Exploring body fluid biomarkers for Diagnosis, Prognosis and Treatment Monitoring in MS

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Exploring body fluid biomarkers for Diagnosis, Prognosis and Treatment Monitoring in MS

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geboren te Hilversum

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copromotoren:	prof.dr. J. Killestein prof.dr. C.E. Teunissen

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General introduction, aim and outline of the thesis

Epidemiology

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). It affects more than 2 million individuals worldwide and twice as many women as it does men, although men appear to have a more negative prognosis.[1] In the Netherlands, approximately 1 in 1000 persons are diagnosed with MS.[2] The onset of MS mainly occurs in early adult life (20-40 years).[3] The cause of MS is unknown, but a complex interplay between environmental and genetic factors contribute to the development of MS. Environmental and lifestyle risk factors such as vitamin D deficiency (related to reduced exposure to sunlight), diet, obesity in early life and cigarette smoking are risk factors for MS development.[4] A genetic predisposition has been established and several genes have been associated in MS (the most significant remains the HLA-DRB1*1501 haplotype).[5]

Pathology

From a pathological point of view MS is a chronic inflammatory, demyelinating and neurodegenerative disease of the brain and spinal cord in which the adaptive immune system plays a key role in the pathogenesis of MS. The immune response to myelin results in a cycle of periodic attacks to the myelin. The myelin is wrapped around the nerve cells by oligodendrocytes and essential for an efficient processing of signals throughout the brain. Why the adaptive immune system attacks the myelin is unclear. Focal inflammatory demyelinating lesions in the CNS represent a process marked by leakage of the blood-brain barrier, influx of immune cells, inflammation of the tissue. These lesions, after an attack, can be seen using magnetic resonance imaging (MRI). In the past, these lesions were only visualized in white matter but with recent developments in MRI techniques, lesions are also detectable in gray matter.[6]

Besides demyelination, axonal and neuronal loss (neurodegeneration) and astrocytic gliosis are important hallmarks of the MS pathology. Neurodegeneration is relevant because it is the main underlying mechanism of permanent clinical disability and cognitive decline.[7]

Clinical features

The MS patient can experience a sudden exacerbation of symptoms, due to the attack of the immune system on myelin. A period of symptoms is called a relapse (or 'schub'). Depending on the localization of the acute inflammatory demyelinating lesions and the extent of the inflammatory process, the patient may experience a variety of symptoms, such as optic neuritis (inflammation of the optic nerve), muscle weakness or sensory deficits. Disability

of patients with MS is usually rated using the expanded disability status scale (EDSS).[8] This scale ranges from 0 (no objective neurological impairment) to 10 (death due to MS).

The majority of MS patients (85%) initially experience a relapsing remitting disease course (RRMS). This course is characterized by an (almost) complete clinical recovery after episodes of neurological deficits. In the initial phase of the disease, patients may present with only one episode of neurological deficits and not fulfill the MS diagnostic criteria (see section 'Diagnosis' below). This disease course is referred as a clinically isolated syndrome (CIS). Almost 60-80 percent of the CIS patients convert to MS. Up to 80 percent of the RRMS patients will subsequently develop a secondary progressive course (SPMS) in untreated populations. After this conversion point the inflammatory component of the disease is less prominent but an increase of neurodegeneration occurs. The remaining 15 percent of the patients have a primary progressive course (PPMS) from onset without relapses. Classifying patients in these phenotypes is important for communication, prognostication, design and recruitment of clinical trials, and treatment decision-making. However, these phenotype categories do not provide information about the ongoing disease process. Using additional information about disease activity detected by clinical relapses or imaging as well disability progression is meaningful. MS patients can be classified as active or not-active and progressing or not-progressing. This classification is now added to the standard disease course description in the new definitions of the International Advisory Committee on Clinical Trials in MS.[9]

Diagnosis

The problem of diagnosing MS is largely solved by using MRI, clinical presentation and neurological assessment, but can still be complicated in some patients. This may occasionally result in an undesirable late diagnosis and subsequently delayed treatment. The diagnosis is based on the demonstration of both dissemination in space and time as reflected in the diagnostic criteria, currently the revised McDonald diagnostic criteria (2017).[10] Dissemination in space is defined as ≥1 lesions in ≥2 typically MS regions: (juxta)cortical, infratentorial and spinal cord. Dissemination in time is defined by development of new MS lesions during follow up or an active lesions with gadolinium enhancement.

Biomarkers: Blood and CSF

In MS, there are only very few body fluid biomarkers that are accepted as reliable and practicable parameters so far. Biomarkers play a role in determining MS (e.g. cerebrospinal fluid-specific oligoclonal bands (OCB)), in differential diagnosis (e.g. anti-aquaporine-4 and anti-MOG serology in neuromyelitis optica spectrum disorders), in monitoring disease

modifying treatment (DMT) (e.g. anti-interferon antibodies and anti-natalizumab antibodies) and predicting adverse effects (screening anti-JCV antibodies in natalizumab treatment for the risk of PML).[11]

Currently, the most promising body fluid biomarker in MS, reflecting acute neuroaxonal damage is neurofilament light (NfL). This is a cross disease axonal damage biomarker[12] that increases in cerebrospinal fluid (CSF) and serum of RRMS patients during relapses and concomitantly to the appearance of new T2 and/or GE lesions, returning to baseline within a couple of months after the acute event.[13, 14] In addition, serum NfL levels decrease in individual RRMS patients after disease modifying therapy (DMT) initiation.[15-19] The magnitude of the decrease in NfL levels correlates with the efficiency of the DMT in reducing relapse rate, and NfL was reported to further decrease in individuals switching from first-line to second-line DMTs.[10] NfL holds high potential to monitor inflammatory disease activity in individual MS patients in the clinical setting. The fact that NfL is detectable in serum makes this biomarker more suitable for treatment monitoring then CSF -NfL. CSF collection is too invasive for longitudinal sampling when monitoring treatment.

The list of promising biomarkers in MS is extensive and the road to clinical application long. Nevertheless, biomarkers reflecting and predicting disease activity and progression are crucial for personalized medicine and may provide guidance in the rapidly evolving treatment armamentarium in MS. MS biomarker research is thriving and data are being generated rapidly.[11]

Aims and outlines of this thesis

The main aim of this thesis was to explore novel biomarkers that are associated with disease progression and to explore biomarkers in monitoring treatment and treatment safety. With this aim, different markers reflecting potential roles in neuroinflammation, demyelination, and axonal damage were analyzed. For monitoring DMT and DMT safety, natalizumab concentrations and neurofilament light were explored. Two other biomarkers with a more diagnostic purpose were also worthy to analyze.

Part 1: Diagnostic biomarkers

In this part two biomarkers with a diagnostic purpose were analyzed: kappa/lambda free light chains and messengerRNA from blood platelets. The main aim of **chapter 1** was to validate kappa free light chains and lambda free light chains indices as a diagnostic biomarker in MS compared with OCB in a large multicenter study including samples from eighteen MS-centers across Europe. This set up was chosen because large scale studies comparing

diagnostic performance of the two methods (free light chains and OCBS) and to define the cut-off of free light chains were lacking.

The need for a reliable blood-based biomarker (less invasive than CSF) for making a straightforward MS diagnosis is obvious. Therefore, in **chapter 2** messengerRNA from blood platelets was analyzed. As a result of their involvement in the immune response and potential role in the progression and development of the disease, we hypothesized that blood platelets of MS patients contain a disease-related RNA-signature which could be used as a diagnostic tool.

Part 2: Disease course biomarkers

Validated and discriminative body fluid biomarkers reflecting and predicting natural disease progression are lacking, therefore in this part, we analyze three potential markers for predicting disease progression.

In **chapter 3** we evaluated if cerebrospinal fluid mtDNA concentration in multiple sclerosis can serve as a marker of ongoing neuropathology and help differentiating between MS disease subtypes. In **chapter 4** acid sphingomyelinase activity and altered sphingolipid metabolism was explored as potential biomarkers in serum of MS patients, to predict active and progressive disease, and response to disease modifying therapy. In **chapter 5** Tissue Transglutaminase was measured and correlated to clinical and MRI parameters to evaluate if this biomarker is a promising marker for progression of multiple sclerosis.

Part 3: Treatment monitoring biomarkers

The main aim of **chapter 6** was to investigate the potential of serum neurofilament light (sNfL) as a biomarker of disability progression with minimal contribution of disease activity, i.e. relapses and new T2 or GE MRI lesion formation, collectively referred to as focal inflammatory activity. We measured sNfL longitudinally in a cohort of natalizumab-treated RRMS patients followed clinically. We examined whether sNfL at baseline (at the initiation of NTZ treatment) predicted disability progression. In **chapter 7** we compared MS disease activity after different washout periods in patients switching from natalizumab to fingolimod. In addition, we investigated several body fluid factors that possibly influence recurrence of disease activity, including serum natalizumab concentration and lymphocyte counts. In the last chapter (**chapter 8**) we wanted to give neurologists insight in natalizumab concentrations at time of re-dosing, therefore we investigated longitudinal natalizumab concentrations in 80 patients in relation to disease activity, with possible influencing factors.

The thesis concludes with an overview of the findings presented in part 1, 2 and 3, including interpretation of the data in light of the available evidence and some future directions.

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Part 1

Diagnostic biomarkers

Chapter 1

Kappa free light chains are a valid tool in the diagnostic of multiple sclerosis: a large multicenter study.

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Multiple Sclerosis, 2019

Abstract

Objective: To validate kappa free light chains (KFLC) and lambda free light chains (LFLC) indices as a diagnostic biomarker in multiple sclerosis (MS).

Methods: We performed a multicenter study including 745 patients from eighteen centers (219 controls and 526 CIS/MS patients) with a known oligoclonal band (OCB) status. KFLC and LFLC were measured in paired cerebrospinal fluid (CSF) and serum samples. Gaussian mixture modeling was used to define a cut-off for KFLC and LFLC indexes.

Results: The cut-off for the KFLC-index was 6. 6 (95% confidence interval (CI) 5.2 – 138.1). The cut-off for the LFLC-index was 6.9 (95%CI 4.5 – 22.2). For CIS/MS patients, sensitivity of the KFLC-index (.88; 95%CI .85-.90) was higher compared with OCB (.82; 95%CI .79-.85; p<0.001), but specificity (.83; 95%CI .78-.88) was lower (OCB: .92; 95%CI .89-.96; p<0.001). Sensitivity and specificity for the LFLC-index were both lower compared with OCB.

Conclusion: Compared with OCB, the KFLC-index is more sensitive but less specific for diagnosing CIS/MS. Lacking an elevated KFLC-index is more powerful for excluding MS compared with OCB but the latter is more important for ruling in a diagnosis of CIS/MS.

