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Detection and kinetics of persistent neutralizing anti-interferon-beta antibodies in patients with multiple sclerosis. Results from the ABIRISK prospective cohort study

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ABSTRACT

Two validated assays, a bridging ELISA and a luciferase-based bioassay, were compared for detection of anti-drug antibodies (ADA) against interferon-beta (IFN-β) in patients with multiple sclerosis. Serum samples were tested from patients enrolled in a prospective study of 18 months. In contrast to the ELISA, when IFN-β-specific rabbit polyclonal and human monoclonal antibodies were tested, the bioassay was the more sensitive to detect IFN-β ADA in patients' sera. For clinical samples, selection of method of ELISA should be evaluated prior to the use of a multi-tiered approach. A titer threshold value is reported that may be used as a predictor for persistently positive neutralizing ADA.

1. Introduction

The introduction of biopharmaceuticals (BP) has been an important step forward in care for multiple sclerosis (MS) patients. In spite of the progress in developing disease-modifying therapy, a suboptimal or even loss of response to BP are frequent. While some therapeutics may not work in a specific individual due to disease heterogeneity, loss of response may arise in an additional fraction of patients due to the development of anti-drug antibodies (ADA). ADA can modify pharmacokinetic and pharmacodynamic properties of the BP and thus decrease efficacy by neutralizing the BP (Sorensen et al., 2003; Sorensen et al., 2006; Polman et al., 2010; Bertolotto et al., 2014). ADA formation may also associate with BP-specific hypersensitivity reactions (Giovannoni et al., 2007). Furthermore, by cross-reacting with the endogenous homologue of the drug, ADA may impair important physiological functions even after therapy cessation (Petersen et al., 2006; Sominanda et al., 2010). Consequently, ADA need to be taken into account not only when estimating the benefit-burden ratio of a therapy for an individual patient, but also when calculating the value of therapeutics on a socio-economic level (Link et al., 2017).

The Innovative Medicines Initiative (IMI; www.imi-europe.org) ABIRISK consortium [Anti-Biopharmaceutical Immunization: Prediction and analysis of clinical relevance to minimize the risk] was formed by clinicians, academic scientists and EFPIA (European Federation of Pharmaceutical Industries and Associations) members (www.abirisk.eu). One of its tasks was to scrutinize currently used ADA methods and, if needed, to re-develop and re-validate assays for detection of ADA following updated guidelines adopted in the pharmaceutical industry, and to test these assays in a prospective study in a clinical setting.

Validations of assays for the analysis of ADA (previously subdivided in the terms binding antibodies (BAb) and neutralizing antibodies (NAb)) towards the drug interferon-beta (IFN-β) (covering therapy with the products of IFN-β: IFN-β1a intramuscularly (IM), Biogen, USA; IFN-β1a subcutaneously (SC), Merck, Germany; IFN-β1b, Bayer AG, Germany, and Novartis, Switzerland) have already been published (Hermanrud et al., 2016; Ingenhoven et al., 2017; Rup et al., 2015). In the ADA assay, a two-tiered approach with a bridging enzyme-linked immunosorbent assay (ELISA) was used. Samples that tested positive in the first assay step (tier 1) were termed ADA reactive (Rup et al., 2015). Such samples were then assessed in a confirmatory assay (tier 2), where samples were tested with and without addition of excess IFN-β. Specific binding of ADA inhibited the reaction with the capture reagent, thus confirming specificity (Ingenhoven et al., 2017). Samples that scored positive in the screen and the confirmatory assay were reported as ADA positive, while samples that scored negative in either the screening or the confirmatory assay were reported as negative (Rup et al., 2015). To compare the results of the two assays, all samples, including those that were reported as ADA negative, were screened for NAb positivity and titers in a cell-based assay (tier 3) (Hermanrud et al., 2016). If ADA in patient sera can neutralize IFN-β by blocking the stimulation of the IFN-β receptors in the cell-based assay the sample is considered positive for NAb (Hermanrud et al., 2016).

The ADA ELISA and NAb assays were established and validated using the same polyclonal positive control (rabbit-anti-human IFN-β), allowing cross-assay comparison, and determination of assay sensitivity. Historically, most routine testing of ADA against IFN-β has deviated from the classical three-tiered approach as outlined in the ABIRISK terms and definition publication (Rup et al., 2015), since the ADA assays for anti-IFN-β were considered unreliable for screening by experience from several investigators, due to low sensitivity and specificity. Thus, in this study we compared the sensitivity and specificity of the newly developed and validated ADA bridging ELISA and the validated NAb assay, with regards to the detection of anti-IFN-β purified human monoclonal antibodies (mAbs) and ADA in clinical samples from a prospective cohort, in order to assess if the three-tiered approach should also be applied to anti-IFN-β ADA testing.

