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Pharmacodynamics of interferon beta in multiple sclerosis patients with or without serum neutralizing antibodies

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Abstract To analyze the *in vivo* biological effect of anti-interferon beta (IFN-beta) neutralizing antibodies (NABs), blood concentrations of neopterin, beta2microglobulin (Beta2-MG), mRNA-dependent myxovirus-resistant protein A (MxA) and dsRNA-dependent protein kinase (PKR) were measured before (pre-dose) and 24 hours after (post-dose) IFN-beta administration in 49 patients with multiple sclerosis (MS) with (n = 25) and without (n = 24) NABs.

The results indicated that pre-dose levels of MxA-mRNA and PKR-mRNA were highly variable [coefficient of variation (CV) > 100%] among patients. A lower inter-individual variability was observed for pre-dose levels of Beta2-MG and neopterin (CVs of 29% and 44%, respectively).

Significantly lower pre- and post-dose blood levels of IFN induced markers, except for post-dose PKR-mRNA (p = 0.09), were seen in NAB+ compared with NAB– patients and between patients with high (> 200 $t_{1/10}$) and low (≤ 200 $t_{1/10}$) NAB titers. A significant inverse correlation between NAB titer and pre-dose levels of the above IFN-induced markers was found. In summary, our findings confirm that NABs affect absolute concentrations of IFN-beta induced markers and suggest that such an effect occurs in a titer-dependent manner.

Key words interferon beta · neutralizing antibodies · multiple sclerosis · MxA · PKR · Beta2-microglobulin · neopterin

Introduction

Interferon beta (IFN-beta) is a first-choice therapeutic strategy for patients with relapsing–remitting (RR) multiple sclerosis (MS) [1] and those with secondary progressive (SP) MS experiencing continuing relapse activity [2, 3]. Neutralizing antibodies (NABs) against IFN-beta may occur in variable percentages of patients [2, 4–8]. The clinical significance of NABs has been a matter of controversy and has been extensively investigated ever since the initial descriptions in MS patients [9–23]. Monitoring blood levels for IFN-beta-induced compounds and expression of IFN type I-inducible genes provides an

Table 1 Demographics and clinics of patients (NABs, neutralizing antibodies; MS, multiple sclerosis; RR, relapsing–remitting; SP, secondary progressive; EDSS, expanded disability status scale)

	Patients without NABs (n = 24)	Patients with NABs (n = 25)
Age (years)*	41.0 ± 11.3	45.2 ± 12.9
Gender (females/males)	19/5	19/6
MS duration (years)*	13.8 ± 8.7	10.7 ± 3.8
Disease course	RR (10); SP (14)	RR (9); SP (16)
EDSS*	5.3 ± 1.6	4.5 ± 1.6

* mean ± SD

interesting approach to studying the *in vivo* biological effects of NABs.

Serum concentrations of IFN-induced compounds have been examined already to some extent, in patients with RRMS undergoing IFN-beta therapy. Decreases in serum levels of neopterin, beta2-microglobulin (Beta2-MG) and in peripheral blood mononuclear cell (PBMC) concentrations of mixovirus-resistant protein A (MxA)-mRNA were found in RRMS patients who developed NABs [24–31]. However, the findings were contradictory [28]. Further questions still remain as to whether lack of efficacy of IFN-beta in NAB+ patients is related to these antibodies in a titer-dependent manner. Indeed, while some studies described an association between NAB titer and loss of IFN-beta biological response[24, 27–29, 31], others failed to demonstrate such an association [25, 30].

In order to gain new insights into these issues, the pharmacodynamic profile of IFN-beta in patients with MS, both with and without NABs, was first defined by measuring neopterin, Beta2-MG and MxA-mRNA concentrations in blood immediately before and 24 hours after IFN-beta injection. In addition, pre- and post-dose intracellular levels of the IFN-induced dsRNA-dependent protein kinase (PKR)-mRNA were also determined.

The changes in PBMC PKR-mRNA have been so far only marginally studied in patients with MS [32].

To address the role of NAB titers in affecting the pharmacodynamics of IFN-beta patients with NABs were finally classified in two different groups based on NAB titers.