Introduction

Cerebrospinal fluid (CSF) assessment is often part of the diagnostic workup for multiple sclerosis (MS) and its value is supported by the latest 2017 revisions of the McDonalds criteria.[1] CSF examination is frequently performed in diagnosing MS for excluding alternative diagnoses although in the current MS criteria[2] assessment of oligoclonal IgG bands (OCB) plays a limited role. However a recent study showed the added value of OCB in the MS diagnostic criteria.[3] In the 2017 revisions the OCB have a prominent role in patients with a clinically isolated syndrome (CIS). The presence of both magnetic resonance imaging (MRI) criteria for dissemination in space (DIS) and CSF-specific OCB will enable to establish the MS diagnosis in patients with a single clinical episode suggestive of CNS inflammatory demyelinating disease. The OCB assessment also has important prognostic value in CIS and MS[4, 5]. However, the assessment of OCB is labor intensive, requires trained personnel and is in some cases examiner- and method-dependent, which may affect its reliability.

Alongside intact immunoglobulins, which are composed of two heavy and two light chains, plasma cells produce and secrete immunoglobulin free light chains (FLC), of either kappa (KFLC) or lambda (LFLC) chains. KFLC and LFLC can be detected in both CSF and serum.[6-10] Since the late 1970s, multiple studies have reported increased CSF levels of KFLC in MS.[6-14] The analytical specificity of the earlier methods (e.g. radioimmunoassay[15, 16], quantitative enzyme-linked immunosorbent assay[8, 17]) was insufficient, but with the recent emergence of the more sensitive nephelometric and turbidimetric FLCs assays, research in this field has been revived. Nephelometric (and turbidimetric) FLC level determination has the additional advantage compared to OCB of being assessed by an automated procedure and being quantifiable.[18]

Using the FLC assay[19], recent studies showed that both CSF KFLC levels and the KFLC index are increased in patients with CIS or relapsing remitting MS (RRMS) compared with controls.[8, 14, 18, 20-22] The use of an index measure is necessary, for example [CSF KFLC/serum KFLC]/[CSF albumin/serum albumin], to include blood-CSF barrier permeability.[10, 14] The KFLC index has comparable sensitivity and specificity to OCB for diagnosis of MS and CIS.[14, 21, 23] However, large scale studies comparing diagnostic performance of the two methods and to define the cut-off of FLC are lacking. The main aim of this study was to validate KFLC and LFLC indices as a diagnostic biomarker in MS compared with OCB in a large multicenter study including samples from eighteen MS-centers across Europe.

Methods

Patients and controls

Eighteen MS centers participated, located in the Netherlands, Spain, France, Belgium, Hungary, Italy, Poland, Turkey, Denmark, Serbia, Austria and Switzerland. We selected 745 paired CSF/serum samples from patients with known OCB status, diagnoses as CIS (n=242), RRMS (n=235), primary-progressive MS (PPMS)(n=41) and secondary-progressive MS (SPMS)(n=8). We also included inflammatory neurological disease controls (INDC)(n=67), non-inflammatory neurological disease controls (NINDC)(n=76), symptomatic controls (SC) (n=49) and healthy controls (HC)(n=27) as defined previously.[24] The different control groups were pooled into one control group (n=219). The CIS and MS patients were also pooled (CIS/MS) (n=526).

The large majority (84%) of the CIS/MS patients fulfilled the 2010 McDonald criteria[2] but in some cases the patients were diagnosed according the 2005 McDonald criteria[25] (16%). Table 1 presents the demographic and clinical characteristics of the patients and controls.

All participants gave written informed consent at the center where the CSF/serum was conducted.

Table 1. Demographic and clinical characteristics of the included patients and controls at the time of lumbar puncture.

Disease group	*N*=745	Sex (female) *N* (%)	Age (years) Mean ± SD	Stage/type of disease RR/SP/PP	Disease duration (months) Median (IQR)	No. of patients using treatment	No. of patients on corticosteriods Median (IQR)	EDSS Median (IQR)	OCB positive *N* (%)
CIS	242	177 (73.1)	35 ± 10	n.a.	0.8 (0.3–3.0)	1	4	2.0 (1.0–2.5)[a]	186 (76.9)
MS	284	170 (59.9)	38 ± 11	235/8/41	13.1 (2.4–48.1)	3[b]	10	2.0 (1.5–3.5)[c]	245 (86.3)
Controls	219	130 (59.4)[d]	42 ± 12	n.a.	n.d.	1	1	n.a.	17 (7.8)
- NINDC	76	49 (64.5)	45 ± 13	n.a.	n.d.	0	0	n.a.	4 (5.3)
- INDC	67	33 (49.3)	42 ± 13	n.a.	n.d.	1[e]	1[e]	n.a.	13 (19.4)
- SC	49	32 (65.3)	39 ± 10	n.a.	n.d.	0	0	n.a.	0 (0)
- HC	27	16 (59.3)	41 ± 10	n.a.	n.d.	0	0	n.a.	0 (0)

RR: relapsing-remitting; SP: secondary progressive; PP: primary progressive; disease duration: time between CSF lumbar puncture and date of onset first neurological complaints; EDSS: Expanded Disability Status Scale, OCB: oligoclonal IgG bands; CIS: clinically isolated syndrome; MS: multiple sclerosis; n.a.: not applicable; n.d.: not determined; NINDC: non-inflammatory neurological disease control; INDC: inflammatory neurological disease controls; SC: symptomatic control; HC: healthy control; CSF: cerebrospinal fluid; CLIPPERS: chronic lymphocytic inflammation with pontine perivascular enhancement responsive to steroids.
All participants gave written informed consent at the center where the CSF/serum was conducted.
[a]In 178 CIS patients, an EDSS was available.
[b]Disease-modifying treatment in combination with corticosteroids was used in two MS patients. In one patient, only disease-modifying treatment.
[c]In 107 MS patients, an EDSS was available.
[d]In six control patients, the sex was unknown.
[e]One INDC (CLIPPERS syndrome) patient had steroids and methotrexate at the time of lumbar puncture.

CSF and serum samples

Only CSF samples that were immediately centrifuged and stored in polypropylene tubes within two hours at -80° C, at the local center, were included. The assessment of OCB had been performed by isoelectric focusing (on agarose or polyacrylamide gel) followed by immunofixation by the centers as part of the diagnostic work-up.

Samples were taken between 2005-2016, with a median age of 2.9 years (IQR 1.7-5.7).

We used fresh aliquots and in our lab we did not freeze and thaw the samples during the analyses. As far as we know, no effects of freezing and thawing have been reported.

KFLC, LFLC and albumin analysis

KFLC, LFLC and albumin concentrations in CSF and serum samples were analyzed using the turbidimetric analyser SPAplus® (The Binding Site, Birmingham, UK) with the serum free light chain immunoassay (Freelite®, The Binding Site, Birmingham, UK) according to the manufacturer's instructions. All samples were measured centrally in the Neurochemistry Laboratory of the Department of Clinical Chemistry of the VU University Medical Center (VUmc), Amsterdam, The Netherlands. All samples were run blinded for the clinical data.

To verify the QC data supplied by the manufacturer we calculated intra-assay coefficient of variation (CV) by taking the mean CVs of 4 replicates of 5 samples (CSF/serum) within one run. We calculated inter-assay CV based on n=5 samples (CSF/serum) measured in 5 different days. CV values for KFLC and LFLC were all found to be lower than those supplied by the manufacturer (suppl. Table 1). CV values for albumin were comparable to those supplied by the manufacturer. Assay linearity was experimentally confirmed for albumin (serum and CSF) and the FLC assays in serum and showed that recalculated values varied by 25.7% (KFLC) and 14.2% (LFLC) from the original value.

For 29 samples CSF albumin results were below detection. Here, we assigned a random uniform value between 35 mg/ml (lowest detected value in undiluted rerun) and 175 mg/mL (formal detection limit).

FLC indices

We determined the CSF/serum quotients (Q FLC) of KFLC and LFLC and calculated indices, in order to take possible blood-CSF diffusion into account. The FLC indices were defined as [Q FLC] x [serum albumin/CSF albumin].

Statistics

Differences in demographics, clinical characteristics, FLC concentrations and FLC indices were tested via the Mann-Whitney U test for comparison of 2 groups of non-normally distributed data. For comparison of more than 2 groups, Kruskal-Wallis test with post hoc Dunn's multiple comparison test was applied. For normally distributed continuous variables, an independent samples *t*-test, or one-way ANOVA with post hoc Bonferroni correction was applied. For binary variables, a Chi-square test was performed. These statistical analyzes were performed in SPSS 22.0 (IBM Crop., Armonk, NY, USA).

Gaussian mixture modeling was used to define cut-offs for abnormal FLC indices using the R statistical software program version 3.2.1 mixtools package. First, the number of

distributions that best described the data was determined with the R boot.comp function. Next, we defined a data-driven cut-off as the point where the lines of two fitted normal distributions crossed each other. The main analyses included all subjects. Data were log-transformed because FLC indices were not normally distributed. Based on the defined cut-off subjects were classified as positive or negative for kappa or lambda FLC as binary result, similar as the available results for OCB status. As extra comparisons we combined the different tools to compare with the single measurement OCB. The three combinations were: KFLC with OCB, LFLC with OCB and KFLC with LFLC. We defined the outcome of the combination as followed: when one of the measurements is positive the combination is positive, when both are negative the combination is negative.

To compare the sensitivity, specificity and accuracy between the two different diagnostic tools (OCB and FLC), the McNemar test was used (SPSS 22.0 (IBM Crop., Armonk, NY, USA). The positive and negative predicted values (PPV and NPV) were compared using the R package DTcompair. *P*-values <0.05 were considered statistically significant.

Results

Paired CSF and serum samples from a total of 745 patients were included in this multicenter study (see supplementary figure 1 for the flow-chart of patient selection). The patient groups (n=526) consisted of 242 CIS and 284 MS patients. The control group (n=219) consisted of 76 NINDC, 67 INDC, 49 SC and 27 HC. The control group was older (mean 42 years ±12) compared to the CIS/MS patient group (mean 35 years ±10, p<0.001). There was no significant difference in sex distribution between the two groups (p=0.20). Only 7.7% of the control group had a positive OCB status. The diagnosis of these patients are: Tolosa hunt (n=2), meningitis (n=1), neurosarcoidosis (n=2), lupus (n=1), transient global amnesia (n=1), stroke (n=1), post-infectious myelitis (n=1), myelitis (n=1), encephalitis (n=1), epilepsy (n=1), ADEM (n=1), neurodegeneration (n=1), INDC, exact diagnosis unknown (n=2), neuritis optica (n=1).

For a detailed list including the diagnoses of non-inflammatory neurological diseases and inflammatory neurological diseases included in the groups, see supplementary tables 2A and 2B.

FLC concentrations and FLC indices

Table 2 shows the levels and indices of the KFLC and LFLC in CIS/MS patients and controls. CSF KFLC and CSF LFLC concentrations were significantly increased in CSF of CIS/MS patients compared to controls (both p<0.001). In addition, KFLC and LFLC concentrations in CSF were higher in MS compared to CIS patients (both p<0.001).