2. Material and methods

2.1. Patients and blood samples

Patients were recruited to the ABIRISK-MS-P01 study (EudraCT Number: 2012-005450-30), a multicenter, non-interventional, prospective observational cohort study of IFN-β treatment with subsequent evaluations of the patient's ADA formation against the therapy. The study enrolled patients at ten centers (Karolinska Institutet, Stockholm, Sweden; Innsbruck Medical University, Innsbruck, Austria; Department of Neurology, Medical University of Graz, Graz, Austria; Department of Neurology, Kepler Universitätsklinikum GmbH, Linz, Austria; Department of Neurology, Technische Universität München, Munich, Germany; University of Düsseldorf, Medical Faculty, Department of Neurology, Düsseldorf, Germany; Hospital Universitari Vall d'Hebron, Centre d'Esclerosi Múltiple de Catalunya, Barcelona, Spain; Department of Neurology, Hospital Regional Universitario de Málaga, Spain; University Hospital Basel, Basel, Switzerland; MS Center, Department of Neurology and Center for Clinical Neuroscience, Charles University in Prague, 1st Faculty of Medicine and General University Hospital, Prague, Czech Republic) in six European countries, for whom IFN-β had been prescribed in the usual manner and in accordance with the terms of the marketing authorization. Patients eligible to enter the study were those treated with non-pegylated IFN-β products (IFN-β1a IM, IFN-β1a SC, and IFN-β1b), 18 years of age and older, and diagnosed with MS according to McDonald criteria (Polman et al., 2011) or clinical isolated syndrome (CIS) at the time of diagnosis. Patients who previously had been treated with any IFN-β preparation were excluded. Blood sample

collection was performed during follow-up visits as part of the clinical routine at the responsibility of the treating physician and following national guidelines. Where possible, serum samples were collected prior to therapy initiation and post therapy start on the same day after the first injection of IFN-β, and then at month 1, 2, 3, 6, 9, 12 and 18 of therapy. Blood samples were drawn between 4 and 14 h after last injection of IFN-β and were stored at −80 °C until use. Prior to the patient's participation in the study, written informed consent was obtained from all subjects.

2.2. Assays to detect ADA towards IFN-β in clinical samples

The two assays developed as part of the ABIRISK project were applied to the samples of the patients obtained from the prospective study.

In short, the ADA bridging ELISA was developed as a two-tiered assay with immobilized IFN-β1a IM and use of biotinylated IFN-β1a. After incubation with patient serum, bound ADA was detected using horseradish peroxidase (HRP)-labelled streptavidin and 3,3′,5,5′-tetramethylbenzidine substrate. To identify ADA reactive samples a floating-cut point was used for screening (tier 1) (Ingenhoven et al., 2017). The confirmation assay of the ADA ELISA was performed in the presence of excess IFN-β1a IM, and the percentage of inhibition of ADA binding was calculated (tier 2). All details of analysis, assay set-up, reagents, and validation parameters have recently been described (Ingenhoven et al., 2017), and performed at the Department of Neurology, University Hospital in Düsseldorf, Germany.

The NAb assay was developed as a cell-based assay using the luciferase bioassay, in which the binding of IFN-β to the cell surface IFN-β receptors (in competition with binding of ADA in the serum) generate production of luciferase enzyme that was quantified by use of the Steady-GLO Luciferase Assay System (Lam et al., 2008). Samples screened NAb positive were titrated and samples that did not pass the threshold value for titer in the titration step were considered as negative for NAb. All details of re-development of the assay, analysis, assay set-up, reagents, and validation parameters have been published (Hermanrud et al., 2016), and all analyses were performed at the Danish Multiple Sclerosis Center, Department of Neurology, Rigshospitalet, University of Copenhagen, Denmark. The previously described confirmation step of NAb, using antibody binding columns (Hermanrud et al., 2016), were omitted in this study, since all samples were also run on the ELISA and all NAb positive samples were confirmed by NAb titration.

Persistently ADA positive patients were defined as having at least two consecutive positive samples and not followed by any negative samples, and transiently ADA positive as patients with at least one positive sample followed by at least one negative sample. Patients with one ADA positive sample only at the end of the study (at 18 months) were grouped as undetermined regarding positivity, as they did not fulfil the requirements for the persistently or transiently positive groups.

2.3. Generation of human mAbs towards IFN-β

Five high affinity human mAbs were generated towards IFN-β1a SC in the Immune Regulation Laboratory of the Institute for Research in Biomedicine, Bellinzona, Switzerland. A sample of a peripheral blood was obtained from a relapsing-remitting MS-patient who developed ADA after receiving therapy with IFN-β1a SC at the Clinical Department of Neurology, Medical University of Innsbruck, Austria. Memory B cells were isolated from cryopreserved PBMCs using anti-FITC microbeads (Miltenyi Biotec) following staining of PBMCs with CD22-FITC (BD Phamingen), and were immortalized with Epstein-Barr virus and *CpG* in multiple wells as described previously (Traggiai et al., 2004). Culture supernatants were tested for binding to IFN-β1a SC by indirect ELISA.

All mAbs were produced by recombinant technology as IgG1 molecules by transient transfection of Expi293 cells (Invitrogen) using polyethylenimine (PEI). mAbs were purified by protein A or protein G chromatography (GE Healthcare) and concentrated by Amicon Ultra filter units (100 K, Millipore). Total IgG was quantified by Pierce BCA protein assay (Thermo Fisher Scientific).