Materials and methods

Patients and study design

49 patients with clinically definite MS according to Poser criteria [33], who were undergoing therapy with recombinant (r) IFN-beta1b (Betaferon®, Schering, Berlin, Germany) given at 8×10^6 IU subcutaneously (SC) every other day were included consecutively in this cross-sectional study. All patients were required to sign an informed consent form prior to inclusion. Local Ethics Committees and/or Institutional Review Boards approved the study.

In Table 1 the demographics and clinics of patients at the time of sample collection are summarized. Patients with NABs had been undergoing therapy for 2.8 ± 1.9 years, while NAB– patients had been taking IFN-beta for 4.1 ± 1.7 years (p = 0.08).

Samples for each individual were collected according to the following scheme: (i) pre-dose, that is immediately before an rIFN-beta1b injection, corresponding to 48 hours (up to 72 hours in a few cases) after the previous rIFN-beta1b injection; (ii) post-dose, that is 24 hours after an rIFN-beta1b injection (18 hours in a very few cases). Pre-dose samples served for NAB measurements, whereas pre- and post-dose samples were collected for measurement of blood concentrations of Beta2-MG, neopterin, MxA and PKR.

The timing of sample collection was based upon both ethical and biological considerations representing a trade-off between feasibility and optimal data acquisition. First, only two sample collections (i.e. pre- and post-dose) were considered to be acceptable by a local ethics committee. Second, previous studies showed that changes in type I-IFN-induced compounds (i.e. Beta2-MG, neopterin and MxA protein) are still sustained 24 hours after IFN-beta injection in patients with RRMS [26, 28, 31, 34].

In the light of the above-mentioned considerations, samples were collected at 24-hours post-dose in the present study.

Biological evaluations

Venous peripheral blood from each patient was drawn into tubes containing ethylenediaminetetraacetic acid and into anticoagulant-free tubes. PBMCs were separated using Ficoll-Hypaque gradient sedimentation; 5×10^6 PBMCs were collected, pelleted and frozen at –80°C until required. Serum samples, obtained after centrifugation, were stored at –80°C until used.

Detection of IFN-beta NAB

Antibody titers were determined by neutralization against 10 IU of rIFN-beta1b as previously described [6]. Sera were routinely inactivated at 56°C for 30 minutes before titration. Then 60μl of twofold serial dilutions (starting from 1:10) of sample or control sera were incubated at 37°C with 60 μl containing 20 IU/ml of rIFN-beta. After 1 hour, 100 μl of individual mixtures were added to duplicate monolayers of human lung carcinoma (A549) cells in 96-well microtiter plates. After 18–24 hours of culture and extensive washing, the cells were challenged with the encephalomyocarditis virus and incubated at 37°C for 24 hours. Controls included a titration of the IFN preparations used in the respective assays and of a reference standard antibody to IFN-beta (National Institutes of Health, Bethesda, MD; code GO38-501-572). Any antiviral activity and its neutralization were assessed on the basis of the virus-induced cytophatic effect (CPE), and to quantify this, the cells were stained with crystal violet in 20% ethanol. The dye taken up by the cells was eluted with 33% acetic acid and its absorbance measured in a microdensitometer at 540 nm (OD_{540}). The extent of virus-induced

CPE, its inhibition by IFN-beta, and the reversal of this by NABs were shown by the amount of dye eluted from each well. Titers were calculated using Kawade's method and expressed logarithmically as $t_{1/10}$, namely the dilution of serum reducing 10 laboratory units (LU) per ml of IFN to 1 LU/ml [35].

Serum samples were routinely assayed for, and all were found to be free from, endogenous or residual IFN activity.

The neutralizing activity of the titred sera (≥ 80 $t_{1/10}$ that corresponds to (≥ 1.9 Log) was characterized as being due specifically to the presence of IFN antibodies as previously described [36].

Patients were assigned to one of three groups according to their NAB status: NAB-negative (NAB–), NAB low positive (≤ 200 $t_{1/10}$, which corresponds to ≤ 2.3 Log; LP-NAB+) and NAB high positive (> 200 $t_{1/10}$, which corresponded to >2.3 Log; HP-NAB+).

