAB titres >150 TRU/mL as ‘high-titre NAb positive’. The cut-off level of 150 for ‘high-titre’ NAb positivity is based on a recent study suggesting that NAB titres up to 150 TRU/mL generally have a well-retained MxA mRNA response. For MxA RNA analysis, peripheral blood was collected in PAXgene tubes, and left at room temperature for at least 2 hours before freezing at -20 degrees. Automated RNA isolation was performed, within 3 months after freezing, in the VU Medical Center Amsterdam (the Netherlands) on the Bio robot MDX (Qiagen) according to the manufacturers instructions (PAXgene Blood RNA Mdx kit). Yields and purity of isolated RNA were determined by spectrometric analysis (Nanodrop). Samples were diluted to 100 ng/µl and stored as aliquots at -80°C. MxA mRNA expression was assessed by one-step real-time quantitative RT-PCR with Taqman probes and normalised to the expression level of ‘housekeeping gene’ glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to correct for experimental variations. The primers for the Mxa and GAPDH assays were intron-spanning. The MxA primer sequences were 5’-ACACACCGTGACGGATATGGT-3’ (forward) and 5’-AATTTTGGACTTGGCGGTTCT-3’, and the dual-labelled fluorescent probe was 5’-(FAM) CGGCTTGCTTTACAGATGTTTCGATAAA -(TAMRA)’-3’ (FAM, 6-carboxyfluorescein; TAMRA, 6-carboxy-N,N,N’,N’-tetramethylrhodamine). The GAPDH primer sequences were 5’-ATTCCACCCCATGGCAAATTC-3’ (forward) and 5’-AGCATCGCCCCACTTGATT-3’ (reverse)
and the dual-labelled probe was 5’-(FAM) CCCATCACCATCTTCCAGGAGCGAG-(TAMRA)-3’.
Calibration curves for MxA and GAPDH were prepared by serial dilutions of double-stranded DNA specifying the MxA and GAPDH amplicons, respectively with amounts ranging from 1 x 10⁹ to 1 x 10¹ copies. The assays were able to detect 100 copies of the respective calibrator targets. Absolute concentrations were expressed as copies per ng. The RT-PCRs were set up according to the manufacturers instructions (EZ rTth RNA PCR reagent set, Applied Biosystems) in a reaction volume of 50 µl. The fluorescent probes (Eurogentec) were used at a concentration of 500 nM for MxA and of 200 nM for GAPDH. The PCR primers (Eurogentec) were used at a concentration of 300 nM for MxA and of 500 nM for GAPDH. Hundred ng of purified RNA was used for amplification. Calibration curves were run in parallel with each assay. Multiple negative water blanks were included in every assay. The thermal profile used for the MxA and GAPDH assays was as follows. The reaction was initiated at 50°C for 2 min (uracil N-glycosylase), followed by reverse transcription at 60°C for 30 min. After a 5-min denaturation at 95°C, 40 cycles of PCR were carried out by denaturation at 94°C for 20 s and 1 min of annealing/extension at 56°C and 59°C for MxA and GAPDH, respectively. According to Gilli et al. cut-off values for IFN-β bioactivity were based on MxA mRNA expression results of 48 untreated controls. For the first sample, MxA mRNA expression (here always recorded as the MxA/GADPH ratio) of <0.2 was considered negative (0.2 = median value of untreated controls), MxA mRNA expression between 0.2 and 1.0 was considered suboptimal and MxA mRNA expression of >1.0 was considered adequate (1.0 = 2 times SD of untreated controls). All patients with MxA mRNA expression of < 1.0 were invited for assessment of MxA induction at a second visit. Regarding the results of the induction measurements, three categories of patients were specified: adequate biological responders, biological non-responders and suboptimal biological responders. Adequate biological responders were defined as patients with both an expression level > 0.2 four hours post-injection and > 3-fold higher expression 4 hours after injection compared to pre-injection. Patients with both an expression level < 0.2 four hours post-injection and < 3-fold higher expression 4 hours after injection compared to pre-injection as biological non-responders and patients with either an increase of less than 3-fold or a threshold level < 0.2 were called suboptimal responders. Patients with both inadequate MxA mRNA expression at their first visit and no biological response at the second visit were considered as persistently lacking a biological response. Laboratory workers in VU Medical Center were blinded with respect to the CPE results and their Italian colleagues for MxA mRNA expression results.