KFLC and LFLC serum concentrations were significantly higher in the control group compared to CIS/MS (both, p=0.001) but did not differ significantly between CIS and MS (KFLC p=0.33, LFLC p=1.00).

See figure 1A for the KFLC indices per subgroup and figure 1B for the LFLC indices per subgroup. KFLC and LFLC indices were significantly increased in CIS/MS compared to controls (both p<0.001). In addition, FLC indices were higher in MS compared to CIS (KFLC p=0.07, LFLC p<0.001).

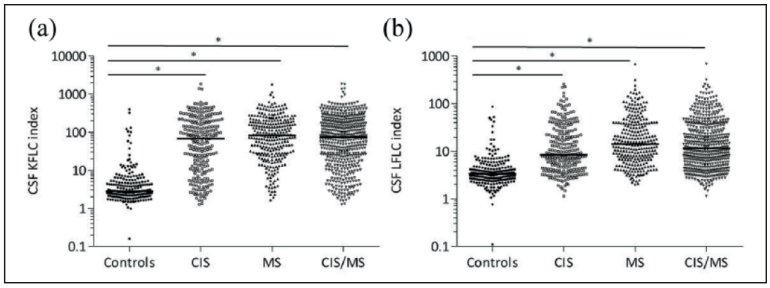

Figure 1. CSF KFLC and LFLC indices of CIS, MS, CIS/MS and controls. (a) Levels of KFLC indices, (b) levels of LFLC indices. Horizontal bars in the scatter dot plot represent the median.
CSF: cerebrospinal fluid; KFLC: kappa free light chains; LFLC: lambda free light chains; CIS: clinically isolated syndrome; MS: multiple sclerosis.
*$p \leq 0.001$.

Table 2. FLC in CIS/MS and controls.

A	CIS/MS (n=526)	Controls (n=219)	p-value
CSF KFLC (mg/L)	3.7 (1.0–10.1)	0.2 (0.1–0.4)	<0.001
Serum KFLC (mg/L)	12.4 (9.8–15.7)	13.7 (10.6–16.9)	0.001
KFLC index	75.6 (21.8–197.0)	2.8 (2.2–4.9)	<0.001
KFLC index ⩾ 6.6	460 (87.5)	38 (17.4)	–
B	**CIS/MS (n=543)**	**Controls (n=202)**	**p-value**
CSF LFLC (mg/L)	0.5 (0.2–1.3)	0.2 (0.2–0.3)	<0.001
Serum LFLC (mg/L)	11.3 (9.2–13.7)	12.1 (9.8–15.4)	0.001
LFLC index	11.6 (5.3–33.3)	3.3 (2.5–4.7)	<0.001
LFLC index ⩾ 6.9	359 (68.3)	30 (13.7)	–

FLC: free light chains; CIS: clinically isolated syndrome; MS: multiple sclerosis; A: KFLC in CIS/MS and controls; CSF: cerebrospinal fluid; KFLC: kappa free light chain; index: FLC quotient/albumin quotient; B: LFLC in CIS/MS and controls; LFLC: lambda free light chains; IQR: interquartile range.
Values are given as n (%) or as median (IQR).

FLC index cut-off

In the total cohort (n=745) (all diagnostic subgroups), a bimodal distribution of the log-transformed KFLC index values fitted the data best. This yielded a cut-off for the log-KFLC

index of 1.89 (95%CI 1.65-4.92) (Figure 2A), which corresponds to an KFLC index of 6.6 (95%CI 5.2– 138.1) on the original scale.

For the LFLC index, a bimodal distribution yielded an optimal cut-off for the log-LFLC index of 1.9 (95% CI 1.5-3.1) (Figure 2B), which corresponds to an LFLC index of 6.9 (95%CI:4.5 – 22.2) on the original scale.

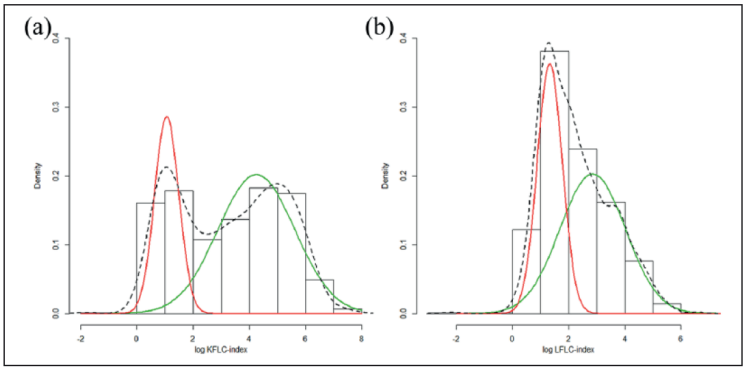

Figure 2. Cerebrospinal fluid (a) KFLC and (b) LFLC cut-off values based on mixture modeling. The red (low values) and green (high values) lines are the individual components of the estimated mixture distributions, the dotted line is the combined estimated mixture distribution. The cut-off is defined as the point where the red and green lines cross.

Diagnostic sensitivity and specificity of the indices compared to OCB

KFLC index

When pooling CIS and MS as one group (CIS/MS), the sensitivity to identify CIS/MS from controls of the KFLC-index (.88) was significantly higher than of OCB (.82; p<0.001) at the cost of a significantly lower specificity (KFLC-index: .83, OCB: .92; p<0.001) (see Table 3).

When identifying CIS patients from controls, the sensitivities did not differ significantly between KFLC and OCB (KFLC-index: .81, OCB: .77; *p*=0.15) but the specificity of OCB was significantly higher (KFLC-index: .83, OCB .92, p<0.001). Identifying MS patients from controls, the sensitivity of the KFLC-index (.93) was significantly higher than of OCB (.86; *p*<0.001) but the specificity (.83) significantly lower (.92; *p*<0.001).

The accuracies were similarly high for both biomarkers for all three comparisons. The positive predictive values (PPV) were higher for OCBs (in all three comparisons *p*<0.001). The negative predictive value (NPV) was the same for both markers (*p*=0.56) in CIS vs controls, but when pooling CIS/MS and in MS alone the NPV were slightly higher for KFLC (*p*=0.010, *p*<0.001). (see Table 3).

LFLC index

When pooling CIS and MS in one group (CIS/MS) the sensitivity to identify CIS/MS from controls of the LFLC-index (.66) was significantly lower than that of OCB (.82; p<0.001). The specificity to discriminate CIS/MS from controls was also significantly lower (.86) than for OCD (.92; p=0.019).

When identifying CIS patients from controls, the sensitivities did differ, the LFLC-index was significantly lower than the OCB (LFLC-index: .60; OCB: .77; *p*<0.001). The specificity of LFLC in CIS was lower compared to OCB (LFLC-index: .86; OCB .92; p=0.019). Identifying MS patients from controls, the sensitivity of the LFLC-index (.75) was significantly lower than of OCB (.86; *p*<0.001). The specificity (.86) was also significantly lower compared to OCB (.92; *p*=0.019). The accuracies and NPV were significantly lower of LFLC compared to OCB for all three comparisons. The PPV was significantly lower for LFLC compared to OCB when comparing MS with controls and in the other comparisons the PPV of LFLC was similar with OCB. (see Table 3).

Table 3. Diagnostic sensitivity and specificity of the FLC indices compared to OCB.

	OCB	KFLC	p-value	LFLC	p-value
CIS/MS vs controls					
Sensitivity	.82 (95%CI .79-.85)	.88 (95%CI .85-.90)	<0.001	.66 (95%CI .62-.70)	<0.001
Specificity	.92 (95%CI .89-.96)	.83 (95%CI .78-.88)	<0.001	.86 (95%CI .81-.91)	0.019
Accuracy	.85 (95%CI .82-.88)	.86 (95%CI .84-.89)	1.000	.74 (95%CI .70-.77)	<0.001
PPV	.96 (95%CI .94-.98)	.92 (95%CI .90-.95)	<0.001	.92 (95%CI .90-.95)	0.001
NPV	.68 (95%CI .63-.73)	.73 (95%CI .68-79)	0.010	.53 (95%CI .48-.58)	<0.001
CIS vs controls					
Sensitivity	.77 (95%CI .72-.82)	.81 (95%CI .76-.86)	0.150	.60 (95%CI .54-.66)	<0.001
Specificity	.92 (95%CI .89-.96)	.83 (95%CI .78-.88)	<0.001	.86 (95%CI .82-.91)	0.019
Accuracy	.84 (95%CI .80-.88)	.82 (95%CI .78-.85)	0.149	.73 (95%CI .68-.77)	<0.001
PPV	.92 (95%CI .88-.95)	.84 (95%CI .79-.88)	<0.001	.83 (95%CI .77-.89)	0.001
NPV	.78 (95%CI .73-.83)	.79 (95%CI .74-.84)	0.56	.66 (95%CI .61-.72)	<0.001
MS vs controls					
Sensitivity	.86 (95%CI .82-.90)	.93 (95%CI.90-.97)	<0.001	.75 (95%CI .70-.80)	<0.001
Specificity	.92 (95%CI .89-.96)	.83 (95%CI.78-.88)	<0.001	.86 (95%CI .81-.91)	0.019
Accuracy	.89 (95%CI .86-.92)	.89 (95%CI .86-.92)	1.000	.80 (95%CI .77-.84)	<0.001
PPV	.94 (95%CI .91-.97)	.88 (95%CI .84-.91)	<0.001	.88 (95%CI .84-.92)	0.002
NPV	.84 (95%CI .79-.88)	.91 (95%CI .86-.95)	0.001	.73 (95%CI .68-.78)	<0.001

KFLC: kappa free light chains; LFLC: lambda free light chains; NPV: negative predictive value; OCB: oligoclonal bands PPV: positive predicted value.

To compare the sensitivity and specificity between the two different diagnostic tools (OCB and FLC), the McNemar test was used.

Combination of the different tools compared to single measurement.
Three combinations were made, KFLC index-OCB, LFLC index-OCB and KFLC index-LFLC index, and all were compared with the analysis of OCB alone. Sensitivity and specificity were calculated for all the subgroups compared to the control group. The sensitivity of the combination OCB with the KFLC-index increased to .88, and the specificity of this combination decreased to .83. The same results were obtained for the combinations of LFLC index-OCB and KFLC index-LFLC index compared to single OCB, showing a slightly higher sensitivity (LFLC index-OCB .87, KFLC-LFLC index .87) and a lower specificity (respectively .83 and .80).

Sensitivities and specificities in alternative subgroups
Including patients with the diagnosis CIS (according to McDonald 2005 criteria) as RRMS patients resulted in lower sensitivities of OCB, KFLC and LFLC were seen when comparing CIS patients with controls. However, the *p*-value did not change relevantly when comparing the new sensitivities for OCB, KFLC and LFLC. No relevant differences were seen when comparing MS to control group.

No relevant differences were seen when we exclude patients with the CIS diagnosis according to McDonald 2005 criteria.