An indirect ELISA protocol, which was not validated to clinical diagnostics, was used to confirm the binding of the newly generated human mAbs towards IFN-β using 384-well SpectraPlates (PerkinElmer) for primary screenings or 96-well MaxiSorp plates (Nunc) for any following test. Briefly, ELISA plates were separately coated with 1 µg/mL of IFN-β1a SC, IFN-β1a IM, and IFN-β1b, blocked with 1% BSA and incubated with titrated samples of mAbs, followed by alkaline phosphatase-conjugated anti-human IgG-Fc gamma specific secondary antibodies (Jackson ImmunoResearch). Plates were then washed, substrate (p-NPP, Sigma-Aldrich) was added and plates were read at 405 nm. EC50 (ng/mL) was calculated for every sample by nonlinear regression analysis using GraphPad Prism 5 software. IFN-β1a IM was used for coating instead of IFN-β1a SC for the comparison of the results of the indirect ELISA assay with those of the bridging ELISA assay.

The rabbit-anti-human IFN-β polyclonal antibodies (pAbs) (Anti-Human IFN-β/500-P32B, PeproTech, USA), which also served as positive control during assay validation of the assays to detect ADA in clinical samples, was analyzed in parallel for comparison to the mAbs.

2.4. Application of the ADA ELISA and the NAb test for re-assessment of the sensitivity using the human mAbs towards IFN-β

Analogous to the sensitivity assessments during validation (Hermanrud et al., 2016; Ingenhoven et al., 2017), mAbs sensitivity experiments were performed by determining the limit of detection (LOD) in both the bridging ADA ELISA and the NAb assay. The LOD was assessed by generation of calibration curves (serial dilutions of the mAbs and pAbs added in pooled human serum) in twelve assay runs by two operators. The concentration at the LOD was interpolated using a 4-parameter logistic fitting model, applying the equation (aiming for a 1% negative rate): mean concentration at cut-point + 2.718*[standard deviation (SD)].

2.5. Statistical analysis and software

Microsoft Excel software (version 2010) was used for calculations of means, medians, %CV and titers for the assays. Further was GraphPad Prism version 6 (GraphPad software Inc., USA) used for curve fitting. The software SPSS statistics 22, IBM, USA, was used to generate Receiver Operating Characteristics (ROC) curves.

For calculations of sensitivity using 4-parameter logistic dose response curves of NAb, Dose-Response Analysis Using *R* was applied: a language and environment for statistical computing (Ritz et al., 2015). *R* Foundation for Statistical Computing, Vienna, Austria (https://www.R-project.org/)). Package *drc* was done according to protocol (Ritz et al., 2015). SigmaPlot Systat Software Inc., USA, was used for the 4-parameter logistic dose response curves for statistics of the ADA ELISA assay.

2.6. Ethics approval

The study was approved by local ethical committees at the clinical sites involved.

For Austria: Ethical committee approval number: AN2013-0040 331/2.1; for Basel, Switzerland: Ethical committee approval number: 305/13; for Barcelona, Spain: Clinical Research Ethics Committee, University Hospital Vall d'Hebron, ethical committee approval number EPA(AG)66/2013(3866); for Dusseldorf, Germany: Local ethics committee, protocol 4451; for Malaga, Spain: Institutional ethical committee CEI Málaga Nordeste, ethical committee approval number 09/2014 6 EPA; for Munich, Germany: Ethical committee approval

number: 335/13; for Prague, Czech Republic: Medical Ethics Committee of the General University Hospital in Prague is 125/12, Evropský grant 1. LF UK-CAGEKID; for Sweden: the Sweden Medical Product Agency Dnr. 5.1-2013-108370 2014-01-27, protocol ABI-MS-P01, Eu-nr 2012-005450-30 and the Stockholm regional ethical committee Dnr. 2013/1034-31/3 and Dnr. 2015/749-32.

3. Results

3.1. Patients included

A total of 151 patients were recruited in Austria (n = 29), Czech Republic (n = 50), Germany (n = 29), Spain (n = 23), Sweden (n = 10), and Switzerland (n = 10). Three of the 151 included patients dropped out prior to therapy start, resulting in a total of 148 patients with 1192 samples (88% of a maximum of 1359 possible samples) available for analysis. Of these, 98 patients finished the study with a complete series of nine samples per patient. The MS-patients enrolled were treated with IFN-β formulations as follows (with completed series and percentage of total patients completed in brackets): IFN-β1a IM 38 patients (24 completed; 24%), IFN-β1a SC 68 patients (47 completed; 48%), (of these patients 14 patients were on 22 μg and 54 patients were on 44 μg, of whom, respectively 8 and 39 patients completed the study; 8% and 40%), IFN-β1b 40 patients (27 completed; 28%). Two patients dropped out after start and were not available.

3.2. ADA ELISA and cell-based NAb analyses

The analyses of the samples by the two methods were performed for ADA and NAb and the collection of the results is shown in Fig. 1, in which the three-tiered approach is included, based on screen (tier 1) and confirmation by ELISA (tier 2) and NAb titer determination (tier 3) by the bioassay.

Table 1 gives the numbers of negative and positive samples respectively for ADA and NAb. For the confirmatory ADA assay only 105 of the 1192 samples were ADA positive (9%) and for the NAb analyses 181 of the 1192 samples were NAb positive with titers (15%). Concordant results between ADA and NAb were found for 93% of the samples, and the discrepant results were mostly due to NAb positive samples being negative in the ADA ELISA.