Statistical analysis
Results did not follow a normal distribution; therefore results are expressed as median values and were analysed using non-parametric statistical tests. Differences in patient characteristics between the different NAb-status groups (negative, low-titre NAb positive and high-titre NAb positive) were tested with the Mann-Whitney test. The correlation
between CPE values and MxA mRNA expression or MxA induction was determined with the Spearman rank test.

**Results**

**CPE results**

Of the 79 patients studied at the first visit, 37 (47%) tested NAb negative, 19 (24%) NAb low-titre positive and 23 (29%) high-titre positive (Table 1). Positive test results were found in 15 patients treated with Betaferon, in 12 patients treated with Avonex and in 15 patients treated with Rebif. There were no statistical differences found in patient characteristics between NAb negative, low-titre NAb positive and high-titre NAb positive patients. In patients with high NAb titres at their first visit (n=23), NAb positivity was confirmed in 14 out of 16. Repeated measurements were not fully complete; in 7 out of 23 patients with initial high NAb titres a second sample has not been available.

**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>NAb (TRU/mL)</th>
<th>NAb negative (≤ 20)</th>
<th>low-titre NAb positive (20-150)</th>
<th>high-titre NAb positive (&gt;150)</th>
<th>Total n (%)</th>
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<tbody>
<tr>
<td>female n (%)</td>
<td>24 (65)</td>
<td>13 (68)</td>
<td>17 (74)</td>
<td>54 (68)</td>
</tr>
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<td>age in years</td>
<td>42.1 (21-63)</td>
<td>41.5 (26-56)</td>
<td>43.2 (16-56)</td>
<td>42.0 (21-63)</td>
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<tr>
<td>median [range]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>disease duration in years</td>
<td>9.7 (2-21)</td>
<td>10.3 (2-18)</td>
<td>9.4 (2-31)</td>
<td>9.7 (2-31)</td>
</tr>
<tr>
<td>median [range]</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>treatment duration in years</td>
<td>5.4 (1-13)</td>
<td>5.3 (1-10)</td>
<td>3.2 (1-12)</td>
<td>4.8 (1-13)</td>
</tr>
<tr>
<td>median [range]</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RR-MS n (%)</td>
<td>29 (78)</td>
<td>17 (89)</td>
<td>21 (91)</td>
<td>68 (85)</td>
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<tr>
<td>IFN-β n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avonex</td>
<td>8 (22)</td>
<td>10 (53)</td>
<td>4 (17)</td>
<td>20 (25)</td>
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<tr>
<td>Rebif</td>
<td>15 (40)</td>
<td>3 (15)</td>
<td>12 (52)</td>
<td>30 (38)</td>
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<tr>
<td>Betaferon</td>
<td>14 (38)</td>
<td>6 (32)</td>
<td>9 (39)</td>
<td>29 (37)</td>
</tr>
<tr>
<td>Total n (%)</td>
<td>37 (47)</td>
<td>19 (24)</td>
<td>23 (29)</td>
<td>79</td>
</tr>
</tbody>
</table>

NAb= neutralizing antibodies; TRU/mL= tenfold reduction units per milliliter; RR-MS= relapsing-remitting multiple sclerosis; IFN-β= Interferon-beta

