RESEARCH PAPER

Neurologia i Neurochirurgia Polska
Polish Journal of Neurology and Neurosurgery
2019, Volume 53, no. 5, pages: 348–357
DOI: 10.5603/PJNNS.a2019.0047

ISSN 0028–3843

Anti-interferon-beta antibodies in Polish multiple sclerosis patients: prevalence and clinical significance in a long-term prospective study

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ABSTRACT

Aim of the study. To determine the prevalence of anti-interferon-β binding (BAb) and neutralising antibodies (NAb), and to investigate whether NAb measured by luciferase-based cell assay can predict treatment response in multiple sclerosis (MS) patients treated with interferon-β-1b (IFNβ-1b).

Clinical rationale for the study. A subgroup of IFNβ-treated MS patients develop NAb directed against the drug. The clinical significance remains controversial, which could be explained to some extent by technical difficulties in NAb detection and quantification. A simple, specific and reproducible test for NAb might help elucidate these uncertainties.

Materials and methods. Sera from 101 consecutive MS patients initiating treatment with IFNβ-1b were collected at baseline and during the first two years, and assessed for BAbNAb with a novel luciferase-based cell assay. Median clinical follow-up lasted 5.1 years.

Results. BAb were present in 97% and NAb in 88% of the study cohort. Unexpectedly, 92% of patients tested positive for BAb and 12.5% for NAb at baseline, before drug exposure. Patients with baseline NAb positivity were more likely to remain free of disease activity in the first three years of treatment. When baseline-positive cases were grouped together with those who remained NAb-negative, and the resulting group was compared to those who became positive after drug exposure, NAb positivity was associated with a higher risk of disease activity during the entire follow-up. Direct comparison of BAb/Nab-positive and BAb/Nab-negative patients only revealed an association of BAb positivity with more active disease after four years of treatment, while NAb failed to predict the outcome.

Conclusions and clinical implications. Antibodies developed after treatment initiation are associated with a worse outcome. Naturally-occurring antibodies appear to predict more benign disease. Their prevalence and specificity require further investigation.

Key words: multiple sclerosis, interferon-beta, neutralising antibodies, treatment outcome

(*Neurol Neurochir Pol 2019; 53 (5): 348–357*)

Introduction

Interferon beta (IFNβ) continues to be commonly used as a first-line disease modifying drug (DMD) in relapsing-remitting multiple sclerosis (RRMS) patients, but its efficacy varies among individual cases.

In response to IFNβ treatment, a proportion of patients develop either binding (BAb) or neutralising anti-IFN antibodies (NAb). IFNβ-1b is thought to be more immunogenic than IFNβ-1a, and a subcutaneous route of administration more immunogenic than an intramuscular route [1]. BAb appears in up to 97% of patients, usually between the third and twelfth months of treatment [1]. NAb appears later (6-24 months) and less commonly (28–42%) [1–3]. For pegylated IFNβ-1A, NAb prevalence has been shown to be below 1% after two years of treatment [4].

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The significance of these findings remains controversial. There is evidence from clinical trials and open-label observational studies that NAb in high titres are persistent [5] and associated with a loss of IFN efficacy [2, 6–8]. In fact, NAb may be the most common cause for early treatment failure in IFNβ-1b-treated patients [9]. In many studies however, Nab's influence was most apparent in NAb-positive periods [3, 10, 11], and a considerable proportion of NAb-positive patients revert to a NAb-negative status over time [3, 10], with restoration of IFN activity [10]. On the other hand, some researchers [8, 12] have observed only sporadic conversion to NAb-negativity and worse outcomes in long term follow-ups.

At least in part, these uncertainties stem from the complexity of NAb measurement. All currently used assays are based on NAb-mediated inhibition of IFN's effects on IFN-responsive cells. The effect can be observed as protection from the cytopathic effect in a viral challenge, as in cytopathic effect assay, a gold standard since 1985 [13], or confirmed via MxA mRNA detection [14]. Attempts to design a non-cellular assay have thus far been unsuccessful.

A new generation of tests uses growth-arrested cells transfected with luciferase under a selective class-I IFN controlled promotor [15]. While this involves live cells, there is no need for continuous cell line culture. Currently, two luciferase-based tests have been validated and are commercially available [16, 17], but no long-term follow-up is available with regards to their prognostic value.

