PhD-Thesis

Immunisation against Interferon-beta and Natalizumab in Multiple Sclerosis patients: Prediction and analysis of Anti-Drug-Antibodies to minimise the risk

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This thesis was generated within ABIRISK: Anti-Biopharmaceutical Immunisation: prediction and analysis of clinical relevance to minimise the RISK.

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1. Introduction

1.1. Multiple sclerosis

Multiple sclerosis is a chronic inflammatory disease of the central nervous system, caused by the auto-immunologically triggered destruction of the myelin layer of neurons, followed by axonal damage[1]. With a prevalence of about 1:1000 with major differences depending on geographical region[2] and median onset of 25-35 years (in women earlier than in men),[3] it is the most common neurological disease that leads to permanent disability in young patients. While the pathophysiology of multiple sclerosis is still poorly understood and a specific mechanism or particular target molecules for the pathophysiological development of the disease have not been identified, despite decades of, clinical patterns and especially treatment options for multiple sclerosis are well established.

About 90% of patients typically show a relapsing disease course at onset, characterised by a rapid development of neurological symptoms, such as optic neuritis, sensory loss, brainstem symptoms, cerebellar ataxia, paralysis and other symptoms related to various functional systems[4]. These attacks are typically followed by a complete or incomplete remission of symptoms. After the relapsing phase in most patients the disease passes into a secondary progressive course with a slow accumulation of neurological failures over time. The most established theory is that at the beginning of the disease there is a predominance of inflammatory mechanisms while later on neurodegeneration becomes dominant. Therefore, so-called immunomodulatory drugs show high efficacy in the relapsing phase of multiple sclerosis, whereas there are still no or only minimally effective treatments for the secondary or primary progressive disease course[5-6].

1.2. Diagnosis of multiple sclerosis

There are clinical and imaging criteria for diagnosis of multiple sclerosis, supported by additional diagnostic tools such as an analysis of cerebrospinal fluid. The McDonald criteria, which were last revised in 2010[7], are widely used for the diagnosis of multiple sclerosis. According to these well-established criteria, the following characteristics are required:

- Two relapses with objective clinical evidence of at least two lesions related to different functional systems
- Two relapses with objective clinical evidence of at least one lesion plus dissemination in space of demyelinating lesions in an MRI
- One relapse with objective clinical evidence for at least two lesions plus dissemination in time of demyelinating lesions in an MRI
- One relapse with objective clinical evidence of at least one lesion plus dissemination in space and time of demyelinating lesions in an MRI

The presence of oligoclonal bands is no longer included in the revised McDonald criteria of 2010. However, CSF oligoclonal band screens are still routinely performed in the diagnostic evaluations of multiple sclerosis since they are present in 95-98% of MS patients and thus still represent an important diagnostic tool in the evaluation of potentially differential diagnoses[8-10]. Furthermore, evoked potentials, optical coherence tomography and laboratory analysis of special markers such as vasculitis markers usually form part of the clinical routine in the primary diagnostic investigation after the first clinical manifestation of MS. Moreover, the exclusion of other inflammatory demyelinating diseases such as neuromyelitis optica (NMO) and MOG antibody (myelin oligodendrocyte glycoprotein) spectrum diseases are of high interest especially in regard to later treatment decisions[11-12].

If a disease in the MS spectrum is suspected but the diagnostic criteria are (still) not fulfilled, the term "clinically isolated syndrome" (CIS) is used[13], although CIS has become a rather rare diagnosis since the introduction of the new and very sensitive MRI-criteria.

1.3. Treatment options

(This section was drafted according to: *"Die neue Therapielandschaft der Multiplen Sklerose". Auer M, Hegen H, Deisenhammer F. Der Mediziner 2014; 7/8, 12-16*)[14]

Nowadays, a large number of immunomodulatory treatments are available for the treatment of relapsing MS, including CIS and relapsing-progressive MS (RPMS), while there is still no established treatment for exclusively progressing disease courses, such as secondary (SPMS) and primary progressive MS (PPMS), although recent positive study results such as those for Ocrelizumab have raised awareness.

The following section comprises a short overview of the available drugs effective in Austria from 2017 (i.e. not including drugs already registered and available in other countries or still in the process of approval).

1.3.1. Interferon beta (IFNβ)[15-18]

This section is only a short overview of IFNβ used in the treatment of MS, because the major part of the thesis will be focused extensively on all aspects of IFNβ therapy. IFNβ was the first treatment available for relapsing MS, as well as for CIS and RPMS. Four IFNβ preparations are available (Extavia® is excluded since it is not approved in Austria): an IFNβ 1b s.c. 250 µg injection every second day (Betaferon®, approved in 1995), an IFNβ 1a s.c. 22 and a 44 µg injection three times weekly (Rebif®, approved in 1998), an IFNβ 1a i.m. 30 µg

injection once weekly (Avonex®, approved in 1997) and an IFNβ 1a s.c. 125 µg injection every two weeks (Plegridy®, approved in 2015). IFNβ is a recombinant 166-amino-acid glycoprotein which affects the modulation of hundreds of immunomodulatory genes; however, there is no deeper knowledge of the exact pathophysiological mechanisms. The reduction of relapse rate is about 30-35% compared to placebos for all IFNβ preparations. The most common side effects are flu-like symptoms and injection site reactions. The development of anti-drug-antibodies (ADAs) is dependent on IFNβ preparation and varies between 2% and 47%, while being additionally dependent on the laboratory assay used and cut-off (see section 1.4). Treatment monitoring includes routine blood tests for liver enzymes and lymphocyte counts, and testing for neutralising antibodies (NAbs).

1.3.2. Glatirameracetate (Copaxone®)[19-20]

Glatirameracetate is a four-amino-acid oligopeptide that was approved in 2003 and is now available in two dosages: 20 mg s.c. daily and 40 mg s.c. three times weekly. As with IFNβ, the pathophysiological mechanisms of drug action for Glatirameracetate are unknown; however, relapse reduction compared to placebo has also been determined to about 30%.The side effects are injection site reactions and rare allergic reactions to single injection margins. No standard treatment monitoring procedure is required for this medication.

1.3.3. Teriflunomide (Aubagio®)[21-22]

Teriflunomide is an oral medication, approved in 2015 and administered once daily at a dosage of 14 mg. It was developed from the precursor drug Leflunomide, which is used in the treatment of rheumatologic diseases. Teriflunomide inhibits dihydroorotate dehydrogenase (DHODH), an enzyme involved in pyrimidine synthesis. This effect slows the maturing process of rapidly proliferating cells such as lymphocytes and thus causes an immunosuppressive effect in MS patients. The relapse reduction rate is estimated at about

30% compared to placebo. The observed side effects are rare and include transient and incomplete alopecia in about 10% of patients as well as diarrhoea. Moreover, teratogenic effects were observed in animal models that were treated with Teriflunomide. Treatment monitoring included laboratory tests with the monitoring of the lymphocyte count and liver enzymes every two weeks within the first six months of treatment, followed by blood tests every three months for further treatment duration.

1.3.4. Dimethyl fumarate (Tecfidera®)[23-24]

Dimethyl fumarate, approved in 2015 and administered orally twice a day, is an inhibitor of the NFκB pathways. Modulating the downstream immunoregulatory cascades, this drug has demonstrated high efficacy in the treatment of MS with relapse reduction rates of about 50% compared to placebo. The most commonly observed side effects are flushing and gastrointestinal effects such as upper abdominal pain or nausea in about one third of patients. Monitoring includes blood sampling with a major focus on the lymphocyte count every three months. Five cases of progressive multifocal leukoencephalopathy (PML) during Dimethyl fumarate treatment were all associated with long-term lymphopenia and an age of above 50 years.

IFNβ, Glatirameracetate, Teriflunomide and Dimethyl fumarate are used as first-line immunomodulatory therapy in mild to moderate relapsing MS.

1.3.5. Natalizumab (Tysabri®)[25-29]

Natalizumab, approved in 2006 for the treatment of high active relapsing MS, was the first monoclonal antibody used in MS therapy and is administered in a dosage of 300 mg intravenously every four weeks. This drug blocks the very late antigen 4 (VLA-4), located on the surface of the lymphocytes, which is necessary for adherence to the blood vessel endothelium and, thereby, for diapedesis through the blood-brain barrier. With this mode of

action, Natalizumab was able to show an immunosuppressive effect restricted to the central nervous system, leading to a relapse reduction of nearly 70% compared to placebo. While common side effects are limited to infusion-related allergic reactions (mostly related to ADAs), PML is an important issue in treatment with Natalizumab, more commonly observed than with other immunomodulatory and/or immunosuppressive drugs. PML is caused by reactivation of the John Cunningham virus (JCV) in the central nervous system; therefore detection of anti-JCV antibodies in the patients' serum before treatment starts as well as during treatment every six months due to possible seroconversion, is of the utmost importance when treating patients with Natalizumab. The current prevalence of PML in Natalizumab-treated patients is 4.20/1,000, whereby, in addition to the JCV status (negative or positive) and JCV antibody index, treatment duration and prior immunosuppressive therapy are important factors influencing the risk of developing PML. Four to 12% of patients develop ADAs against Natalizumab, which is discussed in more detail in section 1.5.

1.3.6. Fingolimod (Gilenya®)[30-31]

Fingolimod, approved in 2011, was the first oral drug used for the treatment of relapsing MS and in Austria it is used only in highly active MS. Fingolimod is an agonist inhibitor of the sphingosine-1-phosphate receptor resulting in downregulation of the receptor expression, which serves the lymphocytes in the process of maturing and migration from the lymph nodes. The therapeutic effect is a lymphopenia in the peripheral blood system and selective immunosuppression. Relapse reduction rates have been described as 50% compared to placebo. The rare but important side effects, which require specified monitoring, are macula edema (prevalence 1:250) and cardiac effects such as bradycardia and atrioventricular blocks. Therefore, ECG-monitoring for six hours after the first drug intake as well as an eye examination three months after treatment commences are required in the guidelines. Additionally, laboratory tests with a focus on treatment-induced lymphopenia every three

months are recommended. Thirteen PML cases have been observed so far during treatment with Fingolimod.