**MxA mRNA expression and correlation with CPE results**

The MxA mRNA expression of all patients (relative to GADPH mRNA expression) varied from 0.01 to 2.02 with a median MxA mRNA expression of 0.49. 24 patients (30%) showed an adequate MxA mRNA expression at screening and 55 (70%) patients were invited for a second visit, because of negative or suboptimal MxA mRNA expression (Figure 2). Mean interval between the first and second visit was 3.5 months (range 3-5 months). Negative MxA mRNA expression levels were associated with high-titre NAb results, with the median NAb titre 16
fold higher in patients considered MxA mRNA expression negative compared to patients with adequate MxA mRNA expression (318 vs. 20 TRU/mL respectively) (Figure 1). Median NAb titre results were similar in patients with MxA mRNA negative expression compared to patients with suboptimal MxA mRNA expression (20 vs. 35 TRU/mL respectively). Median MxA mRNA expression was similar in NAb negative and low-titre NAb positive patients, but significantly lower in high-titre NAb positive patients (0.75 vs. 0.77 vs. 0.06 respectively). The Spearman correlation coefficient showed a significant (negative) correlation between NAb titres and MxA mRNA expression ($r = -0.452; p = 0.001$).

**Figure 1.** MxA mRNA expression results correlate with NAb titres. Median values and 95% confidence intervals are shown for each NAb category. The Spearman correlation coefficient shows a negative correlation between NAb titre and MxA mRNA expression ($r = -0.452; p = 0.001$). MxA, myxovirus resistance protein A; mRNA, messenger RNA; NAb, neutralizing antibodies.

**MxA mRNA induction and correlation with CPE results**

In 45 of the 55 reinvited patients MxA induction was measured (Figure 3); 9 patients had already stopped IFN-β treatment before induction measurement could be performed and 1 patient was not available for a second visit (Figure 2). Lack of MxA induction as previously defined (biological non-responders) occurred in 21/55 (38%) patients. Of these patients, 3/21 (14%) were NAb negative, 1/21 (5%) was low-titre NAb positive and 17/21 (81%) patients were high-titre NAb positive. If only the post-injection measurement was taken into account, the interpretation of the MxA expression results would have resulted in two more NAb negative patients without a biological response, suggesting a false-negative biological response, emphasizing the need of a pre-injection sample. Of the 23 patients with high NAb titres, none had an adequate biological response at induction measurement, confirming that high NAb titres completely abolish IFN-β IFN-β bioactivity. The Spearman
correlation test showed a significant (negative) correlation between MxA induction and NAb titre measurement 3 months before ($r = -0.547$, $p = 0.001$).

**Figure 2.** Study Outline. MxA mRNA expression is measured 4 hours after IFNβ injection. Adequate MxA mRNA expression refers to an expression level of $>1.0$; MxA induction = MxA mRNA expression 4 hours after IFNβ injection divided by MxA mRNA expression directly before IFNβ injection. Adequate biological responders had both a MxA mRNA expression of $>0.2$ four hours post-injection and MxA induction of $>3$. Suboptimal responders showed either a MxA mRNA expression of $<0.2$ or a MxA induction of $<3$. Biological non-responders showed both a MxA mRNA expression of $<0.2$ and MxA induction of $<3$. *One patient was not available for second visit at time of analysis. NAb, neutralizing antibodies; MxA, Myxovirus resistance protein A; NAb –, NAb negative, $\leq 20$ TRU/mL; NAb +, low-titre NAb positive, 20–150 TRU/mL; NAb ++, high-titre NAb positive, $>150$ TRU/mL;

**Adequate MxA mRNA expression or induction in the presence of high NAb titres**

Of the 79 patients who were tested at screening, only one patient had adequate MxA expression and high NAb titres at the same time (CPE titre: 896 TRU/mL). Three of the adequate biological responders at induction measurement had high NAb titres at first measurement. In these patients high NAb titres could not be confirmed at second measurement, one patient tested NAb negative at second time point and in two patients NAb measurement was not repeated. Of the 21 patients with high NAb titres and induction measurement of MxA mRNA, 17 were classified as biological non-responders.
Figure 3. MxA induction results correlated with Nab titre. Median values and 95% confidence intervals are shown for each Nab category. The Spearman correlation test showed a negative correlation between MxA induction and Nab titre ($r = -0.547$; $p = 0.001$). MxA, myxovirus resistance protein A; mRNA messenger RNA; Nab, neutralizing antibodies.