Clinical rationale for the study

We sought to determine the influence of IFNβ NAb, measured by luciferase-based cell assay, on disease activity in RRMS patients treated with IFNβ-1b in a long-term prospective observation, in the hope that their detection could predict drug response and so guide treatment decisions.

Materials and methods

Patients

A total of 101 RRMS patients were recruited consecutively for this study while initiating treatment with IFNβ-1b in the setting of the national MS treatment programme at the outpatient clinic at Heliodor Swiecicki University Hospital in Poznan, Poland, from 2008 to 2013. The study protocol was approved by the Ethics Board of Poznan University of Medical Sciences. Inclusion criteria were: age ≥ 18 years; diagnosis of RRMS according to the 2005 revision of the McDonald criteria [18] (all patients also met the 2010 criteria [19]); no prior disease modifying treatment; fulfillment of the national treatment programme eligibility criteria (Tab. 1); and written informed consent for study participation.

Treatment consisted of interferon beta-1b (Betaferon, n = 96, or Extavia, n = 5) 250μg (8 MIU) subcutaneously every other day.

Baseline clinical information was obtained: sex, age at first relapse, time to second relapse, time to treatment initiation, number of relapses, and EDSS score prior to treatment initiation.

Follow up

The follow up spanned the period from 2008 to 2018 and consisted of monthly neurological assessments.

In addition, 1.5 Tesla head magnetic resonance imaging (MRI, Siemens Avanto, Erlangen, Germany) with a 12-channel head coil, including T1, T2, FLAIR (Fluid Attenuated Inversion Recovery) and PD (Proton Density) sequences, with gadolinium contrast administration, was obtained at baseline and was repeated each year. For individual indications, spinal MRI was performed.

Each year, we recorded the number of relapses, EDSS change, and the presence of new T2/FLAIR or enhancing lesions in MRI.

Definition of NEDA

Consistently with previous works [20], NEDA was defined as no relapses, no disability progression and no MRI activity in a given time period. A relapse was defined as the appearance or worsening of symptoms applicable to multiple sclerosis, with focal neurological abnormality, lasting 24 hours and preceded by ≥ 30 days of neurological stability, in the absence of infection or fever. Progression was recognised when an increase in EDSS occurred and was confirmed after three months. The minimal required increase depended on baseline EDSS: ≥ 1.5 for a baseline score of 0 ≥ 1.0 for scores ranging from 1.0 to 5.0 and ≥ 0.5 for baseline EDSS of ≥ 5.5. New or enlarging lesions in T2/FLAIR or contrast enhancing lesions were considered as MRI activity.

Sampling

Serum samples were collected prior to treatment initiation and again after one, three, six, 12, 24 and 36 months of treatment, then stored at -70°C until further analysis.

The treating and sampling staff were unaware of the patients' antibody status.

Laboratory

Binding antibody detection

We employed a customised indirect ELISA developed at the Department of Neurochemistry and Neuropathology, Poznan University of Medical Sciences, Poznan, Poland [21].

ELISA plates (Nunc, Roskilde, Denmark) were coated with Betaferon (Bayer Pharma AG, Germany) diluted in 0.05M sodium bicarbonate (final concentration: 1 g/mL). The plates were incubated at room temperature for 12 hours, then washed with phosphate-buffered salt (PBS) with 0.05% (vol / vol) Tween 20. Nonspecific binding sites were blocked with 1% bovine albumin solution in PBS-0.05% Tween 20 and the plates were washed with PBS-0.05% Tween 20 again. Then, the

Table 1. National Multiple Sclerosis treatment programme eligibility criteria in Poland 2008–2013