Detection of neopterin and Beta2-MG

Serum concentration of neopterin (DRG Instruments GmbH, Marburg, Germany) and Beta2-MG (S.P.A. Italiana Laboratori Bouty, Milan, Italy) were immunoassayed following the manufacturer's instructions using sera stored at –80 C° until tested. Serum samples were tested in duplicate.

Taqman quantitative RT-PCR for MxA- and PKR-mRNA

MxA and PKR genes transcripts in PBMCs from MS patients were quantified by a real time 5′ exonuclease reverse transcript-polymerase chain reaction (RT-PCR Taqman) assay using the ABI 7700 sequence detector (Applied Biosystems, Monza, Italy). Briefly, total cell RNA and DNA were concomitantly extracted from 5 $X10^6$ PBMCs using Trizol reagent (Gibco BRL, NY, USA) following the manufacturer's instructions. The RNA was dissolved in 50 µl of RNAse-free water and the quantitation of MxA- or PKR-mRNA was performed in a Taqman assay after generation of cDNA. The following primers and probe for MxA (forward primer, 5′-CTGCC TGGCAGAAAACTTACC-3′ reverse primer, 5′-CTCTGTTATTCTC TGGTGAGTCTCCTT-3′ probe, 5′CATCACACATATCTGTAAA TCTCTGCCCCTGTTAGA-3′) or for PKR (forward primer, 5′-TGCTACTACGTGTGAGTCCCAAA-3′ reverse primer, 5′- TGATG TATCTGCTGAGAAGTCACCT-3′ probe, 5′-CAACTCTTTAGTGA CCAGCACACTCGCTTCT-3′) were added to the universal PCR master mix (Applied Biosystems, Foster City, CA, USA) at 300 and 100 nM, respectively, in a final volume of 50 µl. The standards were obtained by cloning the 525 bp MxA fragment or 394 bp PKR fragment into the pCRII plasmid using a TOPO TA cloning kit (In Vitrogen Corporation, San Diego, CA, USA). The DNA from the cloned plasmids was subsequently transcribed using SP6/T7 RNA polymerase under the conditions recommended by the supplier (Promega Corporation, Madison, WI, USA). A linear distribution (r = 0.99) was obtained between 10^1 and 10^8 copies of MxA- or PKR-RNA.

PBMC-MxA and PKR transcripts were normalized to DNA values and expressed as the number of copies of MxA- or PKR-mRNAs/ng DNA. DNA levels were measured by using a spectrophotometer at 260 nm.

Statistical analysis

Descriptive statistics were provided for the entire population to report patient demographics/clinics and blood concentrations of neopterin, Beta2-MG, and MxA- and PKR-mRNA.

The coefficient of variation (CV) was used to measure interpatient variability in blood concentrations of biological products. The CV is the ratio of the standard deviation (SD) of the mean of each variable to the mean, expressed as a percentage [CV = (100) (SD/mean)]. Relative increases in levels of neopterin, Beta2-MG, and MxA-and PKR-mRNA from pre- to post-dose were given as the post- to pre-dose levels ratio. Absolute increases above baseline were given by the differences between post- and pre-dose values. Differences between patient groups in terms of blood concentrations and relative or absolute increases in surrogate biological markers for IFN action were analyzed using the Kruskal–Wallis test. Follow-up Mann–Witney U tests were undertaken when differences were found. Pearson's r coefficient was calculated to assess the correlation between levels of Beta2-MG, neopterin, and Log MxA/PKR-mRNA with Log NAB titers.

Statistical analysis was performed by SPSS v.11 for Windows®. A p-value of ≤ 0.05 was considered significant throughout the statistical analyses.

Results

Inter-patient variability in pre- and post-dose levels of neopterin, Beta2-MG, and MxA- and PKR-mRNAs

Individual levels of neopterin, Beta2-MG, and MxA- and PKR-mRNAs at pre- and post-dose are shown in Figure 1. Pre-dose CVs were 44%, 29% for neopterin and Beta2-MG, and >100% for MxA- and PKR-mRNAs; post-dose CVs were 45%, 28% for neopterin and Beta2-MG and >100% for MxA- and PKR-mRNAs. This finding demonstrates a marked interpatient variability in the steady-state and IFN-induced levels of markers in patients with MS undergoing IFN-beta treatment.