Discussion and conclusions

Several studies independently found evidence that anti-IFN-β Nab interfere with IFN-β bioactivity and can have a diminishing effect on treatment response in MS patients$^{19,20,21,22,23,24}$. Some recent studies concluded that above a certain level of Nab IFN-β bioactivity is completely abolished$^9,24$. More recently, post-injection MxA mRNA also showed prognostic significance for the risk of new relapses in a large Italian IFN-β treated cohort$^{26}$. Most studies use a 12 hours interval between IFN-β injection and post-injection sampling. Obviously, this schedule is not likely to include pre-injection samples if applied in daily practice. Thus, we here present the results of the validation of a more feasible protocol for IFN-β bioactivity measurement using MxA mRNA quantification in samples taken only 4 hours after IFN-β administration. Our data suggest that though bioactivity screening with only post-injection samples seems reasonable, MxA induction measurement based on both pre- and post-IFN-β injection samples is slightly more appropriate to determine ultimate IFN-β bioactivity status. If the pre-injection measurements obtained at the second visit were not taken into consideration, the interpretation of the MxA expression results would have resulted in two more Nab negative patients not showing a high enough level of MxA mRNA, thus suggesting a false-negative biological response. Thus, the incorporation of a pre-injection sample may result in the identification of additional patients that still may benefit from the drug. Nevertheless, one should bear in mind that the number of false-negative biological responders is low and the procedure of pre-injection in addition to the 4 hours post-injection
sampling may be somewhat inconvenient for the patient. The MxA induction measurement after 3 months as applied in our study, both corrects for individual and temporal MxA mRNA fluctuations and also meets the EFNS guideline requirement of measuring sustained NAb positivity before therapy change can be recommended. Both MxA expression at screening as well as MxA induction at the second visit showed a good (negative) correlation with NAb titres. This correlation might even have been stronger when repeated NAb titres would have been performed in all patients. Altogether, these findings confirm results from previous studies that NAb are clearly associated with a reduction of IFN-β bioactivity. Both tests seem useful in the clinical evaluation of patients treated with IFN-β. The mRNA MxA test, however, clearly is a more simple and less time consuming laboratory method. Nevertheless, analysis and optimum interpretation of the MxA mRNA data are still open for discussion. Though calibration curves are highly reproducible allowing highly specific and sensitive data, we here have presented absolute quantifications. For clinical application this method permits adequate comparison between and within patients as analysed on different times, at different sites and at different time points. The recent recommendations of the American Academy of Neurology suggest that there is insufficient information on the utilization of NAb testing to provide specific recommendations regarding when to test, which test to use, how many tests are necessary, or which cut-off titre to apply. We believe that our protocol meets some of the criticism of the AAN task force. We suggest to screen for post-injection MxA mRNA after 6 months of IFN-β treatment and to invite patients for a pre- and 4 hours post-injection sample three months later in case of insufficient MxA mRNA levels at screening. If the result of the induction measurement at the second visit shows a non-biological response we consider the patient as persistently lacking a biological response and reconsider treatment options. In case of a poor biological response we recommend to reinvite the patient for a third measurement (pre- and post-injection sample) three months later. We here present the data of a feasible protocol for IFN-β bioactivity measurement using MxA mRNA quantification in samples taken only 4 hours after IFN-β administration. Despite the individual variation in MxA responses, screening for loss of IFN-β bioactivity with only post-injection samples seems reasonable. However, MxA induction measurement based on both pre- and post-IFN-β injection samples at second measurement is somewhat more precise in determining ultimate IFN-β bioactivity status.

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References