3.3. Sample-based comparison of the ADA ELISA and cell-based NAb assay

The number of samples that were confirmed positive in the two assays, by the three-tiered approach, was 103 of all the 1192 samples (9%) (Table 1). Overall, the NAb assay was more sensitive for detecting positivity in the clinical samples with 6% more NAb positive samples than ADA positive. Accordingly, 98% of the ADA positive samples were NAb positive and conversely, of the NAb positive samples 57% were confirmed ADA positive.

Table 1
Numbers of samples from each assay as negative and positive for ADA and NAb. In brackets are the percentages of negative and positive samples for ADA and NAb, respectively, of the total sum of ADA and NAb.

	NAb negative	NAb positive	Sum ADA
ADA negative	1009	78	1087 (91%)
ADA positive	2	103	105 (9%)
Sum NAb	1011 (85%)	181 (15%)	1192

ADA-measurements of the complete series of the 98 MS-patients resulted in 26 confirmed ADA positive patients (27%). The number of NAb positive patients with at least one positive sample was 42 of the 98 patients (43%) and included the 26 confirmed ADA positive patients.

3.4. Kinetics of ADA and NAb development

No confirmed positive samples were detected at treatment initiation (pre- and post-start samples) or one month after treatment initiation by any of the assays, and all ADA positive patients were therefore having a treatment-induced ADA response (Rup et al., 2015). Starting from month 2 the NAb assay detected positivity earlier than the ADA ELISA that started from month 3 (Fig. 2).

Furthermore, the number of positive samples increased after month 2 measurements and leveled out after 12 months of therapy until the end of the study at 18 months. As expected from ADA affinity maturation processes for the antibodies, both the median and mean titers generally increased over time for the positive samples (Fig. 3).

3.5. NAb transiently and persistently positive patients

The distributions of persistently and transiently positive patients are seen in Table 2. Out of the 98 patients that completed the study, 25 (26%) were confirmed persistently positive in the three-tiered approach, and 1 (1%) only of the 98 patients was transiently NAb positive. When only the results from the more sensitive NAb assay were considered, 33 of the 98 patients (34%) were persistently positive, 5 (5%) transiently positive and 4 (4%) undetermined.

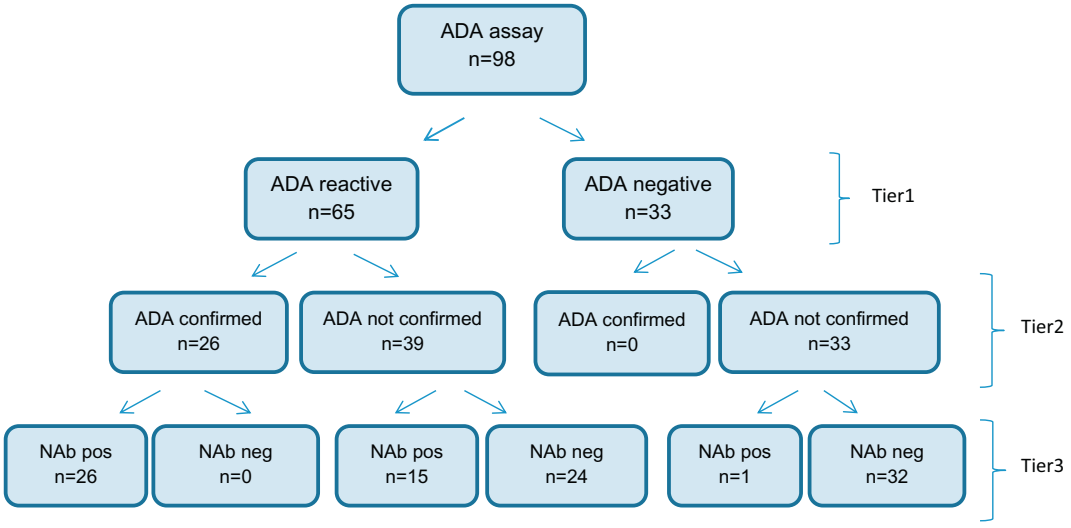


Fig. 1. Combined ADA and NAb anti-IFN-β assays structured as a three-tiered approach including analysis of all samples.

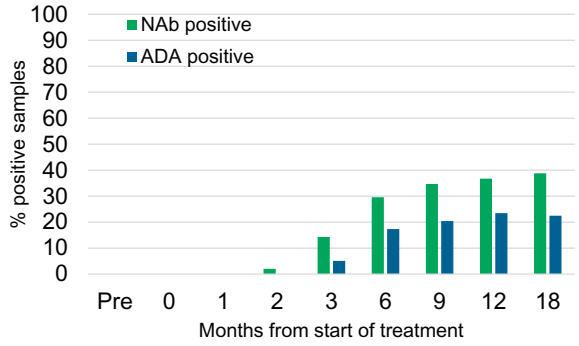
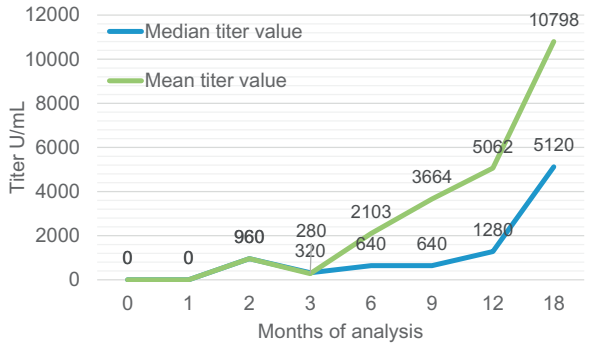


Fig. 2. The prevalence of ADA confirmed and NAb positive samples, respectively, for each month after treatment. All NAb positive samples were confirmed to be positive by titration.