Criterion	2008	2012
Diagnosis	2005 McDonald criteria and contrast-enhanced head MRI consistent with MS	
Disease activity	At least two relapses within the past two years	not specified
Required score	21 pt	15 pt
Scoring system	Age: 16–40 years — 6 pt 40–60 years — 3 pt over 60 years — 1 pt Disease duration: 0–3 years — 6 pt 3–6 years — 3 pt 6–10 years — 2 pt over 10 years — 1 pt RRMS with no neurological deficit — 5 pt. Number of relapses in the last year: 3–4 — 5 pt 1–2 — 4 pt 6–7 — 2 pt none (less than 1/year) — 1pt over 7 — 0 pt EDSS score: 0–2 — 6 pt 2.5–4 — 3 pt 4.5–5 — 2 pt over 5 — 1 pt	Disease duration: 0–3 years — 6 pt 3–6 years — 4 pt 6–10 years — 2 pt over 10 years — 1 pt RRMS with no neurological deficit — 5 pt Number of relapses in the last year: 3 and more — 5 pt 1–2 — 4 pt none — 1pt EDSS score: 0–2 — 6 pt 2.5–4 — 5 pt 4.5–5 — 2 pt over 5 — 1 pt
Exclusion criteria	1. hypersensitivity to IFNβ 2. primarily or secondarily progressive MS 3. pregnancy 4. decompensated liver disease (aminotranferase levels ≥ 2x upper reference limit) 5. thyroid disease (no euthyreosis) 6. intractable depressive mood disorder or history of suicidal ideation 7. epilepsy	

MRI — magnetic resonance imaging; MS — multiple sclerosis; RRMS —relapsing–remitting multiple sclerosis; pt — points; EDSS — Expanded Disability Status Score

standard and patient sera were added. As standard, we employed goat anti-human interferon antibodies (Sigma-Aldrich) in dilutions of 1:20, 1:50, 1:100, 1:200 and 1: 400. Patient sera were assessed at a dilution of 1:100. After incubation at room temperature, the plates were washed with PBS-0.05% Tween 20. Then, secondary antibodies were added (goat anti-rabbit IgG and rabbit anti-human IgG conjugated with alkaline phosphatase, Sigma-Aldrich). P-nitrophenyl phosphate was used as the alkaline phosphatase substrate (Sigma-Aldrich) and 1 M HCl as the inhibitor. Absorbance was measured at wavelength = 405 nm with ELx800 ELISA reader (Bio-TEK), as optical density, in arbitrary units (AU/ml), following the formula:

(10 × tested sample absorbance) / (cut-off values absorbance)

The cut-off was defined at the absorbance's 95th percentile. A standard curve, based on log-log regression (R2 = 0.978), was prepared for the correlation between absorbance and reciprocal standard dilution. Reciprocal serum dilutions were then read from the standard curve. A standard curve was also drawn for correlation between concentration of standard and absorbance.

A patient was considered BAb positive, BAb (+), if BAb were detected at any time, and BAb negative, BAb (-), if no sample tested positive and at least one was obtained after ≥ 6 months of treatment. A patient was considered persistently BAb (+) if he or she did not revert to BAb (-) in further samples, and transient BAb (+) otherwise.

Neutralising antibody detection

To detect NAb, we used a luciferase reporter gene assay (iLite® Type I IFN Assay Ready Cells, Euro-Diagnostica, Sweden) which involves live cells transfected with luciferase under a selective IFN type I-controlled promotor. When exposed to

Table 2. Study cohort baseline characteristics; P values for gender difference

	All	Female	Male	p
Age at first relapse, years, mean ± SD	29.9 ± 8.7	31.3 ± 9.2	25.7 ± 5.2	< 0.001
Time to second relapse, months, median (IQR)	10.0 (4.0 to 24.25)	10.0 (4.0 to 24.0)	11.5 (4.5 to 31.5)	0.401
Time to treatment initiation, months, median (IQR)	18.0 (12.0 to 35.0)	23.0 (12.0 to 35.0)	16.5 (7.5 to 34.5)	0.294
Relapses before treatment, median (IQR)	2 (2 to 3)	2 (2 to 3)	2 (2 to 3)	0.568
EDSS at baseline, median (IQR)	1.0 (0 to 1.5)	1.0 (0 to 1.5)	1.0 (0 to 1.25)	0.445
OCB in CSF, % positive	94%	95%	93%	1.000
Follow-up duration, years, median (IQR)	5.1 (2.9 to 7.15)	4.7 (2.9 to 6.7)	5.75 (2.9 to 8.25)	0.446

SD — standard deviation; IQR — interquartile range

IFNβ, the cells synthesise luciferase which generates bioluminescence. In the presence of IFN-neutralising antibodies, the intensity of luminescence decreases.

We used standard recombinant human IFNβ protein (Abcam) and standard positive and negative control sera (Euro-Diagnostica).