Influence of NAB on the expression of IFN-induced markers

Absolute pre- and post-dose levels of each IFN induced marker in NAB+ and NAB– patients, expressed as mean (± SD) and median, are shown in Table 2. NAB+ patients had significantly lower concentrations of each marker at each time point except for Beta2-MG blood post-dose concentrations (p = 0.09). When patients were grouped together as NAB–, LP-NAB+ and HP-NAB+ (see Table 2), the highest (p < 0.02) mean values of neopterin, Beta2-MG, and MxA- and PKR-mRNAs were observed in NAB– patients compared with either LP-NAB+ or HP-NAB+ patients. The exceptions were the values of PKR-mRNA at post-dose, which were found to be lower, although not significantly (p = 0.09) in HP-NAB+ patients versus LP-NAB+ patients. The lowest values were found in HP-NAB+ patients compared with each remaining group.

Significant inverse correlations between the Log titer of NABs and the pre-dose expression of neopterin (r = –0.54; p < 0.05), Beta2-MG (r = –0.34; p < 0.05), Log MxA-mRNA (r = –0.49; p < 0.05), and Log PKR-mRNA (r = –0.35; p < 0.05) were recorded.

Upon examining IFN beta-induced modifications of the values of each compound, it was found that no

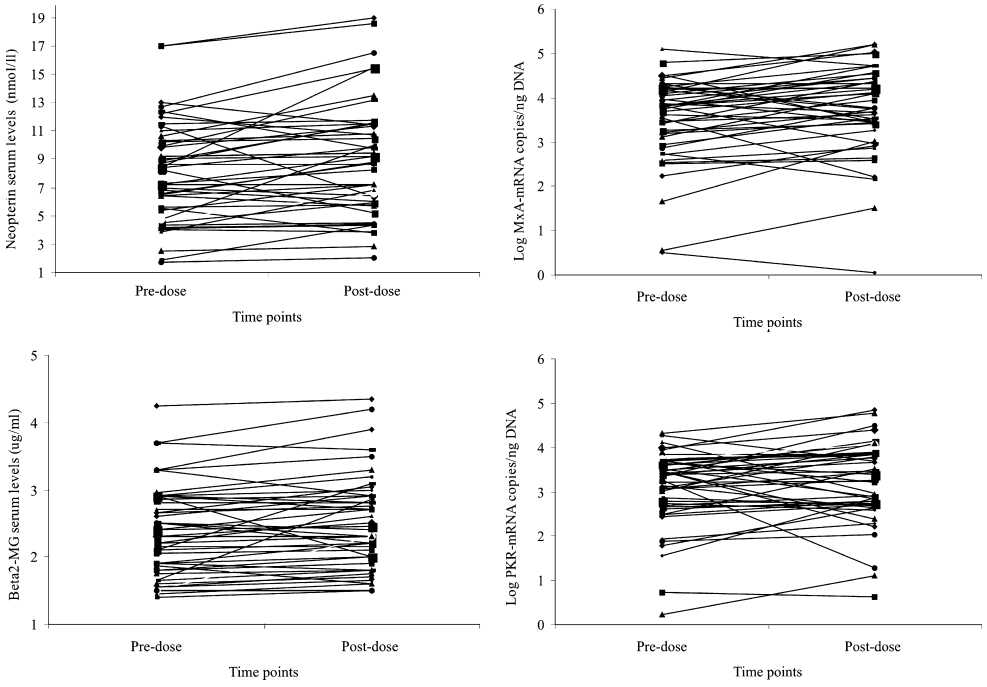

Fig. 1 Change of neopterin (panel A), Beta2-MG (panel B), Log MxA-mRNA (panel C) and Log PKR-mRNA (panel D) expression levels after rIFN-beta1b injection in the entire patients group. (Beta2-MG, beta2-microglobulin; MxA, mixovirus-resistant protein A; PKR, dsRNA-dependent protein kinase; rIFN, recombinant interferon)

Table 2 Influence of NABs on expression of interferon-induced markers (NABs, neutralizing antibodies; LP, low positive; HP, high positive; Beta2-MG, beta2-microglobulin; MxA, myxovirus-resistant protein A; PKR, dsRNA-dependent protein kinase)