1.3.7. Alemtuzumab (Lemtrada®)[32-34]

Alemtuzumab, approved in 2013, is a lymphocyte-depleting monoclonal antibody that is used for the treatment of highly active relapsing MS. Alemtuzumab is directed against the CD52 surface glycoprotein on the T- and B-lymphocytes and has major effects on the immune system. The high effectiveness of the drug was demonstrated by a relapse reduction rate of about 50% compared with IFNβ 44 µg s.c. three times weekly. The dosage regimen is five infusions of 12 mg on consecutive days during the first treatment cycle and three more infusions on consecutive days after 12 months. However, there are highly prevalent side effects to be taken into account during this treatment, namely infusion-related reactions such as urticaria in 90% of the patients, autoimmune reactions such as immune thyroiditis (38%) and, rarely, immune thrombocytopenic purpura (1%) and autoimmune renal failure (0,2 %), which can occur even years after treatment begins. Therefore continuous monthly monitoring with blood tests including thyroid markers, platelet counts and renal function markers, is required over four years following the last administration.

1.4. Pharmacokinetic considerations regarding Interferon-beta and Interferon-beta antibodies

(This section was drafted according to: *Pharmacokinetic considerations in the treatment of multiple sclerosis with interferon-β. Hegen H, Auer M, Deisenhammer F. Expert Opin Drug Metab Toxicol. 2015;11(12):1803-19*)[35]

Human IFNβ is a 166-amino-acid glycoprotein with a complex three-dimensional structure consisting of five α-helices, a disulfide bond and a glycosylation site[36]. Recombinant IFNβ is expressed either in E. coli (termed IFNβ 1b)[37-38] or in Chinese hamster ovary cells (termed IFNβ 1a)[39]. IFNβ 1b has a molecular weight of 18.5 kDa and has some differences to the natural form: a lack of glycosylation, the deletion of methionine at position 1 and cysteine to serine substitution at position 17[40-41]. IFNβ 1a (22.5 kDa) is glycosylated and has an identical amino acid sequence to natural IFNβ; only the glycosylation is different[42]. The biological activity of IFNβ 1a is approximately 10 times higher than that of IFNβ 1b.

IFNβ is not absorbed from the gastrointestinal (GI) tract after oral application, because proteins undergo denaturation by acidic pH in the stomach and proteolytic degradation within the GI tract. Only minimal diffusion of proteins occurs through the GI epithelium[42]. Therefore, IFNβ has to be administered parenterally. The parenteral route of administration requires sufficient solubility of the compound and, in the case of an i.m. or s.c. injection, a small volume of fluid for the purpose of tolerability at the injection site[43].

At the site of the injection, the extracellular matrix (ECM) is a physiological barrier consisting mainly of collagen and glycosaminoglycans[43]. For the transport of a protein through the ECM, both diffusion and convection are important. Diffusion is inversely related to the molecular size and is a relevant determinant for convection protein charge [44-45]. From the interstitial space, there are two principal pathways for the systemic absorption of a protein: transport through the lymphatic vessels and diffusion across the blood vessels[43]. IFNβ is supposed to be absorbed mainly via the lymphatic system[46].

Eventually, i.m. and s.c. administration of IFNβ exhibit protracted absorption, which results in peak plasma concentrations after several hours[47-52]. IFNβ concentrations are detectable for approximately 1-2 days after injection[53-58]. The slow release of IFNβ from the injection site might explain the long apparent half-life of i.m. and s.c. administered IFNβ[59].

A pegylated form of IFNβ 1a was recently developed that shows a significantly longer half-life than the conventional IFNβ 1a enabling less frequent dosing at similar treatment efficacy[18].

Once the drug has entered the blood stream, it is systemically spread followed by a process of extravasation; that is transportation across the vascular wall into the tissue.

Little effort has been made to research the tissue distribution of IFNβ. IFNβ itself is not thought to enter the CNS[60]. This is implied by several studies that showed no or scarce IFNβ concentrations in CSF[61].

Current knowledge of the mechanism of the action of IFNβ includes immunomodulatory properties in the peripheral compartment,[60] such as reduction of antigen presentation (by down-regulation of major histocompatibility complex class II on antigen-presenting cells)[62], shifting cytokines to an anti-inflammatory response[63-64] and reducing leukocyte migration through the blood-brain barrier by, for example, decreasing VLA-4 expression on the lymphocytes[65] or by decreasing the activity of matrix metalloproteinases such as MMP-9[66-67].

Regarding the influence of immunomodulatory pathways, IFNβ displays a broad spectrum of activity comprising antiviral, antiproliferative and immunoregulatory functions. After the binding of IFNβ to the type I IFN receptor, a signalling cascade is induced, resulting in the expression of various genes[68]. Some of these gene products are 2',5'-oligoadenylate (2-5A) synthetase[69-70], neopterin[71-74], β2-microglobulin[71,72,74] and MxA[69,70,74-76].The latter is well established as an IFNβ bioactivity marker and has been used for NAb detection assays[77].

IFNβ treatment is associated with the development of anti-drug antibodies (ADAs), which are referred to as binding antibodies (BAbs) if detected by ELISA or Westernblot. A subset of BAbs is capable if interfering with the interaction between IFNβ and its cellular receptor, therefore preventing the biological effects of IFNβ. These NAbs develop at different frequencies depending on the administered IFNβ preparation[77].

In previous literature a prevalence of NAbs caused by IFNβ preparation was estimated as follows: 28% to 47% for IFNβ 1b, 12% to 28% for IFNβ 1a s.c. three times weekly, 2% to 6% for IFNβ 1a i.m. once weekly[78] and lower than 1% for peg-IFNβ 1a s.c.[18] The broad range is result of different assays being used for the detection of NAbs and differing cut-offs (neutralising units) within the assays and between different laboratories. All the studies show a higher frequency of NAbs for IFNβ 1b, which may be explained by its molecular structure, which shows more differences to human IFNβ than IFNβ 1a and facilitates aggregate formation, as described above. Furthermore, IFNβ 1a was shown to be less immunogenic if administered i.m. once weekly rather than s.c. three times weekly. However, NAb titres in IFNβ 1a-treated patients were higher than those in IFNβ 1b-treated patients. The time to NAb development ranged from six to 18 months, with later onset for IFNβ 1a. Patients who still remained negative after 18 to 24 months were likely not to develop Nabs at a later time. Low titres do not significantly impact the bioavailability of IFNβ and tend to disappear over time, so a treatment switch is not necessarily obligatory. High titres after 18 months of treatment in most cases predict persisting antibodies, with a long-term reduction of treatment efficacy[79-80].

It is well known that high titres of NAbs are able to abolish the bioavailability of IFNβ and thereby reduce the clinical efficacy of IFNβ treatment.[76] Relapse rates in high-titre NAb positive patients approach similar high levels as in untreated patients. Furthermore EDSS-progression and MRI-activity is significantly higher in NAb positive patients than in NAb negative subjects.[81]

Several studies found a cross-reactivity of IFNβ-Nabs, which necessitates a switch between IFNβ preparations in the case of NAb development counteracting treatment efficacy.[82] A switch to a non-IFNβ therapy should be highly recommended for high-titre NAb positive patients.[83]

1.5. Natalizumab and Natalizumab antibodies

In contrast to IFNβ, the mode of action of Natalizumab at a molecular level is well defined. Natalizumab is a humanised IgG4 monoclonal antibody, directed against the α4 subunit of α4β1 and α4β7 integrins (so called "very late antigen 4 = VLA4") on the surface of the lymphocytes and monocytes. The masking of VLA-4 blocks the interaction with the vascular cell adhesion molecule 1 (VCAM-1), which is expressed on the surface of vascular endothelial cells in brain and spinal cord blood vessels, and mediates the adhesion and migration of lymphocytes in areas of inflammation. Due to the inhibition of the diapedesis of leukocytes through the blood-brain barrier, Natalizumab results in a targeted immunosuppression in the areas affected by demyelinating processes within the central nervous system. The drug is administered at a unified dosage of 300 mg intravenously once per month or every four weeks.[25-26]

ADAs were observed in relation to treatment with Natalizumab as well, in which, there is no differentiation of BAbs and NAbs.

The prevalence of ADAs against Natalizumab was 9% in the SENTINEL- and AFFIRM-studies, of which 6% were persistent and 3% permanent[84], while Sorensen et al. found a lower occurrence of ADAs, namely 4,5% (3,5% persistent and 1% transient)[85] and Oliver et al. described a prevalence of 14,1% (9,4% persistent and 4,7% transient)[86]. High-titre ADAs are associated with treatment failure and, in many cases, with infusion-related events[87] such as urticaria or even more severe anaphylactic reactions[88]. ADAs against Natalizumab develop soon after the first infusions, usually within six months after the commencement of treatment.[86]

2. Study rationale

Biopharmaceutical drugs; that is, polypeptides such as IFNβ and monoclonal antibodies such as Natalizumab, gain ever more importance in the treatment of various diseases. One problem with most of these drugs is immunisation against the therapy due to the development of ADAs, which may diminish the efficacy of treatment by neutralising the biological effect of the drug.[89] Especially in the field of multiple sclerosis, it seems important to identify patients who develop ADAs as early as possible or even before treatment starts, because a broad spectrum of immunomodulatory therapies has been approved. This could help to facilitate individualised treatment decisions in terms of choosing the most appropriate therapy for each patient.

The prevalence and kinetics of ADAs against IFNβ and Natalizumab have been well described in previous literature (see sections 1.4 and 1.5); however, studies on large databases that help to identify risk factors for ADA development are still missing.

The aim of the ABIRISK project is to establish collaboration between countries across Europe in order to develop a common database, gathering ADA test results together with the clinical data of MS patients who have been tested for ADAs. The current work indicates a part of the analysis of this multinational database, as well as an extract of individual analyses of local projects foung in the laboratory database of the neuroimmunology laboratory of the department of neurology, Innsbruck, Austria, together with data from the MS database of the neuroimmunology outpatient clinic of the department of neurology in Innsbruck.