Month	*0*	*1*	*2*	*3*	*6*	*9*	*12*	*18*
Mean SD	0	0	453	226	4605	5993	7357	9017
Median IQR	0	0	640	240	1120	3520	6720	18560

Fig. 3. Development of median and mean NAb titers for persistently NAb positive patients with time. For each of the months (0, 1, 2, 3, 6, 9, 12 and 18) the number of samples are: 0, 0, 2, 10, 23, 25, 25 and 25, respectively. The respective standard deviations (SD) and interquartile ranges (IQR) are shown in the table.

A ROC curve was generated for the results of the NAb titers predicting the persistently positive NAb patients (Fig. 4). With an AUC of 0.95, a threshold value of titer 320 U/mL gives a sensitivity of 94% (specificity 83%), and a threshold value of titer 640 U/mL a sensitivity of 85% (specificity 100%). At the threshold value of 320 U/mL the number of persistently NAb positive patients (n = 25, 26%) and confirmed ADA positive was the same, and transiently positive patients were eliminated. For the NAb titers alone the numbers of persistently and transiently positive patients were slightly reduced from 34% to 32% and 5% to 1%, respectively. The discrepancy between the ADA and NAb patient results was reduced from 16 to 8 of the 98 patients, suggesting a better correlation between the two assays when using a higher titer cut-off. By use of a threshold value of 320 U/mL, 82% of the persistently NAb positive patients were identified by NAb analyses at

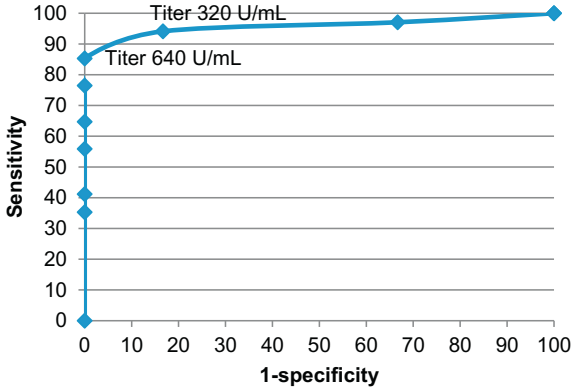


Fig. 4. A ROC curve of persistently positive NAb patients.

months 3, 6 and 9 after therapy started, and a further 15% of patients after 18 months.

3.6. Comparison of assays with mAbs and pAbs analyses

Due to the unexpected observed higher sensitivity of the NAb assay than for the ADA ELISA assay in the analysis of clinical samples, we re-determined the sensitivities of the assays from the validation processes using the rabbit pAbs as control in both assays and then five mAbs towards IFN-β1a SC. All the five mAbs showed high-affinity binding to IFN-β by indirect ELISA with EC50 values ranging from 10 to 50 ng/mL.

The five mAbs gave similar results regarding sensitivities in the bridging ADA ELISA with 0.4–3.6 ng/mL being the LOD. In the NAb assay experiment again the NAb assay was shown to be less sensitive than the ADA ELISA, with a LOD for the five mAbs of 41–747 ng/mL. This was confirmed for the rabbit pAbs as well with a LOD of 26 ng/mL for the ADA ELISA and 1218 ng/mL for the NAb assay, giving 50 times higher sensitivity of the ELISA over the NAb assay. The discrepancy when compared to the results obtained for patient sera was not due to a matrix effect, since the dilutions of mAbs in negative ADA MS serum still gave the same LOD (data not shown). Also, the result was not due to the binding of several mAbs at the same time to the IFN-β molecule that could otherwise give an avidity effect exceeding individual affinities of the mAbs, since mixtures of the mAbs gave the same results.

3.7. Comparison of indirect and bridging ADA ELISAs

Four selected patients (NAb negative, NAb transiently positive, NAb persistently positive (medium titer) and NAb persistently positive (high titer)) were tested for comparison in ELISA by OD-values between the indirect ELISA and the validated bridging ELISA. The results indicated that a significantly higher ADA detection was seen in the indirect ELISA than in the bridging ELISA and detected ADA earlier than both the bridging ELISA and NAb assay (Fig. 5).

4. Discussion

In this study the analysis of ADA and NAb in a prospective cohort of IFN-β treated MS-patients is presented where it was found that,

Table 2
Collection of positive and negative patients according to the assay. In brackets are the percentages of persistently and transiently positive patients for ADA and NAb, respectively, of the total sum of 98 patients. **pos* is undetermined type of positivity.