When excluding INDC from the control group, higher specificities were seen for OCB, KFLC and LFLC when comparing CIS/MS with the controls. However, the *p* value did not change relevantly when comparing the new specificities for OCB, KFLC and LFLC (data not shown).

Discussion

Our study indicates that the KFLC-index is a valid test for diagnosing CIS/MS. Compared to OCB the KFLC-index is more sensitive at the cost of a lower specificity. This trade off resulted in a higher NPV for the KFLC-index compared to OCB, but a lower PPV. In addition, our results indicate that the LFLC-index is not a valid test for diagnosing CIS/MS.

Our sensitivity and specificity for the KFLC index in CIS/MS were lower than a few much smaller previous studies[18, 23, 26], which reported sensitivity in the range of .93-.95 and specificity in the range of. 91-1.00. The study of Desplat-Jégo et al[9] showed a lower sensitivity of .70 and a lower specificity of .82 for the KFLC index in MS patients. The more recent study of Vasilj et al[27] showed a lower sensitivity of 0.71, however a higher specificity of .98. The results of the comparison of KFLC and OCB are in line with results of a recent multi-center study[14], where a cut-off of 5.9 was employed to validate KFLC in CSF as a diagnostic biomarker in 60 CIS patients, 60 MS patients compared to 60 OND, reporting a higher sensitivity of the KFLC

index (.78) compared to OCB (.72) for diagnosis of CIS. In MS, the sensitivity of the KFLC index and OCB were comparable (.93 vs .93). However, the specificity (.95) in CIS and MS was higher compared to our study. Another paper used the same 5.9 cut-off, this resulted in a sensitivity and specificity of .96 and .98 in MS patients.[21]

This discrepancy in the crude sensitivity and specificity of the KFLCs may be due to the more heterogeneous control group in our study compared to the previous studies, due to pooling of the CIS/MS group or the inclusion of not only clinical definite MS patients. For example, similar as for the OCBs, KFLCs can be elevated in inflammatory controls[28], and thus specificity will be lower when included. Nevertheless, this is a very relevant control group in differential diagnosis of MS. The unprecedented large number of patients and the large heterogeneous control group in this study gave us a reflection of the real-life clinical situation thus avoiding spectrum bias and allowed us to give a more representative sensitivity and specificity for OCB, KFLC and LFLC indices.

Noticeable is that the sensitivity and specificity of OCB to discriminate MS patients from controls were lower in our study than previously reported.[10, 29, 30] However, a meta-analysis published in 2013[31] (13,467 patients) showed that the diagnostic specificity of OCB diminished if other inflammatory etiologies were considered. Therefore, the lower specificities in our study may also be due to inclusion of various control groups.

The cut-off in this study was calculated using a data-driven Gaussian mixture modeling approach. We chose a different approach compared to other studies that applied for example receiver operating characteristics (ROC) curve analysis and area under the curve (AUC) values[9, 10, 18, 23, 27, 32], because we reasoned that the cut-off should be defined by biological levels (data driven) and not based on clinical diagnosis, which is an imperfect golden standard. We determined a cut-off of 6.6 for abnormal KFLC indices and 6.9 for abnormal LFLC indices. Our cut-off for KFLC is in line with a previous multi-center study showing a KFLC-index cut-off of 5.9.[10] This almost comparable cut-off for the KFLC index in two multi-center studies supports its robustness and implies that it can be used as an universal cut-off.

There are some limitations in this study. One limitation was that not all patients were diagnosed based on the same MS criteria; most patients by McDonald 2010 (84%) but a few with McDonald 2005, which may have influenced the diagnosis of CIS patients particularly. CIS patients diagnosed before 2010 may very well be MS patients according to McDonald 2010, because in the 2005 criteria MS diagnosis was more stringent. None of the patients were diagnosed by the new 2017 criteria, because of the retrospective set-up, and thus imaging information was not collected. We addresses this problem by pooling all CIS and MS patients. Another reason for pooling CIS and MS is that we did not have the data to test CIS

converting to MS versus non-converting CIS, because we did not have follow-up data. We performed several sensitivity analyses in CIS or MS patients separately, and by reclassifying and excluding specific clinical groups (Table 3). In these analyses, similar results were observed, suggesting that our results are robust for the total population. Another limitation is that we did not repeat the OCB analysis per patient centrally, but relied on the original local outcomes. However, we received the samples and OCB status from expertise MS centers (participating in the BioMSeu consortium) using standardized protocols.[33] Moreover, inter-laboratory agreement is reported to be good for OCBs, for example an inter-laboratory agreement of kappa>0.8 between 19 participating laboratories in Spain was observed.[34]

One more important note is that the best set up for the study would have been if the test population should be suspected MS cases and not already diagnosed with MS. Still, as provided in supplementary table 2B, we included various inflammatory neurological disease controls and quite some patients initially suspected for demyelinating disease.

Alongside the sensitivity and specificity results of the indices, we found significantly increased FLC concentrations and quotients in CSF of CIS/MS patients compared to the control groups. However, our main focus in this study were the FLC indices and not in the concentrations of the FLC. To control for blood-CSF barrier function, we used indices instead of concentrations.

Combination of different markers (KFLC index-OCB, LFLC index-OCB and KFLC index-LFLC index) compared to the single measurement OCB, showed that the combination KFLC index-OCB compared to single OCB gave a slightly higher sensitivity (.88). However, the specificity became lower (.83). The same results were seen in the combination LFLC index-OCB and KFLC index-LFLC index compared to single OCB. By definition the sensitivity become higher and the specificity lower when you decide beforehand that the combination test will be positive when the test is positive in one of the two.

For clinical practice, the KFLC index is more accurate in excluding CIS/MS compared to OCB but for ruling in a diagnosis of CIS/MS, analysis of OCB appears to be more accurate. If we replace OCB by KFLC in diagnostic practice there is a slightly higher chance that a patient with a diagnosis different from MS will get the diagnosis of MS and maybe unnecessarily exposed to potential negative side effects of early treatment. Since the KFLC-index is more sensitive at the cost of a lower specificity, we should stress that replacement of OCB by the KFLC-index is not optimal to arrive at high diagnostic certainty. However, with the higher sensitivity of KFLC, an earlier treatment start may be considered. Whether it is an option to start treatment based on the KFLC result and clinical/MRI findings according to the novel McDonald criteria, or whether the treatment may be adapted after a first year evaluation, is subject of further studies and discussions.

In conclusion, this study indicates that the KFLC-index is a valid tool in the diagnostic process of MS. Since this marker is measured by a faster and rater-independent analytical procedure, it should be considered as a potential cost-effective replacement of the OCB, especially when CSF analysis will regain a more prominent role in the 2017 revisions of the McDonald criteria.

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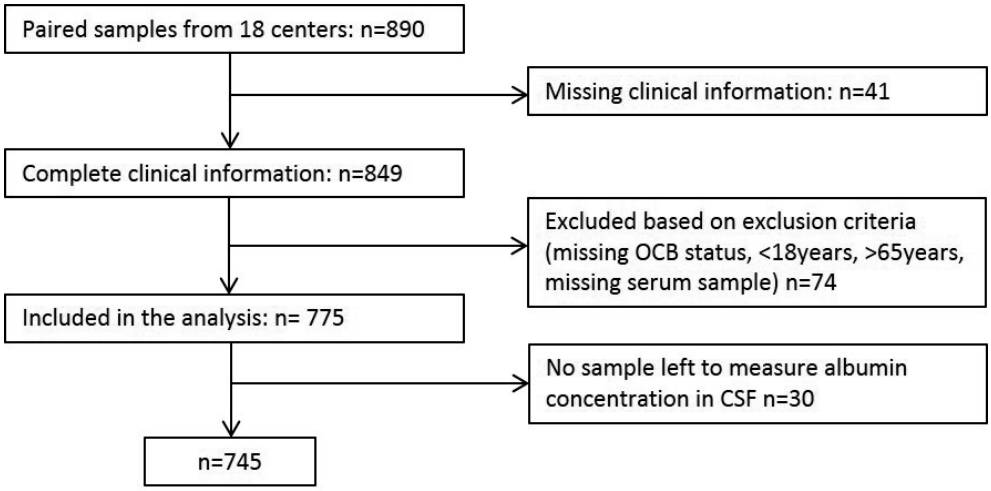

Supplementary Figure 1. Flow-chart of patient selection.

Chapter 2

Blood platelet RNA enables the detection of multiple sclerosis

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Abstract

Objective: In this proof of concept study, we evaluate the diagnostic potential of spliced blood platelet RNA for the detection of MS.

Methods: We isolated and sequenced platelet RNA of blood samples obtained from 57 MS patients and 66 age- and gender-matched healthy controls (HCs). 60% of the matched samples were employed to develop a particle swarm-optimized (PSO) support vector machine classification algorithm. The remaining 40% of the samples served as an independent validation series. The MS patients are part of an early inception cohort, in which patients were included at diagnosis and followed annually.

Results: In total, 1249 RNAs with differential spliced junction expression levels were identified between platelets of MS patients (n=57) as compared to HCs (n=66), including EPSTI1, IFI6, and RPS6KA3, in line with reported inflammatory signatures in the blood of MS patients. The spliced platelet RNA was subsequently used as input for the development of a diagnostic MS classifier capable of detecting MS with 80% accuracy in the independent validation series (n=50, AUC: 0.87, 95%-CI: 0.77-0.97, $p<0.001$).

Conclusions: Spliced platelet RNA may enable the blood-based diagnosis of MS, warranting large-scale validation.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder affecting the central nervous system. Clinical assessment, magnetic resonance imaging (MRI), and cerebrospinal fluid (CSF) analysis play important roles in the diagnostic process.[1] The field of MS biomarker discovery is thriving and the search for precise diagnostic tests continues. So far, no blood-based biomarker for MS has been confirmed.[2] Minimally invasive blood-based biomarkers would complement current MS diagnostics and monitoring.

During the final stages of thrombopoiesis, platelets are loaded with pre-mature messenger RNAs (pre-mRNAs) before being released from the megakaryocyte.[3] As a result, platelets contain a rich RNA repertoire that can change during megakaryocyte development but also during platelet formation and platelet circulation (Figure 1A). Especially the change of RNA transcripts during circulation, possibly achieved by specific splicing queues, is of relevance in the present study. Platelets respond to activating signals from their environment with specific splicing of their pre-mRNAs and potential uptake of RNA from different cell types, leading to a unique and dynamic RNA repertoire.[4–6] It has been shown that RNA isolated from tumor-educated platelets, platelets subjected to RNA changes in patients with cancer, may lead to highly accurate identification of traces of the tumor in blood,[7] independent of inflammatory conditions.[4]

Blood platelets are able to participate in inflammatory responses by secreting different cytokines and interact with different cell types, such as leukocytes and vascular cells.[6] Furthermore, platelets may be involved in the progression and pathogenesis of MS.[8,9] It has been observed that platelets from MS patients exhibit high levels of activation.[10–12] Interestingly, in vivo murine studies demonstrated that platelet depletion reduces MS disease severity, suggesting that platelets could serve as target for new therapeutic approaches.[12] As a result of their involvement in the immune response and potential role in the progression and development of the disease, we hypothesize that blood platelets of MS patients contain a disease-related RNA-signature which could be used as a diagnostic tool (Figure 1A).