The aim of this work is to present actual knowledge about IFNβ- and Natalizumab ADAs, their clinical impact and, particularly, the possible association of ADAs with demographic and clinical variables.

2.1. Personal contribution

The research presented here was performed as part of the ABIRISK project. However, only the results of the author's personal research and analysis will be presented in this paper. The current PhD thesis includes a summary of analyses of the multinational retrospective ABIRISK database, which represents a major part of the activity within the PhD-related research. These results were published as a collaboration of all national data custodians in the form of two papers listing all the involved researchers as co-authors. Furthermore, a local sub-project about one possible risk factor for ADA development is presented. The latter was published by this researcher as the first author in a separate research paper. Additionally, two more publications outside the PhD thesis project were chosen for use as part of the introduction to this thesis. Thus, the present work is an attempt to summaries the best possible part of the author's personal contribution to the multinational ABIRISK project. It does not include an analysis of the prospective ABIRISK study (see methods section), which is scheduled to end in November 2017.

3. Methods

3.1.The ABIRISK project (see also www.abirisk.eu)

ABIRISK (=Anti-Biopharmaceutical Immunisation: prediction and analysis of clinical relevance to minimise the RISK) was launched in March 2012 and funded by the IMI (Innovative Medicines Initiative). The project is scheduled to be completed by November 2017. The aim of this multinational European venture is to elucidate the factors leading to immunisation; that is, the development of ADAs that counteracts various biopharmaceutical drugs with the purpose of facilitating the development of less immunogenic drugs in the

future. It includes research groups on various diseases in which biopharmaceutical drugs are used: multiple sclerosis, haemophilia A, rheumatoid arthritis, juvenile inflammatory arthritis and inflammatory bowel diseases. The MS group is composed of research members in Austria, Sweden, Germany, Denmark, Switzerland, the Czech Republic, Spain and the United Kingdom, as well as at a supportive research centre in France that coordinates the common database analyses.

The ABIRISK members are organised into five work packages (WPs), of which WP-1 was responsible for assay development and conducting the prospective trials; WP-2 was involved in genetic research on collected samples in the prospective study, WP-3 conducted basic research on animal models, WP-4 was responsible for the development of the retrospective ABIRISK database and the eCRF for the prospective study, and WP-5 represented the organising committee and took care of financial issues.

The two main parts of the PhD project within ABIRISK consisted of collaborative work within WP-1 and WP-4. We drafted the protocol for the prospective study in the MS group, which included patients treated with IFNβ, in telephone conferences and annual meeting, and compiled the application to the national ethics committees, as well as major parts of the eCRF (mostly drafted by the Innsbruck group and technically implemented by WP-4). Furthermore, we managed the clinical visits of the prospective patients, of whom 20 were Innsbruck patients, and took care of sample withdrawal, processing, storage and use for further research. The work within WP-1 concerning the prospective study will not be presented here, because it is still in progress and results will be generated over the next few years.

Here, we focus on the work performed within WP-4, which encompasses the development of the retrospective database, data collection, drafting the data load plan and, particularly, analysis of the data.

3.2. The retrospective database

The retrospective ABIRISK database was developed collaboratively within WP-4. It includes data from seven European cohorts. All these data were already present in the distinct institutions and comprised laboratory and clinical data gained during routine visits by MS patients tested for ADAs against IFNβ – starting in 1995 – and Natalizumab – from 2006. In Innsbruck we had to search and analyse wo existing databases: firstly, the laboratory database of the neuroimmunology laboratory, containing ADA test results as well as demographic data and information about the assays used for ADA measurement and, secondly, the clinical MS database of the MS outpatient clinic, which contained the clinical data of the patients who had been tested for ADAs. The two databases had to be harmonised and missing data were sourced elsewhere if possible. This step was necessary in order to facilitate statistical analysis of a previously inhomogeneous database. Once the local database was acquired, a data load plan was developed within WP-4, enabling standardisation of all national databases with all available variables. The data were standardised to a common data format loosely based on the CDISC standards (www.cdisc.org). First eTRIKS®, then tranSMART® provided the data loading platform on which all the data custodians could upload the clarified data. Following further queries and adaptations, all the national data-sets could be integrated in a large single database, including the data relating to 20,695 MS patients in Europe and 42,555 ADA test results (for IFNβ and Natalizumab). Common analyses were performed on this multinational dataset and the results were published by the WP-4 group in two collective papers[90-91].

Statistical analyses were conducted using R software (version 3.0.2)[92]. Descriptive statistics include calculations of median (range) or mean (standard deviation) as appropriate and counts of various data subsets. For group analyses between ADA positive and negative patients, Fisher's exact test was used. For testing of sample numbers across ages, the significance of the slope of the regression line was assessed. For detailed analyses of the clinical variables

and outcome parameters, univariate and multivariate analyses were performed using Cox model analyses and the Cox proportional hazards model. A more detailed description of the statistical analyses is presented in references 90-91.

3.3. ADA and NAb assays

For IFNβ, ADAs (=binding antibodies, BAbs) were not tested routinely, because NAbs, which are solely of interest for clinical routine monitoring, can be tested by established assays. BAb assay, a capture ELISA[93], was mostly performed for previous scientific interest and is not included in the results presented here.

For Natalizumab; standardised bridging ELISA was developed and provided by Biogen Idec[84] immediately after the release of the drug, which is able to detect ADAs (whereby a differentiation of BAbs and NAbs is not needed, since all antibodies are thought to be neutralising). Therefore, the same assay was performed in all laboratories for the detection of Natalizumab ADAs.

The testing for NAbs is of major interest for treatment monitoring during IFNβ therapy. However, various assays have been used, depending on the year and the country in which the tests were conducted. Also, different cut-offs regarding the neutralising units defining negative, positive and, furthermore, low, medium and high positive patients were used, creating a certain non-homogeneity when trying to standardise assay results between countries over a time-frame of nearly 20 years. In Austria nowadays, the Luciferase assay (LUC) is used according to a previously described protocol[94-96]. Until 2008, NAb testing was performed with the MxA protein expression assay (MPA) or MxA gene expression assay (MGA)[97-99]. In other countries the cytopathic effect assay (CPE) method[100] and iLite anti-human IFNβ-1a bioassay[101] were used as well. Additionally, in Munich an in-vivo assay[102] served for detection of NAbs.

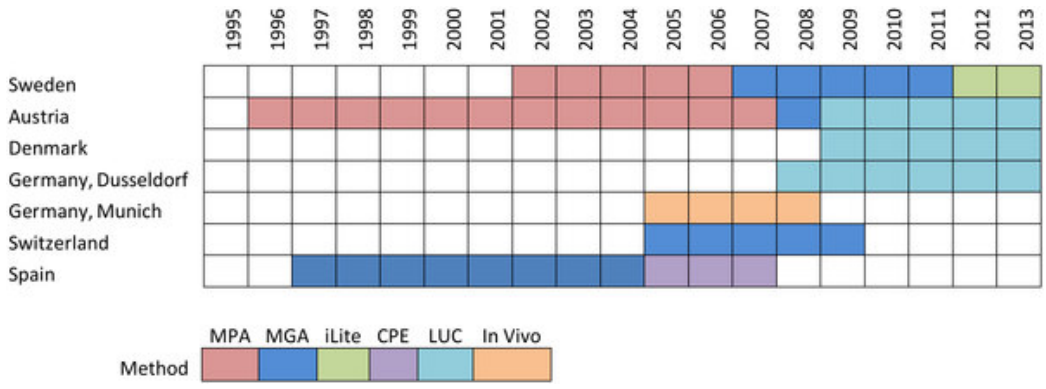

Fig. 1: Main methods used for NAb testing in different ABIRISK cohorts over time.

The neutralising units in all cohorts are displayed as TRU/ml (=ten-fold reduction units per millilitre). The cut-off for a positive NAb result is 20 TRU/ml, except in Sweden, where it is 10 TRU/ml. In Austria, Sweden, Spain and Dusseldorf in Germany a further differentiation of low positive (<100 TRU/ml) and high positive (≥ 100 TRU/ml) is made.

The patients were defined as NAb positive whenever at least one result of >20 or 10 TRU/ml (the latter in Sweden) was obtained. To be categorised as NAb negative, the patients had to be tested negative at least twice, once before and once 12 months after treatment started, and no positive result at all times if more samples during follow-up were available.

3.4. The local database and cotinine test

Additional analyses were performed on the local database in Innsbruck and presented at annual meetings of the ABIRISK consortium. In favour of the above-mentioned collaborative papers, these sub-analyses were not separately published, but included in the multinational statistical analyses. However, some results obtained in the local analyses will be presented in the results section. Additionally, a single project was performed focusing on the particular risk factor of smoking and its possible influence on IFNβ-NAb development[103].

Therefore, we screened our local MS and laboratory databases for patients who were tested for IFNβ-NAb for routine purposes, usually every three months after treatment initiation and for whom smoking habits were recorded. We identified 37 patients who met both criteria and used these patients for a preliminary test of cotinine by means of the enzyme-linked immunosorbent assay (ELISA) and the subsequent power calculation for a larger study cohort (type I error 0.05; type II error 0.20).

Following this calculation, a minimum of 120 samples was required. Finally, we chose 123 stored sera of patients who were tested for NAbs for routine purposes – 62 NAb negative and 61 positive, of whom the smoking status was unknown. These samples were tested for cotinine and used for anonymised analysis. NAb negative patients were defined as having at least one negative test result within 12 months and one confirmatory negative result later than 12 months after treatment commenced. In the NAb positive group, only patients with a result of >100 ten-fold reduction units (TRU) were included in order to exclude borderline NAb results.

We obtained separate approval for this study from the ethics committee.

NAb had been tested for routine purposes in freshly withdrawn samples or in samples sent to the local laboratory on dry ice, using the luciferase assay (LUC)[94]. The intra-assay CV was between 6.3% and 15.2%, and the inter-assay CV ranged from 17.8 to 29.3%.

Residual serum was stored continuously at -20°C until it was used for the cotinine test.