		NAB– (n = 24)	All NAB+ (n = 25)	LP-NAB+ (n = 13)	HP-NAB+ (n = 12)
Neopterin (nmol/l)	Pre-dose	9.6 ± 3.3 (9.5)	6.2 ± 2.8 (5.6)	7.6 ± 2.8 (7.3)	4.7 ± 2.4 (4.2)
	Post-dose	10.7 ± 4.1 (10.8)	6.9 ± 2.7 (6.8)	8.2 ± 2.4 (8.6)	5.5 ± 2.7 (4.5)
Beta2-MG (μg/ml)	Pre-dose	2.5 ± 0.6 (2.5)	2.1 ± 0.5 (1.9)	2.3 ± 0.7 (2.4)	1.9 ± 0.5 (1.9)
	Post-dose	2.6 ± 0.7 (2.6)	2.2 ± 0.5 (2.2)	2.6 ± 0.6 (2.5)	2.0 ± 0.5 (1.9)
MxA-mRNA (copies/ng DNA)	Pre-dose	20433.7 ± 27671.3 (11980.6)	6016.5 ± 11076.9 (1744.5)	6712.2 ± 6831.9 (4789.1)	1340.4 ± 1442.0 (636.3)
	Post-dose	37486.4 ± 48604.3 (16498.6)	9699.4 ± 14488.1 (2955.1)	15217.3 ± 17184.1 (5998.6)	1815.1 ± 1879.8 (914.9)
PKR-mRNA (copies/ng DNA)	Pre-dose	5629.0 ± 5832.3 (4224.7)	2077.3 ± 2481.5 (1190.5)	1978.9 ± 1761.0 (1385.6)	1415.0 ± 1543.8 (1095.6)
	Post-dose	11600.3 ± 19439.9 (4985.0)	3324.7 ± 4414.9 (778.1)	3777.8 ± 5003.0 (1729.0)	2620.1 ± 3951.2 (587.3)

Values are expressed in mean ± SD (median)

differences in increases relative to baseline were present in NAB–, LP-NAB+ and HP-NAB+ patients (Figure 2). The latter suggests that NAB probably do not abolish completely the capability of IFN beta to exert its biological activity.

Discussion

Several approaches have been used to establish whether the development of NABs affects the pharmacodynamics of IFN-beta in patients with MS. Our results confirm that IFN-beta NABs reduce pre- and post-dose blood concentrations of several IFN-induced markers. There were, however, some further findings, which we consider, deserve some attention.

The present study indicates, for the first time to our knowledge that the measurement of PKR-mRNA is worth taking into account when studying the pharmacodynamic profile of IFN-beta in MS patients. Questions remain as to whether PKR protein measurements provide a clinically useful marker for monitoring and predicting the effectiveness of IFN-beta in MS patients, as this study was not powered and designed for this purpose.

High inter-patient variability was observed in neopterin and Beta2-MG pre- and post-dose serum levels, as well as in PBMC MxA and PKR pre- and post-dose transcripts. This finding is not new and is most likely due to heterogeneity in the immunology of patients with MS. Indeed, Chieux and co-authors [37] reported that, in contrast to moderate differences in MxA expression in healthy individuals, clinically