Results

Platelet collection

Blood was collected from healthy controls (HCs) (n=66) and MS patients (n=57) in EDTA-coated Vacutainer tubes. All patients were diagnosed according to the revised McDonald criteria[1] and had relapsing remitting MS for at least 10 years. Of the MS patients, 17 were male and 40 were female, resulting in a ratio of 1:2.4 (Table 1 and S1). All 66 HCs were age- and gender-matched. At the time of blood draw, the disability status of MS patients was

examined according to the Kurtzke Expanded Disability Status Scale (EDSS) and an MRI scan was acquired. Although all patients were in clinical remission, 29 of them showed new T2-hyperintense lesions compared to an earlier performed MRI scan (Table 1).

Platelet RNA as a diagnostics tool for MS

Platelet RNA was sequenced and analyzed according to a previously published protocol[13] (Figure 1B). Briefly, platelets were isolated from whole blood by differential centrifugation, platelets were lysed, and RNA was isolated. The RNA was subsequently subjected to RNA amplification and prepared for RNA-sequencing on the Illumina platform.

We employed our previously published thromboSeq classification software for algorithm development.[4,13] First we randomly selected from the full dataset 17 MS and 19 HCs samples (30% of the total sample size) and assigned those to the training series, employed for biomarker RNA panel selection. Then, the algorithm optimized the biomarker panel towards accurate prediction by a readout of the randomly selected evaluation series (n=17 MS and n=20 HCs). This resulted in a total of 1,249 spliced RNAs which were found to be optimal for blood-based MS diagnostics (Table S2). Of this subset, 645 RNAs were increased in platelets of MS patients as compared to HCs, including the RNAs EPSTI1 and IFI6, whereas RPS6KA3 had decreased levels in MS patients as compared to HCs (Table 2; Table S2).

The classification algorithm reached an accuracy of 84% (area under the curve (AUC): 0.87, 95% confidence interval (95%-CI): 0.76-0.99; Figure 2) in the evaluation series. We subsequently locked the threshold parameters of the algorithm prior to validation, employing a separate MS (n=23) and HCs (n=27) sample series, who were not included in algorithm development, resulting in an accuracy of 80% (AUC: 0.87 95%-CI: 0.77-0.97, sensitivity: 83%, specificity 78%; Figure 2). Post-hoc leave-one-out cross validation analysis of the training series resulted in accuracy of 86% (AUC: 0.96, 95%-CI: 0.91-1.00; Figure 2). We confirmed the sensitivity of the spliced RNA panel for the detection of MS by randomly selecting other training and evaluation series with similar sample sizes (n=1000 iterations, median AUC: 0.89, IQR: 0.08), and confirmed the specificity of the spliced RNA panel by randomly shuffling the groups of the individual samples (n=1000 iterations, median AUC: 0.50, IQR: 0.17).

Discussion

We provide evidence that processes involved in MS result in alterations of platelet RNA profiles. Furthermore, we were able to demonstrate that RNA derived from circulating blood platelets may act as novel blood-based biomarker for MS. Specific splicing of platelet RNA in the presence of tumors has already been proposed in previous studies.[4,7,14] In the case of

MS, platelets appear to play an important and active role in the disease. Platelets seem to not just be involved in inflammatory and immune responses but may also contribute to the pathogenesis of MS.[10,12,15] Here we show that blood platelets isolated from patients with MS show a distinctive RNA signature potentially of value for blood-based MS diagnostics.

This study has several drawbacks. First, although we enrolled age- and gender-matched healthy controls, no individuals were included with other auto-immune or neuroinflammatory disease, potentially reducing the diagnostic accuracy. Second, the sample size was still small, potentially resulting in suboptimal algorithm development. Additional samples should be collected and evaluated. To reach true clinical relevance, follow-up studies should also focus on early-stage MS cases and patients presenting with a clinically isolated syndrome to assess the potential for early detection. All patients in the present study displayed a relapsing-remitting disease course. Future studies should include a broad spectrum of MS subtypes. Furthermore, additional studies are needed to gain insight into its ability to potentially predict disease progression, and DMT response prediction.

To our knowledge, this is the first study utilizing RNA found in circulating platelets as a blood-based biomarker for distinguishing MS patients from healthy individuals. The technique's potential for early diagnosis and treatment-response prediction, however, still need to be assessed in further studies.

Methods

Patients

MS patients participated in a prospective Amsterdam MS cohort study. They were included in this cohort at diagnosis and subsequently followed annually untill year six and had additional follow-up at year 11. Patients have been diagnosed with MS according to the revised McDonald criteria 2017[1] and were relapse-free and without steroid treatment for at least two months (Table 1 and Table S1). All 66 HCs were age- and gender-matched. This study was conducted in accordance with the principles of the Declaration of Helsinki. Approval of sample collection was obtained from the institutional review board and the ethics committee.

Wet- and dry-lab procedures

Blood processing resulting in platelet RNA seq data, and subsequentially algorithm development was preformed according to previously described methods.[13] For particle swarm-optimized (PSO) enhanced algorithm development, we applied the predefined settings; libsize correlation between -0.1 and 1.0, FDR between 0.00001 and 1.0, correlated transcripts between 0.5 and 1.0 and ranked transcripts between 200 and all detected

transcripts (4,812). We selected the particle (algorithm settings) with best performance in the evaluation series following evaluation of 100 particles during 10 iterations (1000 particles in total).

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Figure Legends

Figure 1

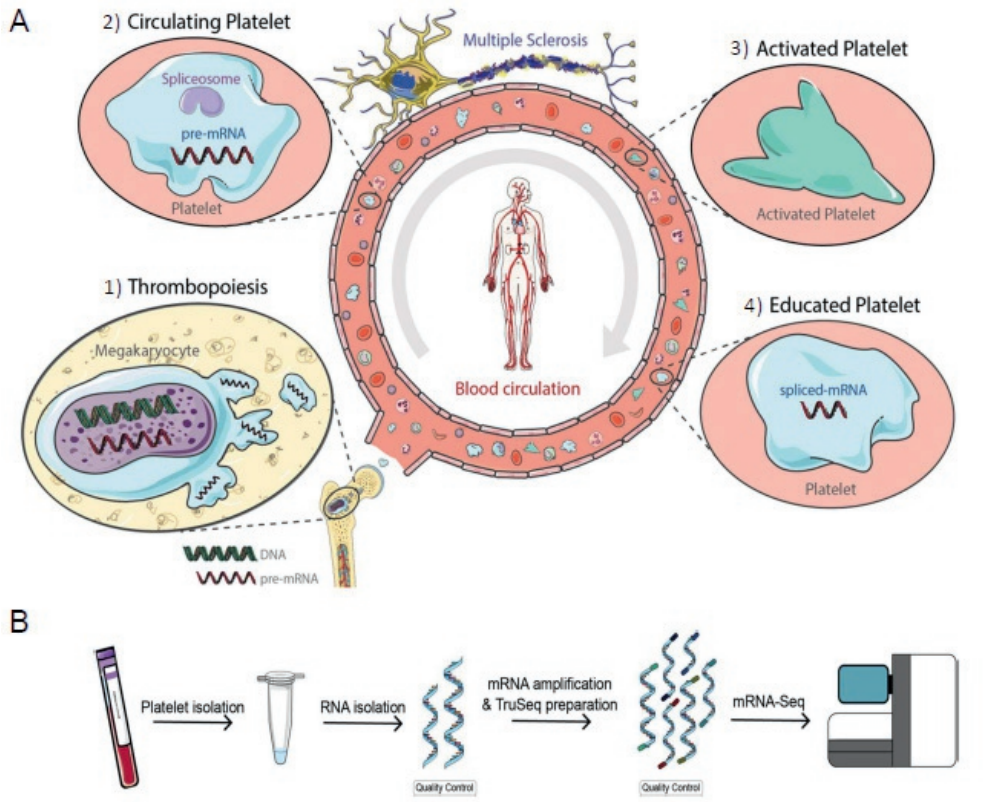

Figure 1. Schematic overview of platelet generation, circulation and possible alteration/education in the presence of MS.

(A) 1) Blood platelets are generated from megakaryocytes residing in the bone marrow. During the final stages of thrombopoiesis, platelets are loaded with pre-mRNAs before budding from the megakaryocyte. 2) Circulating platelets respond to activating signals from their environment with specific splicing of their pre-mRNAs and uptake of RNA from different cell types. 3) Blood platelets in MS patients show increased levels of adhesiveness and activation. 4) Processes involved in MS could potentially lead to specific splicing of platelet RNA, resulting in a disease-specific RNA signature.

(B) Schematic overview of the thomboSeq workflow. Blood was collected in 6 ml EDTA-coated tubes after which the platelet RNA is isolated, amplified, and labeled for sequencing. The RNA isolation and amplification steps are subjected to quality control using Bioanalyzer analysis.

Figure 2

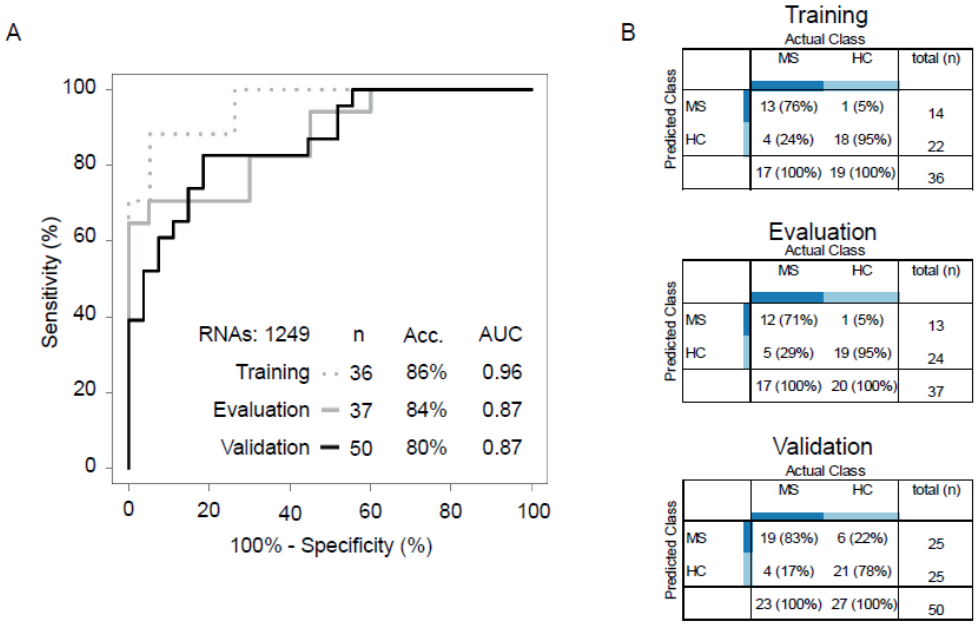

Figure 2. Platelet RNA profiles for MS diagnostics.