Cotinine was tested by means of a commercially available and CE-certified ELISA (AlereTM Serum Microplate EIA Kit Cotinine, Alere Toxicology Plc, Oxfordshire, UK), based on an inhibition principle: Microtitre-plates were coated with cotinine antibodies and incubated with the patients' sera. Then horseradish peroxidase (HRP)-labelled cotinine was added and detected by an enzyme-substrate-reaction, as described in the manufacturer's manual. The intra-assay CV ranged between 5.7% and 9.5% and the inter-assay CV between 6.0% and 7.5% for different cotinine concentrations.

For statistical analyses, we performed a chi-square-test for comparison of the NAb positive and negative with cotinine-positive and -negative groups and, additionally, performed the Spearmen correlation test for NAb titres and cotinine concentration, as well as for the correlation of cotinine levels with sample storage time. Cotinine levels between NAb positive and -negative patients were compared by means of the Mann-Whitney test. P-values of <0.05 were considered significant. Demographic variables are displayed as mean ± standard deviation or median (range), depending on the distribution of values calculated by the D'Agostino-Pearson normality test.

4. Results

As a first step, we analysed the retrospective ABIRISK database for data concerning ADA tests relating to IFNβ and Natalizumab in order to investigate the clinical practice of ADA testing in different countries.

We were able to reproduce the previously described prevalence of NAbs (see section 1.4) with the following rates of NAb positive patients, depending on treatment and preparation in the case of IFNβ (range of all cohorts shown; for details see table 1): for IFNβ 1a i.m. 2.8% to 13.3%, for IFNβ 1a s.c. 11.5% to 34.0%, for IFNβ 1b s.c. 13.3% to 48.7% and for Natalizumab 2.5% to 6.1% and, in the case of IFNβ, the broad range results from the different assays used (see fig. 1) in single cohorts and, moreover, the implementation of NAb testing in clinical routines in different countries (in all patients vs. in patients with treatment failure only).

In a sub-analysis of the Innsbruck MS and laboratory database, where only patients with continuous NAb testing during the first two years were included (this explains the deviation from the percentages displayed in table 1), we found NAbs in 4.8% of patients treated with IFNβ 1a i.m., whereas 13.8% of patients with IFNβ 1a s.c. and 32.3% of patients with IFNβ 1b s.c. were NAb positive, which results in an overall positivity of 14.8% for all IFNβ treated patients at Innsbruck. For Natalizumab, we found an ADA positivity rate of 6.1%.

Treatment	Austria	Denmark	Germany/ Dusseldorf	Germany/ Munich	Spain	Sweden	Switzerland
IFNβ-1a i.m.	6.9	3.5	7.8	2.8	8.8	9.9	13.3
IFNβ-1a s.c.	23.4	15.0	26.9	11.5	29.4	34.0	23.6
IFNβ-1b-Betaferon	25.3	14.8	40.4	13.3	68.3	48.7	28.6
IFNβ-1b-Extavia	-	23.9	37.5	-	-	40.2	-
Natalizumab	6.1	5.4	-	-	-	2.5	-

Table 1: Percentage of IFNβ-NAb and Natalizumab-ADA positive patients for each treatment stratified by country

Furthermore, we analysed the overall number of NAb/ADA tests conducted for IFNβ- and Natalizumab-treated patients. Fig. 2 shows the number of yearly ADA tests and reflects the introduction of ADA testing to clinical routines.

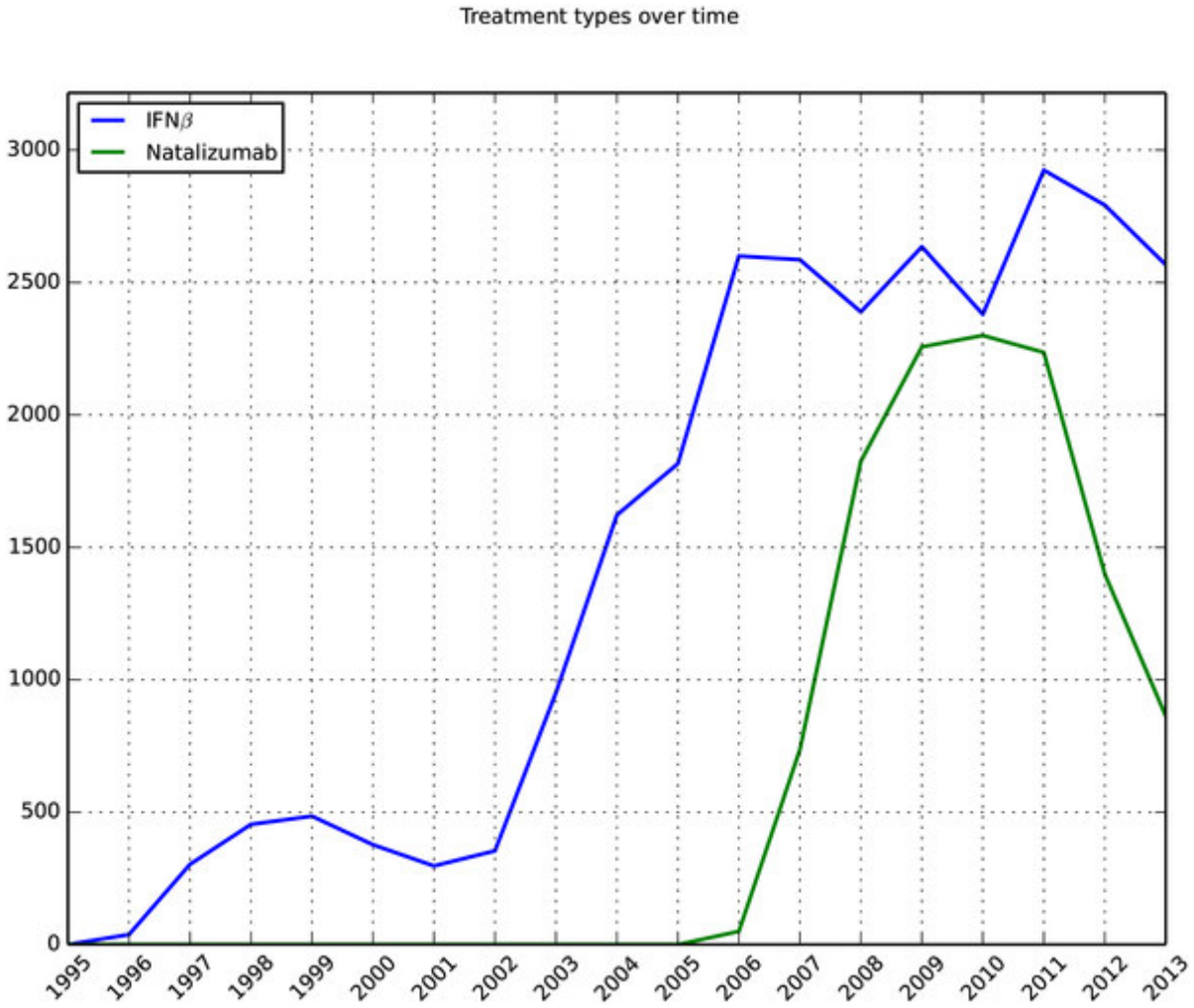

Fig. 2: Overall number of NAb tests against IFN and ADA tests against Natalizumab over time, separated by year and including all retrospective ABIRISK cohorts.

In the ABIRISK database, a mean of 2.05 samples per patient were tested in all countries with a range of 1.0 in Munich (where only patients with treatment failure were tested) to 2.8 in Denmark (where ADA testing is routinely performed in newly-treated patients, which is the clinical practice in Austria as well).

The time from the start of treatment to the first ADA test varied from one month to several years, depending on when ADA testing was routinely introduced into clinical practice.

For Natalizumab, a more detailed analysis was performed on the Innsbruck cohort, including 1,280 patients on Natalizumab, of which 82 tested ADA positive at any time point. We included only those patients who had had ADA tests every month after the commencement of treatment which is common practice in our outpatient clinic. We were able to show that the vast majority of patients who eventually presented ADA positive (>80%) developed ADAs right after the first infusion, showing a positive result in the sample taken in month one, i.e. before the second infusion. All the patients except one presented with ADAs within six months of treatment. As previously described (see section 1.5), 31 out of 82 patients (37.8%) reverted to a negative ADA status, which happened mainly in patients with low ADA titres, whereby reversion in most cases occurred during the first six months as well. Therewith, we were able to demonstrate, even if only in a small cohort, that for Natalizumab we were able to predict a long-term ADA status after six months for nearly all patients without any further testing at later timepoints. Furthermore, exclusively in the Innsbruck cohort, we associated IFNβ-NAb status with differing potential demographic and clinical variables that were thought to potentially influence the development of NAbs (for Natalizumab in the single cohort, not enough data was available to achieve sufficient statistical power). The following data were available for statistical analyses (using the Chi-square test for non-parametric and the Mann-Whitney-Test for parametric variables) between NAb negative and NAb positive patients: age, gender, pre-treatment vs. treatment-naïve, positive or negative family history of MS, presence of oligoclonal bands, cerebrospinal fluid (CSF) cell count, IgG-Index, smoking history and number of pregnancies. In this cohort of 300 to 400 patients we were able to detect a significant direct association with age only, estimating higher NAb risk in older patients. All the results are displayed in table 2:

	Male	Female	total	p=0,133
NAb-	982	2140	3122	
NAb+	207	517	724	
total	1189	2657	3846	
	17.4%	19.5%		

	No pretreatment	Pretreatment	total	p=0,932
NAb-	260	78	338	
NAb+	48	14	62	
total	308	92	400	
	15.6%	15.2%		

	OCB-	OCB+	total	p=1,000
NAb-	17	317	334	
NAb+	3	58	61	
total	20	375	395	
	15.0%	15.5%		

	No family history	Familiy history	total	p=1,000
NAb-	287	34	321	
NAb+	54	6	60	
total	341	40	381	
	15.8%	15.0%		

	Non smoker	Smoker	total	p=0,212
NAb-	169	112	281	
NAb+	27	26	53	
total	196	138	334	
	13.8%	18.8%		

	NAb-	NAb+	p<0.001
age [median(range)]	38 (9-86)	40 (12-72)	

	NAb-	NAb+	p=0.449
CSF cell count [median(range)]	19.5 (0-722)	18.5 (0-113)	

	NAb-	NAb+	p=0.152
IgG index [median(range)]	0.81 (0.46-1.64)	0.87 (0.38-1.66)	

	NAb-	NAb+	p=0.244
Number of pregnancies [mean]	0.928	1.176	

Table 2: Association of IFNβ-NAb status with different demographic and clinical variables with p-values. In the section for non-parametric variables, patient numbers per groups and NAb rates stratified by single specific variables are shown. For parametric variables median (range) for NAb positive and -negative patients is shown.