To inactivate native IFNβ, serum samples were incubated at 56°C for 30 minutes. The samples were then diluted with the diluent provided (Diluent D): 100 µL for a 60 µL sample. Pre-diluted samples and control sera were added to wells on a microplate, which were then closed with a lid, mixed by swirling, and left for 30 minutes at 37°C with 5% CO_2.

iLite Type I IFN Assay Ready Cells were thawed in a 15-minute 37°C water bath, then diluted according to the manufacturer's instructions and 50 µL of the cell suspension was added to each well. The microplate was mixed and incubated at 37°C with 5% CO_2 for 18 hours. Thirty minutes before the incubation completion, Bright-Glo™ Luciferase Assay System was thawed. The substrate was prepared as instructed by the manufacturer and added to each well in 50 µL portions. The microplate was mixed and incubated for two minutes in darkness at room temperature, then placed in a luminometer (FLx800, Bio-TEK). Luminescence intensity was recorded.

A calibration curve was prepared for the correlation between light intensity in relative light units (RLU) and standard concentration. A sample was considered NAb-positive if the ratio of luminescence intensity of the sample and standard mean was ≤ 1.0. If the ratio was >1.0 the sample was labelled negative.

A patient was NAb positive, NAb (+), if NAb were detected at any time during follow-up, and NAb negative, NAb (-), if all samples, including at least one collected at ≥ 24 months, were negative. If a NAb (+) patient did not test negative later, the patient was considered to be persistently NAb (+). Otherwise, he or she would be labelled transient NAb (+).

Statistical methods

Variables are presented as either mean ± standard deviation (SD) for normally distributed variables, or median with interquartile range (IQR) for variables without normal distribution.

Baseline characteristics were assessed for mutual correlations and compared in subgroups depending on BAb and NAb status.

Associations were considered between antibody status and the results of the follow-up (NEDA, relapse occurrence, disability progression, MRI activity) in each subsequent year, for each period up to a given year and after a given year, and for the entire follow-up. They were compared between the subgroups: BAb (+) and (-), persistent BAb (+) and (-), NAb (+) and (-), persistent NAb (+) and (-).

We used Fisher's exact test for nominal variables and Mann-Whitney *U* test for ordinal variables. For interval variables, normality of distribution and equality of variances was assessed with d'Agostino-Pearson and Levene's tests. For normally distributed variables, comparisons were made with the use of t-test. For non-normal data distribution, Mann-Whitney *U* test was employed instead.

P values of ≤ 0.05 were considered statistically significant.

Statistical analyses were performed using StatSoft STATISTICA version 13 [22] and MedCalc, version 15.8 [23].

Results

Baseline

A total of 101 RRMS patients, 77 females and 24 males, were recruited for the study. In Table 2, the baseline characteristics of the study cohort are presented. Women were older than men. There were no other significant gender-specific differences.

Of 101 patients included in the study, nine had compensated thyroid disease, including six with subclinical hypothyroidism, one with Hashimoto disease, one with non-toxic goitre treated with levothyroxine, and one on levothyroxine following thyroidectomy due to toxic goitre. Two subjects had asthma, one had a history of uveitis, and another one of hepatitis of unknown aetiology (both without treatment at the time of our study). Four patients had a history of anxiety and/or mood disorders, including one who was treated with mirtazapine. In two patients, arterial hypertension was present.

Table 3. Proportion of patients with positive and negative anti-IFNβ antibody status

	All	Female	Male
BAb: All tested	97	74	23
BAb (+) at any time	95 (98%)	72 (97%)	23 (100%)
transient BAb (+)	7 (7%)	5 (7%)	2 (9%)
persistent BAb (+)	88 (91%)	67 (91%)	21 (91%)
BAb (-)	2 (2%)	2 (3%)	0 (0%)
baseline BAb (+)	66 of 72 (92%)	47 of 53 (89%)	19 of 19 (100%)
NAb: All tested	77	58	19
NAb (+) at any time	68 (87%)	50 (84%)	18 (95%)
transient NAb (+)	1 (1%)	1 (2%)	0 (0%)
persistent NAb (+)	67 (86%)	49 (84%)	18 (95%)
NAb (-)	9 (12%)	8 (14%)	1 (5%)
baseline NAb (+)	9 of 72 (12.5%)	8 of 53 (15%)	1 of 19 (5%)

BAb — binding antibodies; NAb — neutralising antibodies
No difference in baseline characteristics was found between BAb (+) and BAb (-) or between NAb (+) and NAb (-) cases

Antibody prevalence

200 samples were included in the final analysis. This allowed us to determine BAb status in 97 and NAb status in 77 patients.