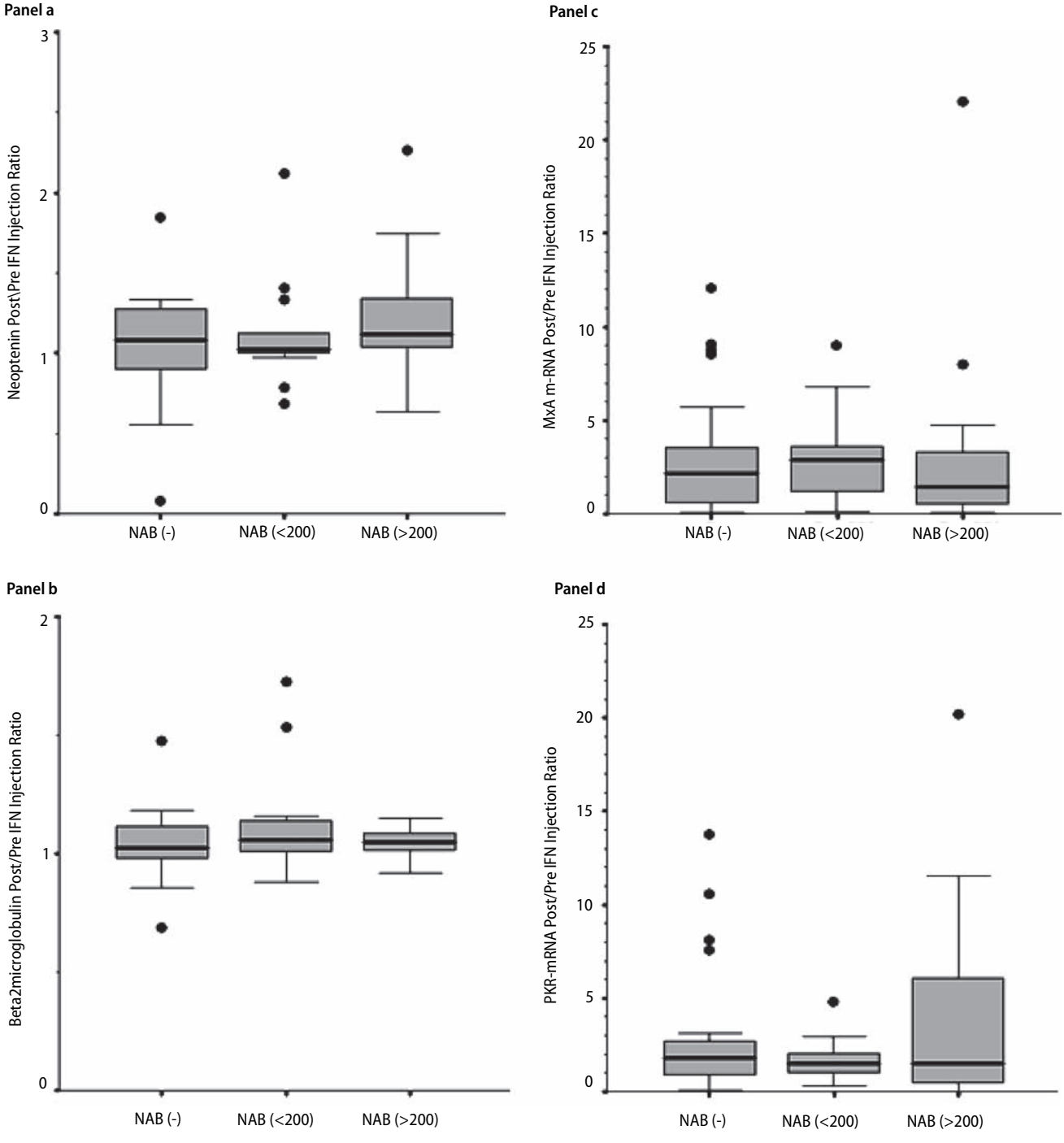

Fig. 2 Post/pre-dose ratios of neopterin (panel A) and Beta2-MG (panel B), peripheral blood mononuclear cells MxA-mRNA (panel C), PKR-mRNA (panel D) in NAB– (< 10 $t_{1/10}$), LP-NAB+ ((200 $t_{1/10}$) and HP-NAB+ (≤ 200 $t_{1/10}$) patients. (Beta2-MG, beta2-microglobulin; MxA, myxovirus-resistant protein A; PKR, dsRNA-dependent protein kinase; NAB, neutralizing antibody; LP, low positive; HP, high positive) Box and whiskers plots are used to present data. In these graphs, boxes represent interquartile ranges; horizontal bars stand for median value; vertical bars extend to the smallest and the largest observations within 1, 5 interquartile range from the extremes of the box. Outliers are displayed as individual points

stable patients with RRMS have high inter-individual variance in MxA expression. This observation has been extended here and our results support the idea that each MS patient might possess a specific and individual capacity to respond to IFN-beta. Several factors could contribute to this wide range of vari-

ability, including the immunological (e.g. change in Th1/Th2 ratio or cross-talk from other cytokine-activated pathways) and genetic characteristics of patients [38–41].