(A) ROC-curve of diagnostics of healthy controls and multiple sclerosis patients. Training, evaluation and validation series are indicated separately.
Acc = Accuracy, AUC = area under the curve.

(B) Cross-tables of diagnostics with the optimum point from the ROC-curves.

Table 1. Patient characteristics.

	Healthy controls (n=66)	Multiple Sclerosis (n=57)	P value
Gender			
Male n(%)	22 (33)	17 (30)	0.70
Female n(%)	44 (67)	40 (70)	
Age (mean ±SD, year)	46.5 ±7.4	46.6 ±6.9	0.91
DMT n(%)	NA	27 (47)	
New T2 lesions n(%)	NA	29 (51)	
EDSS (mean ±SD)	NA	3.0 ±0.9	

Table 2. Top RNAs with differentials spliced junctions.

	Up in MS	Down in MS
1	EPSTI1	HBB
2	DCUN1D4	AHCYL1
3	MTND1P23	RPS6KA3
4	IFI6	CDK16
5	MTND2P28	ADI1
6	UBE2L6	TADA3
7	MTRNR2L12	TMED4
8	MTND4P12	EFHC1
9	ATF7IP	AMPD2
11	MTATP6P1	TUBB

Part 2

Disease course biomarkers

Chapter 3

Cerebrospinal fluid mtDNA concentration is related to multiple sclerosis disease subtypes and treatment response

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Multiple Sclerosis, 2018

Abstract

Background – Mitochondrial dysfunction is increasingly recognized as an important feature of MS pathology and may be relevant for clinical disease progression. However, it is unknown whether mitochondrial DNA (mtDNA) levels in the CSF associates with disease progression and therapeutic response.

Objectives: To evaluate if CSF concentrations of mtDNA in MS patients can serve as a marker of ongoing neuropathology and may be helpful to differentiate between MS disease subtypes. To explore the effect of disease modifying therapies on mtDNA levels in the CSF.

Methods: CSF mtDNA was measured using a digital PCR CSF mtDNA in two independent MS cohorts. The cohorts included 92 relapsing-remitting (RRMS), 40 progressive MS (PMS) patients (27 secondary progressive and 13 primary progressive), 50 various neurologic disease controls and 5 healthy controls.

Results: Patients with PMS showed a significant increase in CSF mtDNA compared to non-inflammatory neurologic disease controls. Patients with higher T2 lesion volumes and lower normalized brain volumes showed increased concentration of mtDNA. Patients treated with fingolimod had significantly lower mtDNA copy levels at follow-up compared to baseline.

Conclusions*:* Our results showed a non-specific elevation of concentration of mtDNA in PMS patients. mtDNA concentrations respond to fingolimod and may be used to monitor biological effect of this treatment.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder of the central nervous system (CNS) and is characterized by a high degree of heterogeneity in progression and treatment response. To date, validated and discriminative body fluid biomarkers reflecting and predicting natural disease progression, crucial for monitoring patients in clinical trials, are lacking.[1,2] In addition, a biomarker guiding treatment decisions in early disease would be very useful in daily clinical practice. Cerebrospinal fluid (CSF) is an accessible source of CNS-derived products and its composition can reflect molecular changes occurring in the CNS.

In recent years, impaired mitochondrial function is increasingly recognized as a key pathological hallmark of MS.[3,4] Demyelination leads to an increase in energy demand in order to maintain an appropriate intra-axonal ion balance and could thereby affect the number, transport and activity of mitochondria.[5-8] Indeed, the number of mitochondria is highly increased in chronically demyelinated axons as well as in reactive astrocytes[5,7] and extensive neuronal mtDNA deletions have been observed in MS cortical brain samples.[9] Furthermore, significantly higher neuronal mtDNA copy numbers were found in MS normal-appearing gray matter compared to grey matter of non-neurological disease controls.[10] Mitochondria contain circular DNA (mtDNA) encoding 37 genes[11], which is more resistant to degradation by nucleases than nuclear DNA and can thereby be detected in CSF. A significant decrease in circulating cell-free mtDNA in CSF samples of both asymptomatic patients at risk of AD and symptomatic AD patients was observed[12,13] as well as in PD patients[14]. In contrast, CSF mtDNA concentration is markedly enhanced in children with traumatic brain injury, suggesting that mtDNA can be released due to acute cellular degeneration.[15] So far, it is unknown if CSF mtDNA concentrations are related to MS pathology. Based on the observations that mitochondrial dysfunction plays a crucial role in MS pathology and the possible role of mitochondrial dysfunction in clinical disease progression, we here explored the potential of mtDNA levels in the CSF as a candidate biomarker of identify patients with progressive disease. We also explored the effect of disease modifying therapies on free mtDNA levels in longitudinally obtained CSF samples in a Swedish cohort.

Subjects and Methods

Patients and controls: Dutch cohort

Patients were recruited either in response to an appeal in the periodical of the Dutch MS society, or from patients visiting or admitted at our clinic. Patients were invited to voluntarily undergo a lumbar puncture, MRI and clinical testing in the period from September 2000 to November 2005.[16] We included all patients with extensive clinical and radiological data and CSF storage within 2 hours after collection. MS patients were diagnosed according to the

Poser criteria.[17] Patients were classified as having a relapsing-remitting (RRMS), secondary progressive (SPMS) or primary progressive (PPMS) disease course according to the criteria of Lublin and Reingold.[18] In this study we also combined SPMS and PPMS patients in a progressive MS group (PMS). The control group was divided in non-inflammatory (NINDC) or inflammatory (INDC).[19]

2.1.2 Patients and controls: Swedish cohort

The prospective Swedish cohort consisted of longitudinally collected CSF samples in patients on disease modifying treatment (DMT).

CSF samples were collected during routine visits to the neurology clinic at Karolinska University Hospital beginning from the launch of fingolimod (Novartis Pharma AG, Basel, Switzerland) in Sweden 2011. The sampling period for cases was from April 2011 to May 2015 and for controls from February 2014 to December 2014. The MS cohort consisted of RRMS patients with confirmed disease, where a repeated CSF sampling was indicated for example to exclude diseases like Lyme´s disease. In addition, RRMS patients starting fingolimod were invited to donate a CSF sample at baseline and at 6-12 months. All MS patients fulfilled both the 2010 revised McDonald criteria[1]. There were no significant concomitant diseases, such as infections, and corticosteroids had not been given within three months of sampling.

2.2. Clinical examination: Dutch and Swedish cohort

Patients (with MS) underwent clinical examination prior to lumbar puncture, including the Expanded Disability Status Scale (EDSS),[20] which was performed by trained medical doctors. All participants provided written informed consent and the local ethics committee approved the study. Laboratory personnel were blinded for clinical and radiological data at the time of CSF analysis.

2.3.1 CSF collection: Dutch cohort

CSF was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space and collected in polypropylene tubes (Sarstedt, Nurmbregt, Germany). CSF samples were centrifuged within two hours at 1800 g at RT for 10 min and stored at -80 °C in aliquots of 0.5 or 1 ml in Sarstedt polypropylene tubes according to BioMS-eu guidelines.[21]

2.3.2 CSF collection: Swedish cohort

Samples were centrifuged immediately after lumbar puncture at 440g for 10min at room temperature to separate cells from the CSF supernatant. The supernatants were subsequently batched and stored at -80°C until use according to BioMS-eu guidelines.[21]

2.4 MRI acquisition and analysis: Dutch cohort

MRI examination was performed within 3 weeks of CSF collection and only available for the Dutch group. MRI scanning was performed at 1.0 Tesla (Siemens Magnetom Impact, Erlangen, Germany). As earlier described.[22] (see supplementary text-MRI protocol)

2.5 Cell-free mtDNA analysis

The concentration of cell-free circulating mtDNA in CSF was measured directly in 4.5 µl of unpurified CSF by droplet digital PCR in a QX200 platform (Bio-Rad, Hercules, CA, USA) following the digital PCR MIQE guidelines [23] and see the supplementary text about primers. To avoid contamination of CSF with cells that could be a source of cellular mtDNA, we used simultaneous detection of mtDNA and the Bcl-2-associated X (BAX) gene (a single copy nuclear gene) in a multiplex ddPCR reaction. The sequence of the BAX probe is: 6 - carboxy - 2',4,4',5',7,7' – hexachlorofluorescein HEX-CCCGAGCTGGCCCTGGACCCGGT-BHQ1. Primer sequences targeting BAX gene are: Forward, TTCATCCAGGATCGAGCAGG and Reverse, TGAGACACTCGCTCAGCTTC. These primers amplify a single amplicon of 103 base pairs corresponding to apoptosis regulator BAX isoform alpha. We confirmed that either single or multiplex assay of mtDNA-85 and BAX-103 using the procedures described above yields the same results. The ddPCR multiplex assay was performed in a 20 µl reaction volume consisting of 1X ddPCR Supermix for probes (BioRad186-3023), 900 nM forward and reverse primers, 100 nM mtDNA FAM labeled probe, 300 nM BAX HEX labeled probe and 4.5 µl of unprocessed CSF sample. A volume of double distilled water equivalent to CSF sample was used as non-template control (NTC). At least 2 NTCs were included in each ddPCR 96 well plate. If the nuclear BAX gene copies/µl is higher than 1, this indicates contamination of CSF with mtDNA from cells and the CSF samples that contained 1 or more copies of BAX were discarded from this study. The detection limit of nuclear gene using our ddPCR method is 0.5 cells/ µl and more sensitive than CSF cell counts.

Droplet emulsion formation was performed by mixing the 20 µl reaction with 70 µl droplet generation oil using a microfluidic droplet generation cartridge and QX200 Droplet Generator. End point PCR amplifications were performed using C1000 Thermal Cycler with the following conditions: 95 °C 10min; 40 cycles of 94 °C, 30sec and 60 °C 1min; 98 °C 10min. The presence or absence of amplification per droplet was evaluated using QX200 Droplet Reader and analyzed using QuantaSoft protocol. The results were expressed in copies of mtDNA/µl CSF.

The accuracy and linearity of the reaction conditions of this ddPCR method to quantify mtDNA content in CSF has been thoroughly characterized in previous work (linearity of this reaction is 0.999 and the dynamic range is 4-3000 copies/µl).[13]

2.6 Statistical analysis

For statistical analysis, SPSS version 22.0 (Windows) was used. The concentration of mtDNA copies were not normally distributed and therefore a natural logarithm was taken. For correlation analyses subjects were divided in different groups according to their lesion volumes and normal brain volumes based on a median split. We had to make subgroups for the volume related comparisons due to non-linearity of the data.

Based on EDSS scores, patients were divided into three categorical groups (EDSS 0-3.5, 4.0-5.5, 6-10). The three groups largely reflects, respectively, patients with no/limited, moderate and severe walking impairment.