Most patients developed both binding and neutralising antibodies. Overall, 95 (97%) had at least one BAb (+) sample. Of these, seven reverted to BAb (-), making 91% persistently BAb (+). Three cases (2%) remained negative throughout the entire study. For NAb, 68 (88%) tested positive, and among them, one reverted to NAb (-), leaving 87% persistently NAb (+). Nine patients (12%) were NAb (-).

The overwhelming majority of patients tested positive for BAb at baseline (66 out of 72 sampled = 92%). The patients who developed BAb later tested positive between one and 24 months. Notably, many patients with baseline BAb had transient reversal to BAb (-) in the first two years, and became BAb (+) again at 24 months.

The prevalence of NAb positivity at baseline was lower: nine out of 72 (12.5%) tested positive. An additional three patients converted to NAb (+) within the first two years, the rest tested positive at 24 months. Of note, few samples were collected at baseline and 24 months.

The summary of antibody prevalence is shown in Tables 3 and 4.

Follow-up

The patients were followed up for a median of 60 months (range 4 to 110).

Between 70% and 80% of patients maintained NEDA criteria of no disease activity in each year from year 1 to year 6, 60% in year 7, 70% in year 8, and 60% in year 9 (Fig. 1). The cumulative count of patients who maintained NEDA-3 is shown in Figure 2.

Discontinuation statistics

A total of 66 patients stopped treatment during the study. Twenty-three patients (35%) had their treatment revoked after three years because the Polish MS treatment programme was limited to three years until 2012. Seven patients (11%) became pregnant or were considering doing so. Another seven patients resigned for personal reasons. Adverse effects were reasons for discontinuation in 14 patients (21%). In 15 cases (23%), the drug was switched due to lack of efficacy.

No associations between discontinuation for any reason and antibodies or baseline characteristics were found.

Correlations with baseline clinical characteristics

Patients with NEDA for the first seven years had fewer relapses prior to treatment ($p = 0.038$). Likewise, fewer pre-treatment relapses were reported in patients with no disease progression after the first and second years ($p = 0.011$ and 0.014), within the first six years ($p = 0.023$), and at any time during the observation ($p = 0.011$). Patients with progression after the second year were older at the time of their first relapse ($p = 0.025$). Most of the significant differences were in baseline EDSS score, which was higher in cases with progression within the first two, three, seven and eight years (p ranging from 0.017 to 0.041), after the first and the second year ($p = 0.017$ and 0.020), and at any time during the follow-up ($p = 0.017$).

Patients with MRI activity in the first seven years apparently had fewer relapses prior to treatment ($p = 0.041$).

Correlations with BAb and NAb status

For BAb, a significant correlation with NEDA was found: patients with persistent BAb were more likely to experience disease activity after year 4 [$p = 0.014$, RR 7.333 (95% CI 0.511 to 105.267), p for RR 0.143 and year 5 ($p = 0.049$, RR 6.75 (95% CI 0.468 to 97.264), p for RR 0.161]. There were no other associations between BAb status and NEDA, relapses, progression, MRI activity, number of relapses, or EDSS score at any time period.

Table 4. Anti–IFNβ antibody prevalence: by samples and cumulative (with assumption of no change from last known result for missing data)