Patients (n =)	Persistent positive	Transient positive	**pos* positive	Total positive	Negative
ADA confirmed positive	25 (26%)	1 (1%)	0 (0%)	26	72
NAb titer	33 (34%)	5 (5%)	4 (4%)	42	56
All positive confirmed patients	25	1	0	26	72

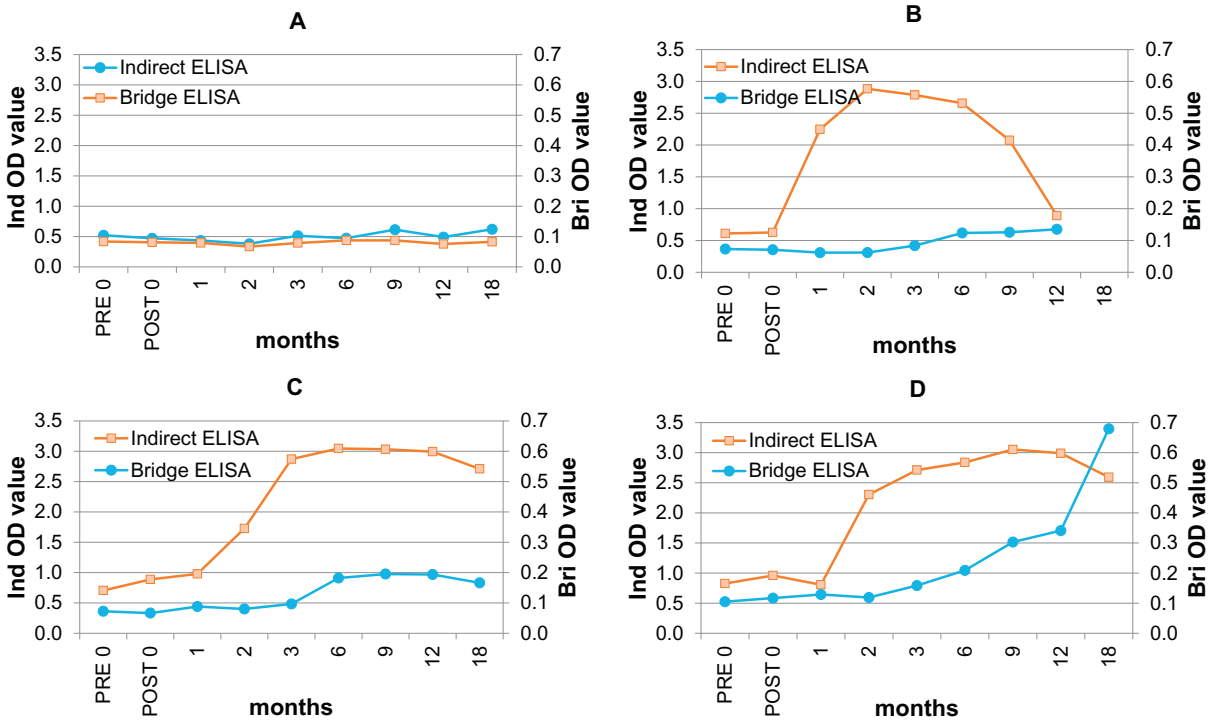


Fig. 5. Comparison of ELISA absorbance (OD) values between the validated bridging ELISA (Bri ELISA OD 405 nm) and the indirect ELISA (Ind ELISA OD 450 nm) of four selected samples from patients A. NAb negative, B. NAb transiently positive, C. NAb persistently positive (medium titer) and D. NAb persistently positive (high titer).

unexpectedly, the NAb assay gave a higher frequency of both positive samples and NAb positive patients than the bridging ELISA method. The standard recommendation for immunogenicity testing of biopharmaceuticals is a multi-tiered approach including a final cell-based bioassay for NAb measurements (European Medicines Agency, 2015; U.S. Department of Health and Human Services, Food and Drug Administration, 2016). The sensitivity of cell-based NAb assays is often found to be lower than the corresponding ELISA assay (Jensen et al., 2012). This can be explained by the fact that only a fraction of ADA is NAb, and that NAbs after maturity processes in general have higher affinities to the drug than ADA. Furthermore, distinguished difference exist in methods of measurements, by which cell-based NAb-mediated inhibition measurements are less sensitive than ADA binding assays (Chatterjee et al., 2018). The use of the multi-tiered testing for overall ADA and NAb ensures that the NAb detected are correlated to the presence of specific antibodies when evaluating the treatment of patients. Here we challenge the three-tiered approach starting with a screening of ADA (tier 1), followed by a confirmatory step (tier 2), and then the positive samples are further characterized for NAb, with a final determination of the titer of NAb for the positive samples (tier 3). This approach is only feasible if the ADA assay, as a screening assay, is more sensitive than the NAb assay. The use of ELISA to measure ADA as an easier method to detect NAb would be a break-through, since neither the direct or capture (sandwich) ELISA's tested have been able to strictly correlate with positive NAb results of bioassays, especially in the low NAb positive titers (Pachner, 2003; Gneiss et al., 2004; Jensen et al., 2012). Our results show that it is important to evaluate a pilot test with both ADA and NAb assays on the clinical patient samples, and not only rely on the positive control before deciding to use ELISA as a screen and confirmation of ADA at first, since some NAb positive samples may otherwise be lost.