Differences between the distinct groups were analyzed using linear regression. All results were corrected for disease duration as a confounder. In all statistical analyses, age and disease duration were both relevant confounders, but due to collinearity only disease duration was included as a covariate (as this was the strongest confounder).

To compare baseline and follow up mtDNA copies in the fingolimod-RRMS group, a Wilcoxon Signed rank test was performed. A p-value <0.05 was considered significant for main effects.

3. Results

3.1 Dutch cohort: mtDNA concentration is significantly increased in progressive MS cases

A total of 120 patients (50 RRMS patients, 40 progressive MS patients (27 SPMS patients, 13 PPMS patients), 23 NIND and 7 IND controls) were included in the study. The study group consisted of 64 women and 56 men with a mean age of 45.5 ± 11.0 years. The median disease duration, calculated as the time lapse between the onset of neurological symptoms and the time of the lumbar puncture, was 6.5(IQR 0.02 -38.77) years. See table 1 for detailed patient characteristics.

Figure 1 shows the relative increase in the concentration of mtDNA copies for each subtype (supplementary figure 1 shows the subtypes with SPMS and PPMS combined as PMS). SPMS and PPMS patients showed a significant increased concentration of circulating cell-free mtDNA in the CSF compared to NINDC (ratio 3.52, p=0.01 and ratio 2.85, p=0.05, respectively). Patients with RRMS showed a trend towards lower concentrations of mtDNA compared to SPMS patients (ratio 0.49, p=0.06) (supplementary table 1).Patients with progressive MS (PMS) showed a significant increased concentration of circulating cell-free mtDNA in CSF compared to NINDC (ratio 3.23, *p*=0.01). There was a trend for increasing concentrations comparing PMS patients with RRMS patients (ratio 1.87, p=0.05). mtDNA concentration did not differ between PMS patients and INDC (ratio 2.66, *p*=0.12) (supplementary table 2).

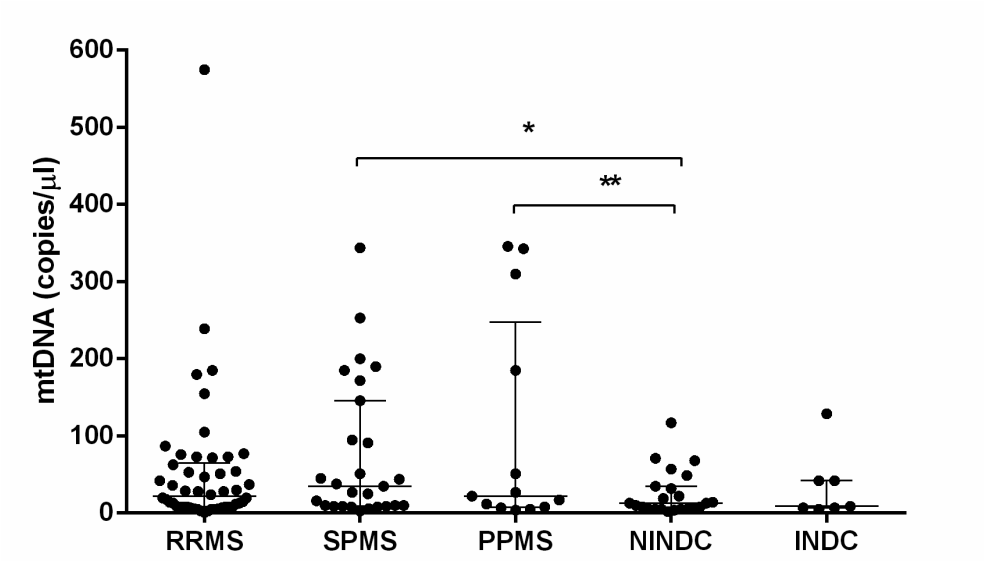

Figure 1. Comparison of mtDNA levels between the subtypes (Dutch cohort).

Scatter plot representing mtDNA concentrations for each individual grouped by diagnosis. The horizontal lines correspond to median and interquartile range. NINDC: median 13 (IQR, 5–35) copies/μL; INDC: median 9 (IQR, 7–42) copies/μL; RRMS: median 22 (IQR, 8–65) copies/μL; SPMS: median 35(IQR, 9–146); PPMS: median 22 (IQR, 7.5–247.5) copies/μL. *p = 0.01, based on linear regression, adjusted for disease duration. **p = 0.05, based on linear regression, adjusted for disease duration. INDC: inflammatory neurologic disease controls; NINDC: non-inflammatory neurologic disease controls; PMS: progressive multiple sclerosis; RRMS: relapsing-remitting multiple sclerosis.

Table 1. Patient characteristics and MRI measures, Dutch cohort.

	All (*n* = 120)	RR (*n* = 50)	SP (*n* = 27)	PP (*n* = 13)	NINDC (*n* = 23)	INDC (*n* = 7)
M:F	56:64	19:31	15:12	8:5	10:13	4:3
Age (years)	45.5 ± 11.0	41.4 ± 9.6	49.6 ± 7.6	50.4 ± 5.3	47.5 ± 15.1	43.3 ± 15.1
EDSS	–	3.0 (2.5–4)	6.0 (4–7)	4.0 (3.5–6)	–	–
Disease duration (years)	6.5 (2.3–6.5)	6.5 (2.9–13.7)	19.3 ± 8.7	10.2 (3.8–19.7)	1.14 (0.3–2.8)	0.07 (0.02–2.8)
DMT:NODMT	–	19:31	4:23	0:0	–	–
T2LV	–	2.79 (0.99–9.23)	7.59 (3.67–15.23)	3.73 (1.19–9.62)	–	–
BHLV	–	0.14 (0.06–0.41)	0.39 (0.06–1.56)	0.53 (0.15–2.92)	–	–
NBV	–	1.25 (1.16–1.31)	1.16 (1.10–1.21)	1.27 (1.17–1.34)	–	–
Gado+	–	8:33	4:23	2:8	–	–

All: all subjects; BHLV: black hole lesion volume; MRI: magnetic resonance imaging; DMT: disease-modifying treatment; EDSS: Expanded Disability Status Scale; F: female; Gado+: gadolinium-enhanced lesions; INDC: inflammatory neurologic disease controls; IQR: interquartile range; M: male; *n*: number of subjects; NBV: normal brain volume; NINDC: non-inflammatory neurologic disease controls; NODMT: no use of disease-modifying treatment; PP: primary progressive; RR: relapsing-remitting; SD: standard deviation; SP: secondary progressive; T2LV: T2 lesion volume.
Values are reported as mean ± SD or median (IQR) (Dutch cohort).

3.2 Validation

To validate the ddPCR results we re-analyzed the high level samples with real time qPCR. The data shown in supplementary figure 2A and 2B show a strong significant correlation between ddPCR and real time qPCR for the quantification of mtDNA in CSF samples from control and the MS subtypes ($r=0.93$, $p<0.05$).

Moreover, there was no correlation between CSF leukocyte count and mtDNA copies (Spearman's rho 0.37, *p*-value>0.05), indicating that high levels of mtDNA copies were not a consequence of cell contamination. Lastly, previous studies have shown there was no correlation between mtDNA content in CSF and the presence of 14-3-3 protein, a cytoplasmic protein that would be increased in the CSF if samples were contaminated with cells.[13]

3.3 Disease modifying treatment: Dutch cohort

Nineteen RRMS patients used disease-modifying treatment (DMT) (all interferon-β) at the time of lumbar puncture. There was no significant difference in the concentration of mtDNA copies between the groups with and without DMT (ratio=1.12 *p*=0.624). The median CSF mtDNA concentration was 16 copies/µl (IQR 7.75-65.25) in the group without DMT and 18 copies/µl (IQR12-77) in the interferon using groups.

3.4 mtDNA copy numbers in relation to EDSS: Dutch cohort

There was no significant correlation between EDSS scores and the number of mtDNA copies/µl, although a trend (*p*=0.08) for a positive correlation was observed when dividing patients into different groups (Category 0-3.5 *n*=39, category 4-5.5 *n*=30, category 6-10 *n*=21, see supplementary table 3).

3.5 mtDNA copy numbers in relation to MRI outcomes in MS patients: Dutch cohort

To study a putative association of mtDNA concentrations and ongoing disease activity, we correlated mtDNA concentration levels in MS patients with radiological parameters, including T2 lesion volume, gadolinium-enhanced lesions, T1-hypointense lesion volumes (BHLV), and normalized brain volume (NBV) as a measure of atrophy.

The T2 lesion volumes were measured in 78 patients and divided into two groups based on the medians. Group 1 had a median T2 lesion volume of 1.53 (IQR 0.13- 4.30) ml and group 2 had a median T2 lesion volume of 10.53 (IQR 6.55- 16.96) ml. The group with higher T2 lesion volumes showed increased concentrations of mtDNA copies compared to group with lower T2 lesion volumes (ratio 2.13, *p*=0.03).

The cohort was divided into two groups based on the presence (n=64) or absence (n=14) of gadolinium-enhanced lesions on MRI. There was no significant difference in mtDNA concentrations between patients with or without gadolinium-enhanced lesions.

Black hole volumes were measured in 66 patients and divided into two groups using the median. Group 1 had a median BHLV volume of 0.063 (IQR 0.03-0.12) ml and group 2 a median BHLV of 0.89 (IQR 0.38-2.34)ml. There was no significant difference in mtDNA copy concentrations between these groups (ratio 0.93, *p*=0.85).

Of 81 patients the normal brain volume (NBV) was available and patients were divided into two groups based on the median. Group 1 had a median NBV 1.14 (IQR 1.09-1.18)L and group 2 with a median NBV 1.29 (IQR 1.22-1.33)L. Comparing these two groups, the group with higher NBV showed significant lower concentrations of mtDNA (ratio 0.42, p=0.02) (table 2).

Table 2. MRI outcomes (Dutch cohort).

	Ratio	*p*-value	95% confidence interval	
			Low bound	Upper bound
T2 lesion volumes	2.13	0.03	1.08	4.18
G^+-enhanced lesions	1.69	0.23	0.71	4.02
Black hole lesion volumes	0.93	0.85	0.44	1.96
Normalized brain volumes	0.42	0.02	0.24	0.90

MRI: magnetic resonance imaging; mtDNA: mitochondrial DNA; IQR: interquartile range; BHLV: black hole lesion volumes; NBV: normalized brain volumes.
The concentration of mtDNA copies was not normally distributed and therefore a natural logarithm was taken. For correlation analyses, subjects were divided into different groups according to their lesion volumes and normal brain volumes based on a median split. Using log transformation of the regression coefficients, ratios of the mitochondrial DNA concentrations between the different subgroups were calculated. Analyzing T2 lesion volumes, patients with higher T2 lesion volumes showed 2.13 times higher concentrations of mtDNA copies (p=0.03). Patients with higher normalized brain volumes showed significant 0.42 times lower concentrations of mtDNA (p=0.02).
T2 lesion volumes. Comparing group 2 with group 1. Group 1: median T2 lesion volume of 1.53 (IQR, 0.13–4.30) mL. Group 2: median T2 lesion volume of 10.53 (IQR 6.55–16.96) mL. Adjusted for disease duration.
Gadolinium-enhanced lesions. Comparing the group with the presence of gadolinium-enhanced lesions with the group with the absence of gadolinium-enhanced lesions. Adjusted for disease duration.
Black hole lesion volumes. Comparing group 2 with group 1. Group 1: median BHLV volume of 0.063 (IQR, 0.03–0.12) mL. Group 2: a median BHLV of 0.89 (IQR, 0.38–2.34) mL. Adjusted for disease duration.
Normalized brain volumes. Comparing group 2 with group 1. Group 1: median NBV 1.14 (1.09–1.18) L. Group 2: median NBV 1.29 (IQR, 1.22–1.33) L. Adjusted for disease duration and gender.