Additionally, we were able to show a significant association of NAb status with EDSS (Expanded Disability Status Scale), used for scaling the neurological impairments in MS patients. This result, adjusted for age, is shown in fig. 3.

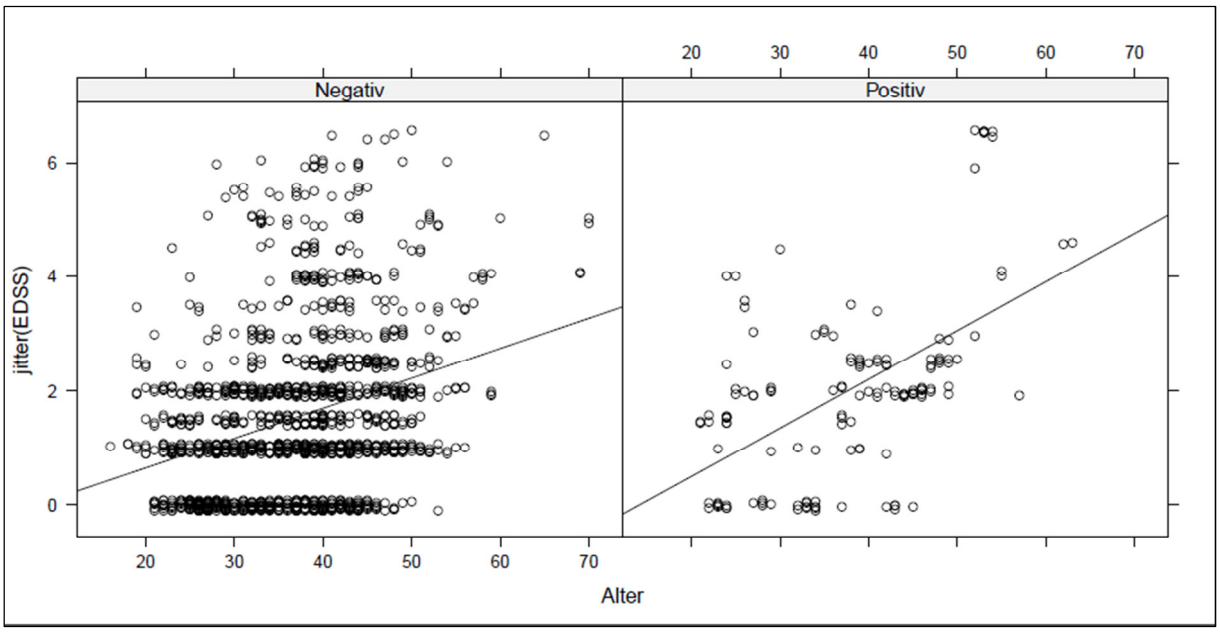

Fig. 3: Linear regression of Expanded Disability Status Scale (EDSS), adjusted for age.

The EDSS-score is significantly higher in NAb positive patients ($p<0.001$).

The age dependent increase of EDSS is higher in NAb positive patients as well, showing an increase of 0.0524 points per year in NAb negative and 0.0849 points per year in the NAb positive population.

The EDSS does not differ between NAb groups for young patients; however, the EDSS difference increases in older patients, shown for age 20 and 60 in table 3:

	age	median EDSS
NAb negative	20	0.5
	60	**2.5**
NAb positive	20	0.5
	60	**4**

Table 3: Exemplary calculation of median EDSS by NAb status and age

Similar analyses on the potential association of demographic and clinical variables with ADA status, now for IFNβ and Natalizumab, were performed in the cumulative retrospective

ABIRISK cohort. Among all 20,695 patients on the ABIRISK database, 5,638 patients on IFNβ and 3,440 patients on Natalizumab were eligible for further analysis; that is, for these patients sufficient ADA test results and demographic and clinical data were available.

Concerning IFNβ, univariate analyses were performed for the association of NAb status with types of IFNβ (IFNβ 1b s.c., 1a s.c. and 1a i.m.), age at the commencement of treatment, gender, month of treatment commencement (with the idea of detecting a potential influence of vitamin D levels, which is known to be directly associated with the seasons of the year due to sun exposure[104-106]), month of birth and assay method used in different countries at different times (see fig. 1). Fig. 4 shows the results of the univariate analysis for different ABIRISK cohorts.

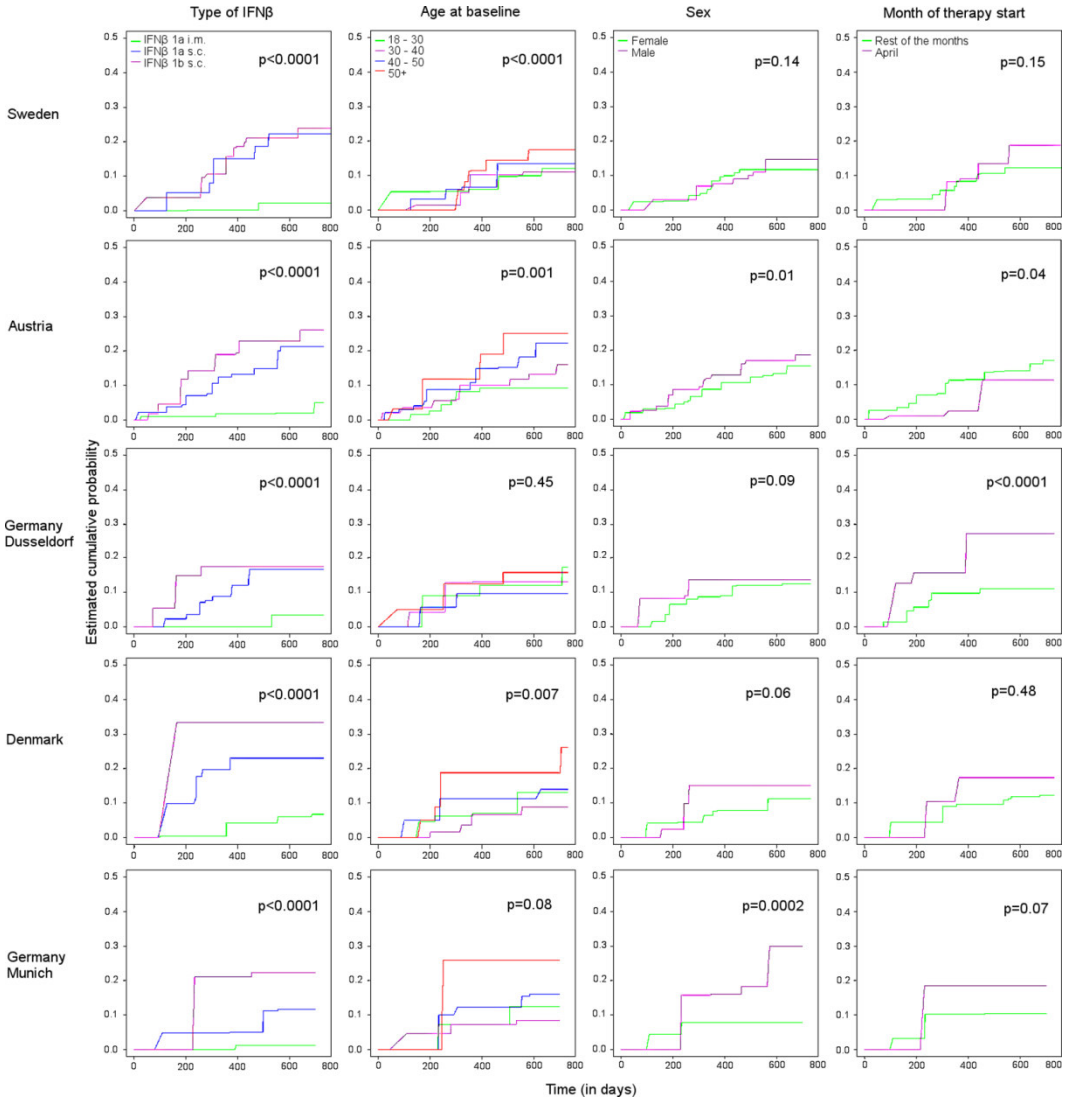

Fig. 4: Cumulative probability of developing IFN-NAb by cohort in relation to categorical variables with p-values

In all the cohorts, as expected, the type of IFNβ preparation was highly associated with NAb development, showing low rates for IFNβ 1a i.m. (1.1% to 4.2%), higher rates for IFNβ 1a s.c. (11.1% to 23.0%) and the highest rates for IFNβ 1b s.c. (17.4% to 33.3%). Most of the patients developed NAbs (independently from preparation) within 18 months after the commencement of treatment.

Age at the commencement of treatment was significantly associated with drug-induced immunogenicity in all the cohorts except Germany; that is, older patients have a higher risk of developing NAb.

Overall, male patients seem to have a higher risk of immunisation against IFNβ compared to females, with significant results in Austria and Germany – Munich and p-values close to significance in Denmark, Germany – Dusseldorf and Sweden.

Controversial results were obtained for month of treatment commencement, where the month of April was chosen (as the season with the lowest average Vitamin D levels) to match against all the other months of the year. While in both German cohorts treatment commencements in April seemed to provoke a higher risk of immunisation, in Austria a reverse result was generated, with a lower risk of NAb development when IFNβ-therapy began in April.

A clear dependency of NAb prevalence was found for assay used, which highlights different levels of sensitivity between the single methods. For instance, the MPA method detected higher rates of NAbs in Austria and Sweden (30% and 20%, respectively) than the MGA assay (8% and 7%, respectively). With the luciferase assay, in Austria 11% of patients were tested positive for NAbs, compared to only 4% in Sweden at the same time using the iLite method.