The sensitivity of antibody assays is inherently related to the affinity and potency of the positive control antibody, and to the determined design of the assay cut-point (Chatterjee et al., 2018). Therefore, in this study the positive controls used in the assays were analyzed, and the rabbit pAbs were compared with newly developed mAbs of human origin, allowing validation of alternative assay formats. During the validation process, the positive control rabbit pAbs indicated that the bridging ELISA was several-fold more sensitive than the NAb assay (Hermanrud et al., 2016; Ingenhoven et al., 2017). These results contrasted the test results from the patient cohort, detecting ADA earlier and at higher frequency applying the NAb assay. When screening by the bridging ELISA was compared with the indirect ELISA, the indirect assay appeared to be more sensitive, suggesting that limitations in the bridging ADA ELISA technique (low signal-to-noise ratio, narrow dynamic range of the assay) may have contributed to this unexpected finding. Indeed, it is known that both FAb-arms of the antibody need to be bound to two different molecules to give a signal in the bridging ELISA while only one arm is needed for detection in the indirect ELISA. Also, ADA at low levels may not be able to bridge the drug since both FAb-arms of the ADA bind to the solid phase due to waste access of coated antigen, resulting in false-negative read-outs (Bendtzen and Svenson, 2011). These limitations in the bridging ADA ELISA could further indicate an issue about the confirmation step, by which sensitivity is lost in the ELISA, resulting in lost ADA positivity of a fraction of the transiently positive ADA. Using the two-tier ELISA protocol includes the requirements of positive results of both techniques for acceptance of samples as ADA positive. This could influence the lower sensitivity of the bridging ELISA analysis as compared to the NAb assay. Nevertheless, bridging ELISA assays are the “gold standard” for immunogenicity screening assays mainly as they are independent from the species of origin of the positive control, and this is the reason why we also used it as format for the IFN-β assay.

The kinetics of the ADA generation were delayed in the less sensitive bridging ADA ELISA as compared to the NAb assay where the first positive samples appeared at measurements after 2 months of therapy, but as expected both ADA responses and NAb titers increased in the persistently positive patients. Both ADA and NAb leveled out after 9 months of therapy, and only 10% of the patients became positive after 9 months. However, it is known from other studies that some patients

generate ADA towards IFN-β later than one year after starting therapy, explaining a recommendation to measure ADA responses at least during the first 24 months of therapy (Sorensen et al., 2005).

The frequency of NAb reported in this study is higher than what has been reported before. The validated NAb assay used in this study was shown to be more sensitive than the commonly used Kawade-assay, increasing the overall positivity by around 14% (Hermanrud et al., 2016). Thus, also the frequency of NAb positive patients with this NAb assay was higher than previously described for historical assays: 17% for IFN-β1a IM treated patients, 26% for IFN-β1a SC, and 96% within the IFN-β1b patients. In comparison to the earlier studies the ADA detection by ELISA have in general demonstrated frequencies from 5 to 30% for IFN-β1a IM, 25–45% for IFN-β1a SC, and 50–80% for patients treated with IFN-β1b (Fernandez et al., 2001; Kivisäkk et al., 2000; Perini et al., 2004; Scagnolari et al., 2002; Prince et al., 2007; Francis et al., 2005; Monzani et al., 2002).

An important question is the clinical relevance of low titer ADA responses. It is well-known that low NAbs do not decrease the clinical efficacy of IFN-β; in contrary low affinity NAb bind reversibly and may protect the IFN-β molecule from degradation and/or consumption and, thereby, prolong the half-life of IFN-β and enhance the effect of an IFN-β injection (Sorensen et al., 2007). A higher sensitivity of the new NAb assay results in early detection of low titer NAbs that may not have clinical importance. In clinical trials, categorization of patients with presence of low titers of NAbs as NAb-positive has obscured the detrimental effect of moderate or high titers of NAbs (Goodin et al., 2012; Sorensen, 2012). However, most patients steadily increased in titers, and the number of transiently ADA positive patients found in this study was low (1–5%). It was documented that the transiently positive patients only reached low NAb titers (lower than 320 U/mL), and only one of them was positive (had a NAb titer higher or equal to 320 U/mL) before they subsequently were analyzed as NAb negative. For NAb titers it is important to distinguish between NAb assay threshold values and clinical relevant threshold values. Patients reaching a NAb titer of 320 U/mL or higher become persistently positive for ADA up to at least 18 months after therapy start, and most of them reached this titer threshold value within 3 to 9 months of treatment, thus enabling early detection of risk for persistent NAb. Setting the threshold value for NAb titers at 320 U/mL and higher did not decrease the number of confirmed ADA positive patients, only decreased the number of NAb positive patients by one patient and eliminated 10 out of the 11 patients with transiently positive low titers. We suggest that this threshold value may be a titer predicting ADA responses of clinical relevance applying this newly validated NAb assay.

In conclusion, the data presented has the following practical implications: first, our data challenges the recommended three-tiered approach in assay development. When a highly sensitive bioassay for the detection of NAbs was applied to samples of a prospective cohort initiated on therapy with IFN-β, the detection rate was higher as compared with the ADA ELISA protocol with formally higher sensitivity. Thus, we suggest to not only rely on formal sensitivity assessments, but also to assess positivity rates in clinical samples prior to decide about a testing approach. Second, as we could define a threshold titer level for the NAb assay that predicts persistency of NAbs, the assay and this titer level should be further assessed with regards to its potential in predicting loss of clinical response, and thus with regards to its clinical relevance.