Apart from these speculations, the reasons for the high level of inter-individual variability remain unclear at present.

While differences were found between patients with and without NABs, in pre and post-dose concentrations of neopterin and Beta2-MG as well as MxA and PKR specific transcripts (Table 2), and in absolute increases over the baseline of the same markers (data not shown), no differences were seen in the relative post-injection increase in any of the examined compounds (Figure 2). The latter has not been previously investigated and was an intriguing finding. Several considerations can be formulated in order to explain our finding. First one could speculate that as soon as IFN-beta is administered it reaches its target cells being free to exert its action. Later on, when the molecule reaches the bloodstream, it can be exposed to the neutralization effect of circulating NABs.

Second, little is known about the reaction of anti-IFN antibody neutralization in terms of molar concentrations. The latter would deserve further investigations in order to understand the neutralization capability of NABs. At present, we may only speculate that following IFN-beta injection, NABs do not neutralize all IFN molecules and those left can still bind to the receptor, thus allowing some effector expression. Third, binding between IFN and NAB is reversible and anti-IFN-beta NABs with different affinity and specificity have been demonstrated *in vivo* [42–45].

It is tempting to speculate that the above factors may influence the efficiency of the NAB neutralization reaction. Recently we obtained evidence that the degree of IFN-beta-induced expression of markers may strictly depend on their pre-dose levels (Scagnolari C, et al., unpublished). This suggests that the ratio post/pre-dose, rather than the absolute increases, may be more representative of the effector mechanism elicited by IFN-beta treatment.

Whatever the explanation is, further studies are warranted in order to better understand these preliminary findings. A more extensive pharmacodynamic profile would possibly provide further insights. It seems also possible that at least in some instances the level of induction of IFN- beta-induced markers decreases during IFN-beta treatment due to a "tachyphylaxis-like" phenomenon [28]. These considerations have to be taken into account to fully understand the capability of NAB to neutralize the activity of IFN-beta.

A third possible source of variability, may derive from circadian variations because in this study, drug injection and sample collection after 24 hours did not always occur at the same time of the day.

Even considering all these limitations, our data provide new insights in the capability of NABs to completely impede IFN action *in vivo*.

Our data also indicate a correlation between NAB titers and the expression of IFN-action markers. More specifically, we observed that HP-NAB+ patients (e.g. > $200t_{1/10}$) were associated with a marked inhibition of the *in vivo* biological response compared with NAB– or LP-NAB+ patients. This is in accordance with previous studies, which showed a significant decrease in Beta2-MG, neopterin and MxA protein in patients with persistently high levels of NABs [24, 27–29, 31] but contradicts others who found that IFN-beta bioavailability was completely abolished, irrespective of NAB titers [25].

This discrepancy can be explained by considering that other factors beside NABs could affect IFN-beta bioactivity in patients with MS. These include abnormal IFN signalling [46], decreased expression of the IFN type I receptor on the cell surface [47], release of free IFN-alpha/beta type I receptors into the circulation [48, 49] and the presence of IFN type I inhibitory proteins [48, 50]. Recently it has been shown that two members of the suppressor of cytokine signalling (SOCS) protein family, SOCS-1 and SOCS-3, also inhibit IFN-induced expression of the antiviral proteins 2′5′-OAS and MxA [51].

In conclusion our data demonstrate that the development of NABs in patients with MS during therapy leads to a decrease in absolute pre- and post-dose levels and changes in surrogate markers for IFN. However, the data also indicate that surrogate markers for IFN increase significantly on IFN-beta administration, even in the presence of NABs. Hence, serum NABs seem to be unable to abolish entirely the effects of IFN-beta administration. Whether this effect of NABs is fully NAB titer-dependent is still debatable and warrants further investigation in larger cohorts of patients and at shorter intervals.

The main aim of this study was to address in detail the pharmacodynamics of IFN-beta in the presence of serum circulating NAB, and not to address the general issue of NAB impact from a clinical point of view. In order to reach definite conclusions on this issue, further studies combining clinical and biological investigations in the same individuals are certainly warranted.

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