				BAb			
Samples	**Baseline**	**1 month**	**3 months**	**6 months**	**12 months**	**24 months**	**36 months**
All	72	30	3	14	4	49	28
Positive	66	14	0	4	1	45	28
Negative	6	16	3	10	3	3	0
% positive	91.7%	46.7%	0.0%	28.6%	25.0%	91.8%	100.0%
% negative	8.3%	53.3%	100.0%	71.4%	75.0%	6.1%	0.0%
				NAb			
Samples	**Baseline**	**1 month**	**3 months**	**6 months**	**12 months**	**24 months**	**36 months**
All	72	30	3	14	4	49	28
Positive	9	3	0	3	0	45	22
Negative	63	27	3	11	4	3	6
% positive	12.5%	10.0%	0.0%	21.4%	0.0%	91.8%	78.6%
% negative	87.5%	90.0%	100.0%	78.6%	100.0%	6.1%	21.4%
Cases				**BAb**			
Cumulative	**Baseline**	**1 month**	**3 months**	**6 months**	**12 months**	**24 months**	**36 months**
Positive	66	67	65	65	64	73	87
Negative	31	30	32	32	33	24	10
% positive	68.0%	69.1%	67.0%	67.0%	66.0%	75.3%	89.7%
% negative	32.0%	30.9%	33.0%	33.0%	34.0%	24.7%	10.3%
Cases				**NAb**			
Cumulative	**Baseline**	**1 month**	**3 months**	**6 months**	**12 months**	**24 months**	**36 months**
Positive	9	11	11	13	12	55	67
Negative	68	66	66	64	65	22	10
% positive	11.7%	14.3%	14.3%	16.9%	15.6%	71.4%	87.0%
% negative	88.3%	85.7%	85.7%	83.1%	84.4%	28.6%	13.0%

BAb — binding antibodies; NAb — neutralising antibodies

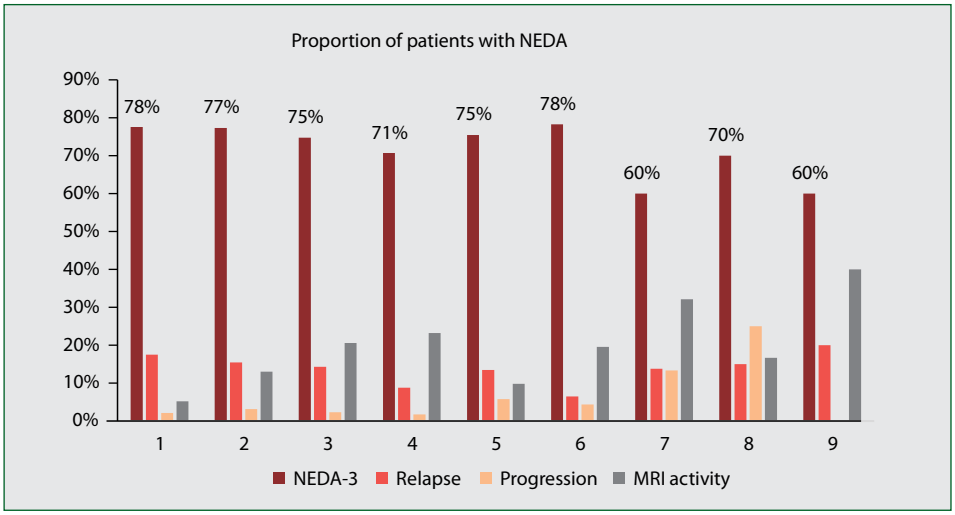

Figure 1. Proportion of patients with no disease activity (according to NEDA-3 criteria) and patients with relapses, progression and MRI activity in subsequent years

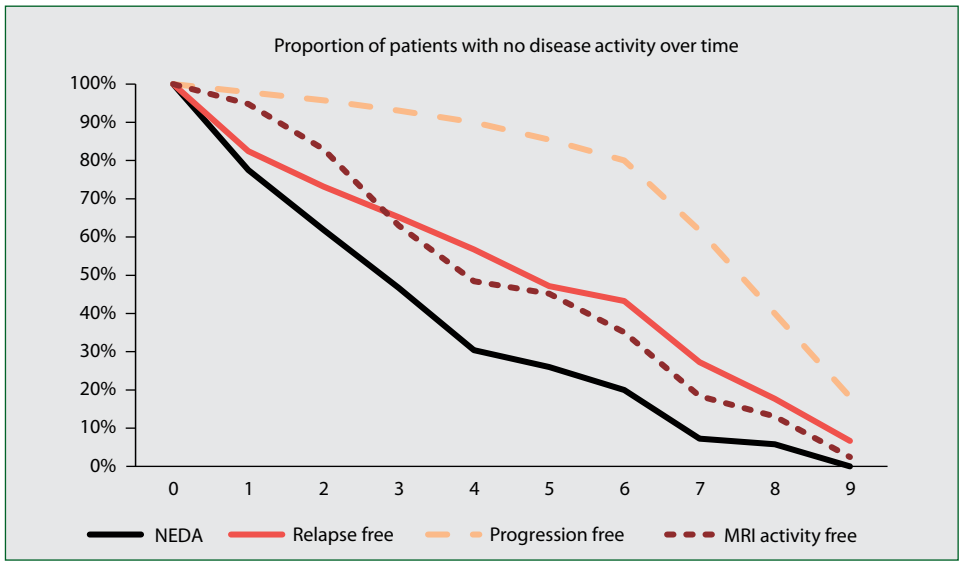

Figure 2. Proportion of patients with no disease activity (according to NEDA-3 criteria), relapses, progression and MRI activity over time

No association was found between NAb and any form of disease activity.