Author contributions

Contribution to study concept and design: PEHJ, CW, KI, LP, CH, MR, DK, JL, FD, AFH.
Contribution to acquisition of data: PEHJ, CW, KI, LP, MaG, CH, BMFR, MR, DK, JL, RR, MA, DB, VG, EB, NF, BOM, PN, MK, MG, SR, CSS, RLPL, SH, DeB, OA, ND, AL, BH, EKH, BK, HPH, MC, XM, TD, FS, PD, MP, SS, PB, FD, AFH, PSS.
Contribution to analysis and interpretation of data: PEHJ, CW, KI, LP, MaG, CH, BMFR, MR, DK, JL, FD, AFH.
Contribution to the critical revision of the manuscript for intellectual content: PEHJ, CW, KI, LP, MaG, CH, BMFR, MR, DK, JL, RR, MA, DB, VG, EB, NF, BOM, PN, MK, MG, SR, CSS, RLPL, SH, DeB, OA, ND, AL, BH, EKH, BK, HPH, MC, XM, TD, FS, PD, MP, SS, PB, FD, AFH, PSS.
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PEHJ, KI, LP, MaG, CH, BMFR, MR, JL, RR, VG, NF, BOM, SR, CSS, SH, DeB, PD, MP, PB, have no conflict of interest to disclose.
AFH have received speaker honoraria and unrestricted research grants from Pfizer, BiogenIdec and Sanofi.
AL is employee of GlaxoSmithKline.
BH has served on scientific advisory boards for F. Hoffmann-La Roche Ltd., Novartis, Bayer AG, and Genentech; he has served as DMSC member for AllergyCare and TG therapeutics; he or his institution have received speaker honoraria from Biogen Idec, Teva Neuroscience, Merck Serono, Medimmune, Novartis, Desitin, and F. Hoffmann-La Roche Ltd.; his institution has received research support from Chugai Pharmaceuticals and Hoffmann-La-Roche; holds part of two patents; one for the detection of antibodies and T cells against KIR4.1 in a subpopulation of MS patients and one for genetic determinants of neutralizing antibodies to IFN-β.
BK has received honoraria for lecturing, travel expenses for attending meetings, and financial support for research from Bayer Health Care, Biogen, Genzyme/Sanofi Aventis, Grifols, Merck Serono, Mitsubishi Europe, Novartis, Roche, Talecris, and TEVA. He is currently also employee of Biogen.
CW has received consultancy or speaking fees from Novartis, Bayer, Biogen, and Teva.
DB has received compensation for activities with Bayer HealthCare, BiogenIdec, Merck Serono, and Novartis.
DK is a fulltime employee at Sanofi-Aventis Deutschland GmbH.
EB is a fulltime employee at Merck RBM S.p.A., Italy.
EKH received consulting fees from Actelion, Biogen, Celgene, Merck, Novartis, Roche, Sanofi Genzyme and TEVA and is supported by Ministry of Education of Czech Republic, project PROGRES Q27/LF1.
FD has participated in meetings sponsored by or received honoraria for acting as an advisor/speaker for Biogen Idec, Celgene, Genzyme-Sanofi, Merck, Novartis Pharma, Roche, and TEVA ratiopharm. His institution has received research grants from Biogen and Genzyme Sanofi. He is section editor of the MSARD Journal (Multiple Sclerosis and Related Disorders).
FS has served on scientific advisory boards, been on the steering committees of clinical trials, served as a consultant, received support for congress participation, received speaker honoraria, or received research support for his laboratory from Biogen, EMD Serono, Merck, Novartis, Roche, Sanofi Genzyme and Teva.
H-PH has received fees for consulting, speaking and serving on steering committees from Bayer Healthcare, Biogen, GeNeuro, MedImmune, Merck, Novartis, Opexa, Receptos Celgene, Roche, Sanofi Genzyme and Teva, with approval by the Rector of Heinrich-Heine-University.
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MK has received funding for travel and speaker honoraria from Bayer, Novartis, Merck, Biogen,
Roche and Teva ratopharm; serves on scientific advisory boards for Biogen and Roche and.
received a research grant from Teva ratiopharm.
ND was at the time of the work employed at Ipsen Biopharm Ltd., United Kingdom.
OA has received advisor fees or honoraria from Almirall, Bayer Healthcare, Biogen, MedImmune, Merck, Novartis, and Teva; research support from Bayer HealthCare, Biogen, Novartis, Roche, and Teva.
PD is an employee at Scicross AB, Sweden.
PN has received speaker honoraria from Biogen and Novartis and has been supported by Ministry of Education of the Czech Republic, project PROGRES Q27/LF1.
PSS has received personal compensation for serving on scientific advisory boards, steering committees or independent data monitoring boards for Biogen, Merck, Novartis, TEVA, GlaxoSmithKline, MedDay Pharmaceuticals, Genzyme, Celgene, and Forward Pharma and has received speaker honoraria from Biogen, Merck, Teva, Genzyme, and Novartis. His department has received research support from Biogen, Merck, TEVA, Novartis, Sanofi-Aventis/Genzyme, and Roche.
RLPL has received research support from the Swiss MS Society, Swiss National Science Foundation, Roche Postdoc Fellowship Program (RPF-program), unrestricted research grants from Novartis and Biogen.
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XM has received speaking honoraria and travel expenses for scientific meetings, has been a steering committee member of clinical trials or participated in advisory boards of clinical trials in the past years with Bayer Schering Pharma, Biogen Idec, EMD Merck Serono, Genentech, Genzyme, Novartis, Sanofi-Aventis, Teva Phramaceuticals, Almirall and Roche.

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