Preparation of IFNβ, age at treatment commencement, gender and month of treatment commencement were included in the multivariate analysis, which was adjusted for the assay method used since this was shown to act as a confounding factor. Fig. 5 shows the result of the multivariate Cox regression model, displaying hazard ratios for single variables.

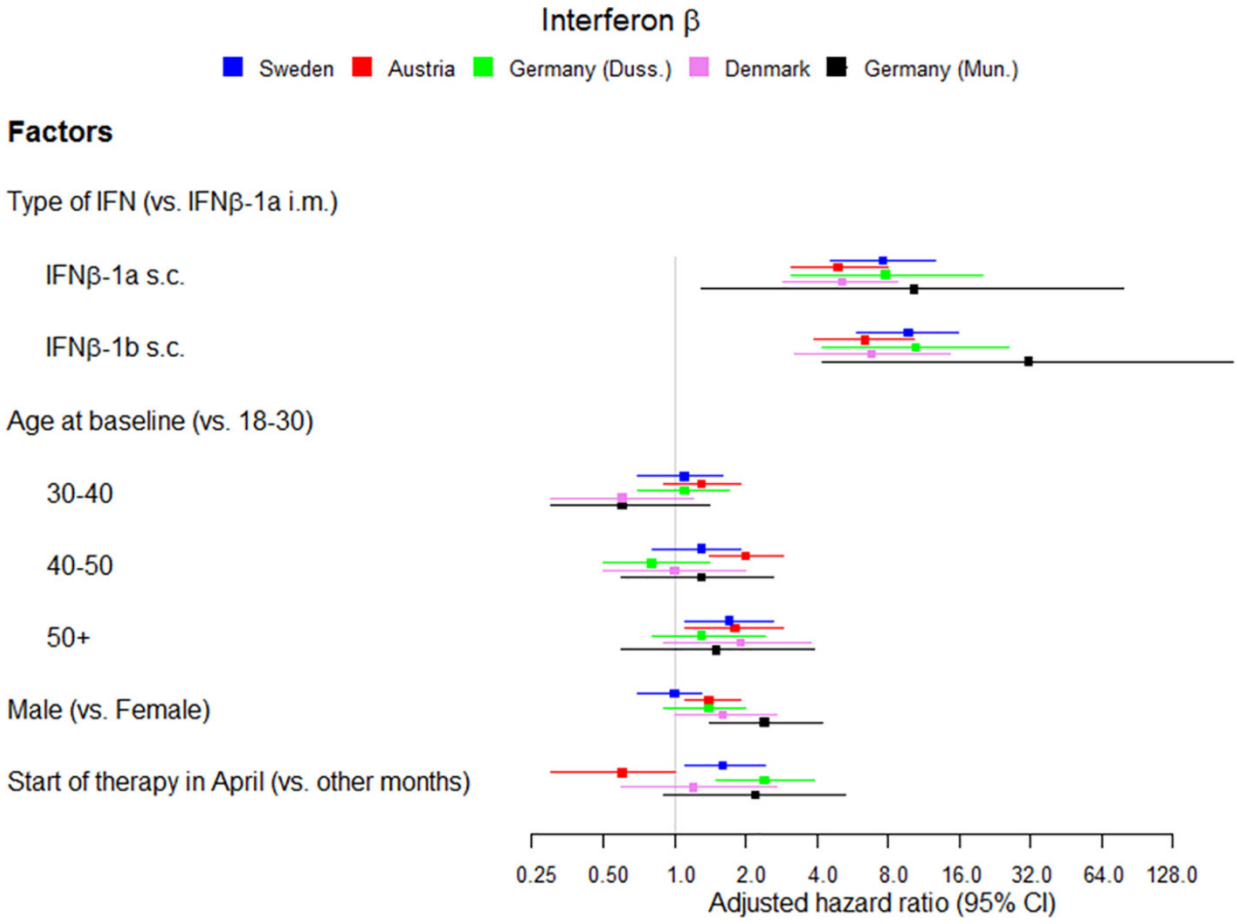

Fig. 5: Multivariate risk factors for IFNβ-NAb development in each cohort

For Natalizumab fewer variables were available for uni- and multivariate analyses. In contrast to IFNβ, there was only one assay method for ADA measurement, namely the bridging ELISA method provided by Biogen, so the results were more comparable between countries. Most of the patients developed ADAs within the first six months after treatment commenced. The variables associated with ADA status were country, age and gender. There were not

enough data available to reach a statistical power of 0.8 for the other clinical variables. The results are shown in fig. 6.

The rate of ADA positivity was similar, but not equal in the countries in which data were available (Austria, Sweden and Denmark). The age of treatment commencement was associated with ADA development, showing a significantly higher risk in older patients (>45 years). Regarding gender, an inverse association was found compared to IFNβ. Natalizumab ADAs were observed at higher rates in female patients (6.6% in female vs. 4.5% in male) at six months of treatment. The multivariate Cox regression model, analysing age and gender as risk factors, adjusted for country, did not show other results, confirming that older patients and women were at higher risk of presenting with Nataizumab ADAs.

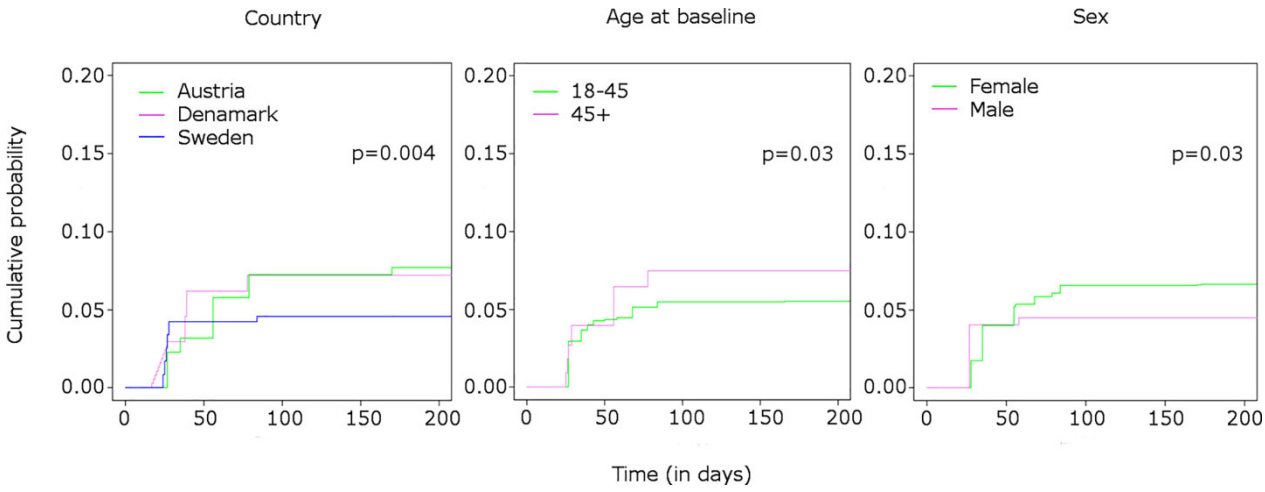

Fig. 6: Cumulative probability of developing Natalizumab ADAs by cohortin relation to categorical variables with p-values

In addition to the above-mentioned results of the joint publications generated within ABIRISK, we conducted a study focusing on smoking as a potential risk factor for NAb development during IFNβ therapy (see methods section 3.3). We used cotinine as an objective, easily measurable surrogate marker in order to distinguish patients by their smoking habits. In a small test cohort of 37 patients, we examined the accordance between cotinine

levels and self-reported smoking habits taken from the local database. In a second cohort we associated NAb status with cotinine levels without having any further information about the smoking habits of patients.

In the test cohort (Table 4, cohort A), of 37 patients (67.6% female, 32.4% male, median age 34 ranging from 21-55, 19 were NAb positive; 10 of these patients were on IFNβ 1b s.c., 8 on IFNβ 1a s.c. and 10 on IFNβ 1a i.m.). We did not find a significant difference of cotinine levels between NAb positive and negative patients. Fifty percent of NAb positive patients were classified as smokers by cotinine levels compared to 33% in the NAb negative group (p=0.32). However, we detected a discrepancy between self-reported (which was assessed at a different time point than cotinine testing) and cotinine test-defined smoking status in seven of the 37 patients (18.9%). Six of these patients were classified as non-smokers by cotinine ELISA, although they claimed to be smokers and one self-declared non-smoker was classified as a smoker.

Of 62 NAb negative and 61 NAb positive patients, of whom the smoking status was unknown (Table 4, cohort B), 29 were being treated with IFNβ 1b s.c., 43 with IFNβ 1a s.c. and 51 with IFNβ 1a i.m. Eighty-six patients (69.9%) were women and 37 (30.1%) men, and the mean age was 37.1±10.4 years. Forty-two patients (34.1%) were classified as smokers by measured cotinine concentration. NAb positive and negative groups did not show demographic differences, except for age, which was higher in NAb positive patients (p=0.019).

We did not find any difference in cotinine concentration between NAb positive and negative patients and, furthermore, the prevalence of smokers defined by cotinine was identical in both groups (p=1.000). Taking cohort A and B together (n=160), we found 34% and 39% smokers respectively in NAb negative and positive patients, as defined by cotinine levels. Fig. 7 indicated the prevalence of smokers and non-smokers dependent on NAb status in cohorts A and B taken together, with a p-value of 0.511. Also, the correlation of NAb titres with cotinine ODs was not significant (p=0.372).

Additionally, we found a significant negative correlation between sample storage time and cotinine levels. However, storage time did not differ between the tested patient groups, with 1.8 (1.0-6.2) years in smokers and 2.3 (1.0-6.0) years in non-smokers. In a regression analysis adjusted for sample age, we did not find an association between cotinine levels and NAb status.