Excluding patients with baseline positivity and with unknown baseline status, attempting to distinguish between the antibodies present at baseline and those developed after drug exposure was impossible due to the small sample size [i.e. no BAb (-) cases were left, three baseline NAb (-) patients]. Therefore, only patients with known baseline positivity were excluded. A trend for higher disease activity was found for NAb (+) in the first year ($p = 0.098$), and for the first two years ($p = 0.069$). In addition, a trend for higher disease activity in BAb (+) cases after years 4 and 5 (both with $p = 0.061$) re-emerged.

After moving the baseline positive patients to antibody negative groups, NAb (+) was associated with a higher risk of disease activity in the first two [$p = 0.019$, RR 3.724 (95% CI 0.990 to 14.017), p for RR 0.052] and three years [$p = 0.041$, RR 2.210 (95% CI 0.928 to 5,260), p for RR 0.073], as well as in the entire follow up [$p = 0.044$, RR 1.575 (95% CI 0.930 to 2.668), p for RR 0.091]. It also correlated with MRI activity in the first three years [$p = 0.043$, RR 6.938 (95% CI 0.458 to 105.035), p for RR 0.162].

In logistic regression analysis, this modified NAb (+) status was a significant independent variable in enter method and was retained as the only significant variable in forward method in models for disease activity within the first two years of treatment.

When considering baseline BAb (+) as BAb (-), BAb (+) was associated with a higher risk of treatment discontinuation, of NEDA-3 loss after the first and the second year, a lower risk of relapse in year 6 although higher in year 4, a higher risk of disease progression in year 6, but again a lower risk after the first and the second year and in the follow-up as a whole. BAb (+) also showed less MRI activity after the first year and in the entire observation.

Comparing patients with and without baseline BAb and NAb [BAb0 (+) and BAb0 (-), NAb0 (+) and NAb0 (-)], regardless of subsequent antibody status, NAb0 (+) was associated with a lower risk of disease activity in the first three years ($p = 0.017$, RR = 0.2239, 95% CI: 0.03611 to 1.3890, p for RR = 0.1080). For baseline BAb, no significant associations were found in direct comparisons, but they remained the only significant variable in a forward model for relapse activity in the first year of treatment, where the absence of baseline BAb increased the likelihood of relapses (Tab. 5).

Logistic regression

For each time period and activity measure, logistic regression models were computed with combinations of BAb and NAb, persistent BAb and Nab, and baseline clinical characteristics.

Neither BAb nor NAb status, persistent or not, was retained as a significant independent contributor. Conversely, pre-treatment EDSS and relapses were abundantly included in models predicting progression, while several relapse and MRI activity models contained age, treatment delay, or time to second relapse. Only two models reached significant goodness of fit, one including NAb, the other persistent NAb (outcome: progression after third year, both with $p = 0.015$ and Hosmer-Lemeshow $p < 0.001$) (Tab. 6). For these variable sets, in forward, backward and stepwise method, only pre-treatment EDSS and relapses were retained.

Discussion

The abundance of NAb and BAb positivity before treatment is the most surprising finding of our study. Previous works have reported naturally occurring BAb and NAb in less than 1% of the healthy population and treatment-naive MS patients [1].