COHORT A	NAb-negative	NAb-positive	total
non-smoker	12 (32.4%)	9 (24.4%)	21 (56.8%)
smoker	6 (16.2%)	10 (27.0%)	16 (43.2%)
total	18 (48.6%)	19 (51.4%)	**37 (100%)**

COHORT A+B	NAb-negative	NAb-positive	total
non-smoker	53 (33.1%)	49 (30.6%)	102 (63.7%)
smoker	27 (16.9%)	31 (19.4%)	58 (36.3%)
total	80 (50.0%)	80 (50.0%)	**160 (100%)**

Table 4: Number of NAb negative and positive patients divided into smokers and non-smokers defined by cotinine level

Cohort A comprises 37 patients with known self-reported smoking status, which were used for power calculation. For these, the distribution of smokers and non-smokers defined by self-reported smoking status is shown.

Cohort A+B includes all 160 patients, 37 of cohort A and 123 of cohort B. For these, the distribution of smokers and non-smokers defined by cotinine level only is shown.

For statistical purposes (power) we analysed approximately the same number of Nab negatives and positives not reflecting general NAb prevalence

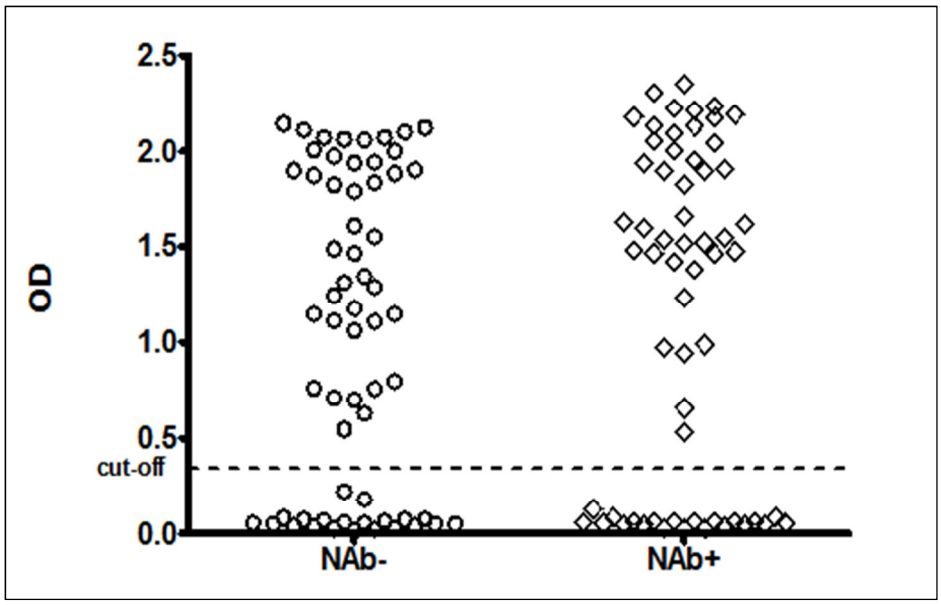

Figure 7: Cotinine levels by NAb status

Distribution of measured cotinine values (expressed as OD=optical density in photometric reading of cotinine-ELISA) in NAb negative and -positive patients. The predefined cut-off distinguishing smokers (OD-value below cut-off) from non-smokers is OD=0.3130. No significant difference between the two groups was detected (p=0.372).

5. Discussion

The aim of the present work was to assess current knowledge and clinical practice regarding ADA testing in MS patients and, in particular, to identify possible risk factors for ADA development in IFNβ- and Natalizumab-treated patients in order to provide an individualised, most effective immunomodulatory treatment for MS patients.

IFNβ was the first medication specifically registered for the treatment of relapsing-remitting MS which, more than 20 years later, is still of high importance in clinical practice[107]. There is sufficient knowledge about immunisation against IFNβ showing a reduced therapeutic effectiveness in NAb positive patients[76-83]. However, no standard practice of NAb testing has been introducted in the MS centres across Europe. The use of ADA testing in routine treatment monitoring varies from routine blood sampling every three months for IFNβ and even monthly testing at the beginning of Natalizumab treatment (which is the current practice in Innsbruck as well) to testing only if treatment failure or adverse effects such as anaphylactic reactions make immunisation against the drug probable. Furthermore, different NAb assays have been used for IFNβ in the different countries taking part in the ABIRISK project, leading to a major variation of NAb prevalence due to the varying sensitivity of the assays used for monitoring.

Especially for research that is focused on predictive factors for ADA development, as well as for the development of new biopharmaceutical drugs with attention to possible immunisation, a sensitive assay is required in order to detect patients who develop antibodies against the drug. In clinical practice, assay sensitivity may be a lesser issue, since treatment failure was reported predominantly for high ADA titres[81-82]. Therefore, a standardised ADA assay would be of high interest in order to facilitate a more consistent monitoring across different sites[108-109]. Our study was able to show the burden of neglecting ADA testing, both for patients and the healthcare system. There were several patients (especially in the 1990s when IFNβ was

introduced, but ADA testing was not commonly applicable) tested positive for IFNβ-NAbs after years of treatment without having any test before. Considering that NAbs usually develop within 18 months after the commencement of treatment[110], such patients could have been protected from an ineffective therapy by testing for NAbs routinely and switching to another treatment when high-titre NAb occurred. Apart from the burden on the patient caused by the administration of injections several times a week, which potentially cause side effects without having sufficient disease control, the costs for the healthcare system have to be taken into account as well, which was clearly shown by a recent cost calculation[90]. Even if the costs of routine Nab testing are considered, a year-long ineffective treatment with possible disease might be lowered by switching to an effective treatment as soon as an immunisation against current therapy is observed[111]. Beside these considerations, one should keep in mind that there are a number of alternative medications for the immunomodulatory treatment of MS. These therapeutic options may at first sight make the application of a potentially immunogenic drug for MS questionable. However, several clinical trials were not able to show a therapeutic superiority of new basic treatments (not considering "escalation treatments" such as Natalizumab or Alemtuzumab) over IFNβ[19-24]. Likewise, with the broader availability of therapeutic options, NAb testing gains even more importance. When other treatment options are available, it would be irresponsible to continue a treatment that has lost its effectiveness due to immunisation. Therefore, NAb testing in IFNβ-treated patients should be enforced more than ever in order to facilitate an individualised MS therapy.

For Natalizumab, we demonstrated that ADAs occur very early after treatment initiation, usually right after the first infusion and nearly always within the first six months of treatment. Additionally, we could show that long-term ADA persistency is highly predictable after six months of treatment. Regarding the risk factors for immunisation against Natalizumab, we were able to demonstrate higher ADA prevalence in older patients, as well as in females. The available data were not sufficient for further analysis due to the low ADA levels of about 6%.

Considering these findings, screening for ADA after treatment with Natalizumab commences can be applied within a limited time interval in order to avoid the additional costs of long-term ADA testing. This will provide an important tool to detect patients with restricted therapeutic effectiveness very early, especially when considering the additional risk of PML. Moreover, ADA are known to be associated with anaphylactic reactions during infusion[87-88]; thus repetitive testing during the first months of treatment may prevent patients from such adverse events when high-titre ADAs are detected early enough. In contrast to IFNβ, for Natalizumab there is the advantage of a unique ADA test[84], introduced together with the release of the drug on the market. This is reflected by better accordance of test results throughout the ABIRISK cohorts in comparison to greater variation of detection of IFNβ NAb. Fig. 2 shows the rapid introduction of Natalizumab ADA testing in clinical routine, while IFNβ NAb testing was largely dependent on reimbursement of the assay and clinical practice in different countries. The decline of ADA testing in the case of Natalizumab in the last few years may be caused by improved knowledge of very early ADA development in this therapy, so that the extensive long-term testing that we observed for some patients in our database in the first years after approval of Natalizumab seems obsolete. However, NAb testing in IFNβ-treated patients, advantageously, showed a consistently high level during the last years once awareness of immunisation was raised at most MS centres.

However, recommendations for ADA testing still vary. For IFNβ there is a belief that one should test only 12 and 24 months after treatment commences, with repetitions every three to six months in the case of a positive NAb result[77,108]. For Natalizumab, recommendations vary from monthly testing to one test only after six months. Based on our findings in the largest ADA database analysed so far, the clinical application of ADA testing could be adapted.

A primary target of the current work was to detect potential risk factors for immunisation, considering all available demographic and clinical data, including modifiable risk factors such

as smoking. For these analyses, we focused on IFNβ because it is only for this treatment that sufficient statistical power was achieved for more variables.

We were able to confirm the previously reported[112] dependence of NAb development from the IFNβ formulations used, showing the highest NAb occurrence in IFNβ 1b s.c. and the lowest in IFNβ 1a i.m. in all cohorts. The higher immunogenicity of IFNβ 1b s.c. is thought to be attributed to its lack of glycosylation, which facilitates formation of aggregates. This process has been described as triggering immunologic reactions[113]. Immunogenicity against IFNβ 1a s.c. was shown to generate higher and more persistent antibody titres[95,114]. Although we observed a switch to less immunogenic IFNβ 1a i.m. in our database over time, IFNβ 1b s.c. and IFNβ 1a s.c. are still in widespread use, and studies have suggested a higher treatment efficacy of these two preparations, especially when considering NAb negative patients compared to IFNβ 1a i.m..[115-117]

For IFNβ as well as for Natalizumab, we found a clear increase of ADA development in older patients, whereby age at the commencement of treatment was used in order to exclude a bias of treatment duration on age analysis. The impact of age on ADA development had been analysed in previous studies[118,119]; however, only one study found a higher median age in NAb positive patients[114]. Due to the large dataset, we were able to show a highly significant association of ADA development with age in all cohorts. The reason for the higher predisposition to immunisation among older patients may be assumed in the mechanisms of the immune system, which change over a life-time. On the one hand, thymic function declines in adults, so that the source of naïve T-cells is reduced in older patients. On the other hand, pathogen exposure over the years causes an increasing number of memory cells which, together with a continuous loss of naïve T-cell repertoire and decrease of total lymphocyte count, influences the adaptive immune responses against novel antigens, increasing the potential of auto-immune reactions. The lymphopenic condition in older individuals is known to favour the proliferation of high affinity autoreactive immune cells[120-122].