Table 5. Logistic regression analysis: relapses in the first year of treatment

Variable	Coefficient	Standard error	Wald's χ^2	p
Baseline Bab (—)	1.974	0.977	4.084	0.043
Constant	-1.569			

Table 6. Logistic regression analysis: progression after third year of treatment

Variable	Coefficient	Standard error	Wald's χ^2	p
NAb (+) / persistent NAb (+)*	17.226	2,881.824	< 0.001	0.995
Age at first relapse, years	-0.001	0.056	< 0.001	0.983
Gender	-0.743	1.182	0.395	0.530
Time to second relapse, months	0.041	0.063	0.424	0.515
Time to treatment start, months	-0.063	0.053	1.437	0.230
Baseline EDSS score	**2.481**	**1.146**	**4.691**	**0.030**
Number of relapses prior to treatment	**1.490**	**0.716**	**4.330**	**0.037**
Constant	-23.075			

*the models were otherwise identical

On the other hand, NAb developed in 11% of patients in the placebo group of a stage III clinical trial of IFNβ-1b [2]. It is understandable that positivity prevalence depends to a great extent on the assay's sensitivity and cut-off thresholds [1, 24]. Therefore these pre-treatment antibodies probably represent naturally occurring, nonspecific antibodies of very low affinity and variable IFN neutralising activity. Alternatively, this could potentially be caused by a methodological issue (i.e. sample contamination) or, less likely, a cross-reactivity with an antigen commonly encountered in the Polish population.

It is plausible that a different kind of anti-interferon antibodies develops in response to drug exposure and undergoes gradual maturation, eventually gaining IFN neutralising properties.

Removal of the baseline NAb-positive cases from the analysis brought a trend towards more disease activity in NAb (+) and, curiously, BAb (+) patients, supporting the hypothesis that the baseline and aquired antibodies represent two different phenomenons. An assignment switch involving moving baseline positive patients to the antibody negative groups revealed an association between early disease activity and NAb positivity, not unlike the previous evidence [2, 6–11]. An analogous grouping shift changed BAb correlations greatly, with contradictory associations with disease activity.

These inconsistent results suggest that the co-occurrence of unspecific and specific binding antibodies is probably common, and renders this analysis invalid.

Interestingly, pre-treatment antibodies predicted a more indolent course in the first three years of treatment. This resembles the observations from pivotal trials of IFNβ in MS [2, 25, 26], where patients who were to develop NAb in later observations had lower relapse rates in the first six or 12 months of treatment. This was thought to be caused by low-affinity anti-IFN antibodies that could initially prolong the drug's half-life, but would eventually develop into harmful, detectable NAb due to affinity maturation [24].

We are aware of our study's limitations. Due to the strict eligibility criteria for the Polish MS treatment programme, patients with low pre-treatment disease activity were preferentially included. This may explain why the study cohort experienced a relatively benign disease course. However, disease activity statistics were similar in other recently described populations [27]. Thirty-five percent of cases were lost after the third year of treatment due to the programme duration limit, regardless of response, while many continued treatment despite sustained disease activity (20–30% non-NEDA in each year), as second-line therapeutics were either not yet registered or out of reach due to programme criteria.

Because of poor patient co-operation and technical limitations, a significant number of scheduled samples were not collected.

Conclusions and future directions

In our RRMS cohort, we observed a high prevalence of naturally occurring anti-IFNβ BAb and NAb.

Exclusion of the NAb0 (+) cases brought results reminiscent of previous ones, with a trend towards more disease activity in NAb (+) and, notably, BAb (+) patients. An assignment switch (moving NAb0 (+) and BAb0 (+) patients to the NAb (-) and BAb (-) groups) revealed an association between

early disease activity and NAb, consistent with previously published research.

Patients with baseline positivity in BAb or NAb experienced less disease activity in the first one or three years of treatment.

We propose that two types of anti-interferon antibodies were detected by our assays: specific, drug-induced antibodies and also low-affinity, naturally occurring antibodies. The naturally occurring antibodies are beneficial or reflect an advantageous immune state. Drug-induced antibodies, once they reach a certain neutralising activity, inhibit IFNβ activity and cause loss of drug efficacy.

Our findings require confirmation in further studies. The prevalence and specificity of low-affinity antibodies should be determined. Also, a titre or anti-IFN activity threshold of predictive significance should be established if possible.

Funding: *This publication was prepared without any external source of funding.*

Conflicts of interest: *The authors declare no conflict of interest related to this work. KO, AK and WK declare that they have no conflict of interest. AP has received travel compensation from Zentiva. AKL received personal compensation for speaking services and/or travel cost compensation and/or research grants from Biogen, Bayer, Merck Serono, Novartis, Teva Pharmaceuticals, CSL Behring, Shire, Sanofi-Genzyme and Roche. SM has received a travel grant from Bayer. These were unrelated to the conduct of this study or the preparation of this manuscript.*

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