We could also demonstrate an association between gender and ADA development, whereby, interestingly, for IFNβ male subjects were more likely to develop NAb, whereas Natalizumab ADAs were more frequently observed in female patients. Generally, autoimmune diseases often show higher prevalence in females[123-125], as is reported for MS as well, whereby genetic factors, such as X-chromosome-linked factors, which lead to a breakdown of immune tolerance[125,126], could play a role, as well as sex hormones, which are thought to be important regulators of the immune system[127]. In women, a slower pharmacokinetics has been reported for several drugs, which may lead to a longer exposure to the drug and facilitate the evolution of autoimmune mechanisms[128]. The different gender relation between IFNβ and Natalizumab could be explained by the differing biochemical structures of the drugs, that is, IFNβ is a cytokine while Natalizumab is a monoclonal antibody. This could result in differing immune responses after administration of the drug; however, the inverse association of gender with immunisation against the two drugs cannot be explained due to scarce knowledge of the association between gender and ADA development. The only study with results on this topic found a higher proportion of women among patients developing IFNβ NAb after 12 months of treatment[129]. However, this result could not be confirmed in a later study[119], in which the number of subjects in both studies was markedly lower than in our ABIRISK cohort. To the best of our knowledge, no study has been conducted investigating the association between gender and ADA development for other monoclonal antibodies.

Since there is evidence in the literature of the association of month of birth with risk of developing MS, albeit with very low effect size,[130-131] we investigated a potential influence of this factor on the development of IFNβ NAb as well. However, no association was found.

The results were controversial for month of treatment commencement. If patients started IFNβ treatment in April they seemed be at higher risk of NAb development in Germany, but at lower risk in Austria when matched against the other months, while in Denmark and Sweden the association was not significant. A possible reason for a seasonal dependence of

immune processes could be allergies caused by pollens or Vitamin D levels, for which there is a seasonal variation and which is directly associated with sun exposure. Since there were such differing results in neighbouring countries (Austria and Germany), it seems unlikely that there is a common seasonal factor influencing NAb development in IFNβ-treated patients. In any case, this fits well with similar controversial results in hundreds of studies investigating a potential influence of Vitamin D on MS and Vitamin D supplementation as possible additional treatments of MS.

In general, the retrospective ABIRISK database is the largest study cohort on collected ADA data in MS so far, which facilitates a high statistical power by comparison with other smaller cohorts. However, there are some limitations to be noticed, generated especially by varying routine practices with respect to ADA testing and the differing ADA assays used in the countries that are participating the ABIRISK project.

Differing follow-up schemes were also observed in the participant countries. For example, while ADA tests were routinely performed during treatment monitoring in Austria, Sweden and Denmark, in Germany ADA testing was left to the discretion of the treating physician, so that in some cases the test was performed only when ADAs were suspected due to treatment failure, which led to higher detection rate of ADAs in tested patients and therefore does not reflect the whole population of MS patients on IFNβ or Natalizumab treatment. Furthermore, the monitoring intervals were different between patients, since the database included patients right from the beginning of ADA testing until the introduction of regular testing every three or six months. Due to long intervals between visits or between the commencement of treatment and the first monitoring, the time point of ADA development after treatment start cannot be exactly determined. This was shown by an own analysis of Natalizumab ADAs in patients in Innsbruck, who had continuous monitoring every month, where we found that most patients developed ADAs right after the first infusion, whereas in the whole cohort we were only able to state that ADAs usually developed within the first six months of treatment. Similar effects

of missing continuous data have to be expected for IFNβ. However, in order to limit the bias due to the lack of sufficient time points of ADA testing, only patients with regular follow-up (at least one ADA test before and one after 12 months after treatment start) were included in the analysis. Moreover, for IFNβ we had to adjust our analyses of variables associated with possible risk for ADA development for the ADA assay because six different assays were used over time in different countries and we found variable sensitivity with different ADA prevalence due to the assays. Additionally, all correlation analyses were performed separately for each country in order to avoid greater bias due to differing follow-up regimes and assays used. Since correlations for single variables did not show significant differences through the countries, analyses of the pooled cohorts were conducted in a second step.

Because recent publications had cited smoking as a potential risk factor for ADA development[132-133], both based on surveys in which patients had to declare their smoking habits, we focused on an alternative way to investigate the influence of smoking on NAb development in IFNβ-treated patients by using cotinine as an easily detectable objective surrogate marker for smoking habits.

After entering the body through the lungs, nicotine is metabolised via hepatic pathways, whereas only a small amount (5-10%) of nicotine gets eliminated by renal excretion. The main hepatic metabolite of nicotine is cotinine (about 80%) and therefore it correlates well with amount of current nicotine intake by tobacco consumption, as shown in various publications[134]. Although passive nicotine intake as well as varying nicotine content in different cigarette trademarks may have a minor influence on cotinine concentration, cotinine has been shown to be a reliable marker to distinguish between smokers and non-smokers[135-136] and is widely used in different businesses, such as in the insurance industry.

Several studies have been conducted investigating the association of nicotine intake stated by patients with measured cotinine concentration and differing results have been obtained. Although there is generally a high agreement of self-reported smoking habits with cotinine

levels[136-139], in some studies discrepancies were found between patients' statements and measured cotinine of up to 17%[140-141]. A study investigating environmental tobacco exposure in non-smokers yielded low sensitivity (89%) and specificity (56%) of questionnaires in comparison to cotinine testing[142]. Therefore, the measurement of cotinine seems to a superior method for epidemiological studies on the influence of tobacco consumption, which is supported by better association of cotinine with typical smoking related outcomes than self-reported smoking habits[143].

In seven of 37 patients we were not able to confirm self-reported smoking habits by measured cotinine concentration. Six of these patients claimed they were smokers, but this could not be detected by the cotinine test. This discrepancy may be due to different time points of assessment. Self-reported smoking status was mostly evaluated at disease onset and sometimes later during the disease, but not exactly at the time of NAb testing. Thus, we might expect some patients to have stopped smoking between disease onset and treatment start or to smoke irregularly with longer interruptions, which are not reflected by the comparatively short half-time of cotinine. However, in a verification study (data not shown) on laboratory employees, cotinine levels matched well with smoking habits, identifying heavy smokers by high cotinine levels and occasional smokers by lower, but still positive cotinine concentration. One of seven patients with discrepant results was identified as a smoker, although the person claimed to be a non-smoker, which might be expected in some cases due to inaccurate statements or passive smoking.

In our analyses we found significantly decreasing cotinine-levels in samples with a longer storage period, even if they were continuously frozen at -20°C, which shows that cotinine might not be stable in serum for many years. We do not believe this fact to bias our results since we used the most recently withdrawn samples (mean time from sampling to analysis of 2.1 years) and storage time did not differ significantly between our study groups.

Regarding general smoking prevalence, official European statistics confirm a higher smoking prevalence (26% according to a recent survey) and higher exposure to passive smoke as well in Austria in comparison with northern European countries, where the prevalence of smokers seems lower, e.g. 11% in Sweden[144].

A recent publication[132] indicated a significantly higher risk of NAb development in smoking MS patients, showing an odds ratio of up to three for risk of NAb during IFNβ therapy in smokers compared to non-smokers. This study was based on a large registry and questionnaires were used to determine smoking habits. The same group found an association between smoking and Natalizumab ADAs as well, again in a questionnaire-based study[145].

Our study contradicts the hypothesis that smoking as a risk factor with respect to NAb development. Despite a relatively small study cohort, the absence of any trend towards smoking or non-smoking in NAb positive patients makes it unlikely that a higher statistical power would have led to a different result.

Our finding is supported by another recent study[133] which did not find any association between smoking and risk for NAb development either. Although performing a retrospective analysis on medical records and relying on self-reported smoking status, the same results were obtained as those for our study, showing a smoking prevalence of exactly 27% in both NAb positive and negative patients with an odds-ratio of 0.94.

Taken together, smoking status defined by cotinine levels does not seem to favour an influence of smoking on development of anti-drug antibodies.

In summary, the research presented here about ADA development in treatment with IFNβ or Natalizumab in MS patients and, particularly the effort to investigate potential risk factors that facilitate the occurrence of ADAs is still of high interest, even considering the newly-approved biological drugs for MS treatment. Both Natalizumab and IFNβ are still used to treat MS and the detection of ADAs seems even more important because switching to other medications is now possible in instances of the loss of therapeutic effectiveness or adverse

events. Based on a local cohort analysis (see fig. 3) we were able to underline that ADAs reverse the therapeutic benefit of a pharmacological treatment and causes more rapid disease progression, if they are not detected early enough.

6. Conclusion

The current thesis comprises a summary of local and cohort-wide analyses generated by retrospective data within the ABIRISK project.

In a large cohort of more than 20,000 patients and over 42,000 samples, we were able to confirm the previously described prevalence of ADAs in relation to the various IFNβ preparations and Natalizumab. Furthermore, it was shown that IFNβ NAbs usually develop between six to 18 months after the commencement of treatment, while Natalizumab antibodies occur within the first six months of therapy. We described the various practices of ADA testing and assays in European countries and advocated a standardised procedure for the future in order to homogenise clinical practice and research into ADAs.

The main target of the analyses was to identify possible risk factors for ADA development. For both IFNβ and Natalizumab, we demonstrated an association between age and the gender of patients with ADAs, while other factors (such as month of treatment commencement, smoking, CSF parameters, family history and pre-treatment) were not associated with ADA status. However, we further demonstrated the influence of ADAs on clinical outcomes and underlined the necessity of implementing ADA testing in clinical routines in order to avoid the ineffectiveness of treatments and adverse reactions, as well as unnecessary costs for the healthcare system.

Furthermore, a locally performed study is presented, measuring cotinine in the serum samples of MS patients treated with IFNβ in order to investigate the possible coincidence of NAb and smoking. We investigated whether smoking could be a risk factor for NAb development in IFNβ-treatment by using cotinine as surrogate marker; however, we did not find any influence of cotinine levels on NAb occurrence.

We believe that, based on these results, further research is necessary, especially for the analysis of the prospectively collected samples within the ABIRISK project.

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