Table 3. Patient characteristics, Swedish cohort.

	Fingolimod-RR (n=23)	RRMS (n=19)	ONDC (n=20)	HC (n=5)
M:F	7:16	5:14	7:13	3:2
Age (years)	42.4±8.1	32.4±8.1	34.15±11.1	29.8±2.9
EDSS	3.0 (2.0–3.5)	2.0 (1.5–3.0)	–	–
Disease duration (years)	10.3±6.1	5.89±4.3	–	–
DMT:NODMT	23:0	19:0	–	–

All: all subjects; EDSS: Expanded Disability Status Scale; F: female; HC: healthy controls; IQR: interquartile range; M: male; *n*: number of subjects; ONDC: other neurologic disease controls; RR: relapsing-remitting; RRMS: relapsing-remitting multiple sclerosis; DMT: disease-modifying treatment; NODMT: no use of disease-modifying treatment; SD: standard deviation.
Values are reported as mean±SD or median (IQR) (Swedish cohort).

3.6 Analysis of cell-free mtDNA content in CSF in the Swedish cohort

A total of 42 RRMS patients, 20 other neurological disease controls (ONDC) and 5 healthy controls (HC) were included in the study. The RRMS patients were divided in two groups: 23 fingolimod-RRMS patients and 19 differently treated RRMS patients.

The control group consisted of 20 patients undergoing a diagnostic work-up for possible neuroinflammatory disease, but where no pathological signs of inflammation were seen on MRI or with established markers of immune activation in the CSF (pleocytosis, oligoclonal bands, albumin quote, increased IgG index). The diagnoses of this group were paraesthesia (n=6), pain syndromes (n=5), psychiatric illness (n=4), vertigo (n=3), essential tremor (n=1), Bell´s palsy (n=1), dysphasia (n=1). In addition, 5 individuals served as healthy controls. See table 3 for further characteristics of the groups.

For fingolimod users, mtDNA copy number concentrations were almost 50% lower on follow-up (median=9.6 copies/µl) compared to baseline (median=17.9 copies/µl), z=-2.52, p=0.012, r = -0.37 (see figure 2 and see supplementary figure 3 for a graph with individual changes).

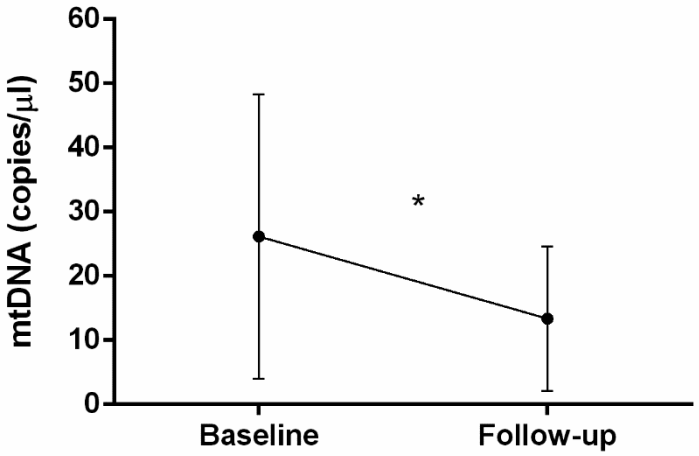

Figure 2. The effect of fingolimod on mtDNA concentration levels (Swedish cohort).

To compare baseline and follow-up mtDNA copies in the fingolimod relapsing-remitting multiple sclerosis group (n = 23), a Wilcoxon signed rank test was performed. The follow-up moment lies after 6–12 months. The horizontal lines correspond to median and interquartile range. *p = 0.012 (z = −2.52, r = −0.37).

There was no significant difference in mtDNA copy levels in the fingolimod-RRMS patients (n=23) compared to the RRMS group using other DMT (dimethyl fumarate: n=7, interferon-beta: n=12)(ratio=1.65 p=0.089).

There were no significant differences in mtDNA copy levels between the RRMS, ONDC and HC groups in this Swedish cohort, see figure 3 and supplementary table 4.

Most of the RRMS patients had an EDSS between 0-3.5. Therefore comparing the different EDSS categories, such as performed for the Dutch cohort, was not possible (category 0-3.5 n=35, category 4-5.5 n=3, category 6-10 n=3, missing EDSS n=1).

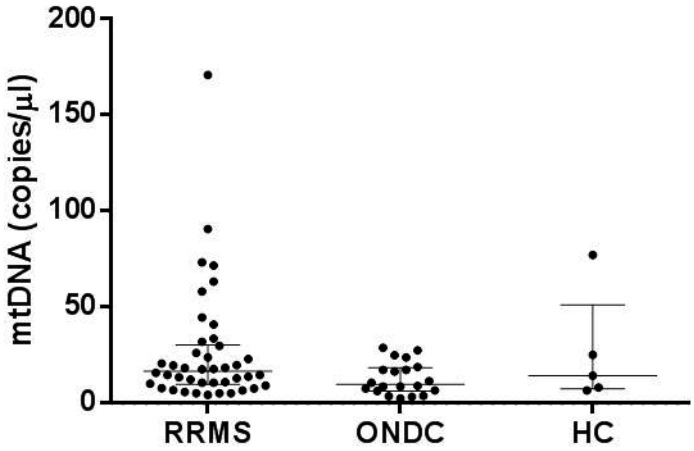

Figure 3. Comparison of mtDNA levels between the subtypes (Swedish cohort).

Scatter plot representing mtDNA concentrations for each individual grouped by diagnosis. The horizontal lines correspond to median and interquartile range.

RRMS: median 16.8 (IQR, 9.9–30.5) copies/µL; ONDC: median 9.8 (IQR, 6.4–18.6) copies/µL; HC: median 14.3 (IQR, 7.5–51.3) copies/µL; HC: healthy controls; ONDC: other neurological disease controls; RRMS: relapsing-remitting multiple sclerosis.

Discussion

The major finding of this study is that concentrations of free circulating mtDNA copies are increased in CSF of patients with progressive MS compared with non-inflammatory control patients. Also, there was a trend for a modest positive correlation with EDSS specifically in progressive MS patients. In addition, we showed that patients with a high T2 lesion volume displayed higher mtDNA concentrations compared to patients with a relative low T2 lesion volume. The group with lower normalized brain volumes showed higher mtDNA concentrations compared to patients with higher normal brain volumes, suggesting a positive correlation between the concentration of free circulating mtDNA copies and brain atrophy. Altogether, our data may suggest that increased concentrations of cell free mtDNA are associated with MS disease activity and progressive disease.

Reduced levels of mtDNA have been reported in both AD and PD cases and it has been speculated that a decrease in mtDNA might be a common phenomenon observed in neurodegenerative diseases. In contrast to this, elevated mtDNA levels as we found in PMS, have also been detected in CSF samples from children with traumatic brain injury and were highly predictive of a poor outcome.[15] This might suggest that high values of free circulating mtDNA in CSF can be seen as a potential biomarker of acute cellular and mitochondrial

stress. It is nowadays widely accepted that neurodegeneration and concomitant brain atrophy are common pathological features of MS, particularly in the progressive phase of the disease. In MS, demyelination leads to an increase in axonal energy demand, which may superimpose effects of neurodegeneration in MS, which is a possible explanation for the higher concentrations of mtDNA in progressive MS in this study.

Enhanced mtDNA concentrations in the CSF correlated with high T2 lesion volumes and were inversely correlated with normal brain volume suggesting that the increased concentration of mtDNA is due to ongoing neuro-axonal damage which is known to be more extensive in progressive forms of MS.[24,25] Even though longitudinal follow-up MRI scans are lacking, the cross-sectional correlations corroborate the finding of higher levels in clinical PMS groups.

Increased mtDNA levels in the CSF might not only reflect disease progression, but might also contribute to the disease process. Mitochondrial DNA is a damage-associated molecular pattern (DAMP), which can bind to glial Toll-like receptor-9 and trigger an inflammatory response.[26] Hence, it is conceivable that enhanced mtDNA levels in the CSF might elicit a glial immune response, however additional research is needed to explore the functional effects of increased mtDNA concentrations in the CSF of MS patients.

The cellular origin of enhanced mtDNA levels in the CSF of progressive MS patients is yet unknown, however it is conceivable that mtDNA is released upon neuro-axonal injury or oligodendrocyte damage, as these are prominent features of progressive MS. Alternatively, mtDNA might be secreted into the extracellular compartment by extracellular vesicles derived from distinct CNS cells, such as reactive astrocytes, which, particularly in lesions that are packed with mitochondria.

Interestingly, an almost 50% decrease in mtDNA copy number was found upon fingolimod treatment. Fingolimod is a sphingosine-1-phosphate (S1P) receptor modulator that limits the egress of lymphocytes from lymph nodes, preventing them from contributing to autoreactive inflammation in the CNS.[27] Fingolimod crosses the blood brain barrier and can also act on S1P receptors present on oligodendrocytes, oligodendrocyte precursor cells, astrocytes, microglial cells and neurons

We observed decreased levels of mtDNA copies in patients using fingolimod (compared to baseline), suggesting a possible role of mtDNA as a biomarker of fingolimod treatment response. It is likely that fingolimod reduces inflammation-mediated cellular damage and subsequent release of mtDNA. However the precise mechanism underlying reduced mtDNA CSF levels upon fingolimod treatment warrants further investigations. Further comparisons with other disease modulatory treatments are needed in order to understand if this effect

is specific for fingolimod or a generic response to reduced inflammation and concomitant CNS cell injury.

Altogether, combining our results of no significant difference between the concentration of mtDNA in CSF between RRMS and SPMS and the significantly decreased levels of mtDNA in CSF in RRMS patients plus the knowledge that fingolimod has been tested in progressive MS trials but with negative outcomes[28], our study showed a non-specific elevation of concentration of mtDNA in PMS patients.

The relatively small sample size and overlap in mtDNA concentrations between patient groups warrants cautious interpretation of our data. It is important to validate our findings in future research, including longitudinal clinical follow-up to further explore the prognostic potential of mtDNA analysis as a tentative biomarker for progressive MS and treatment response.

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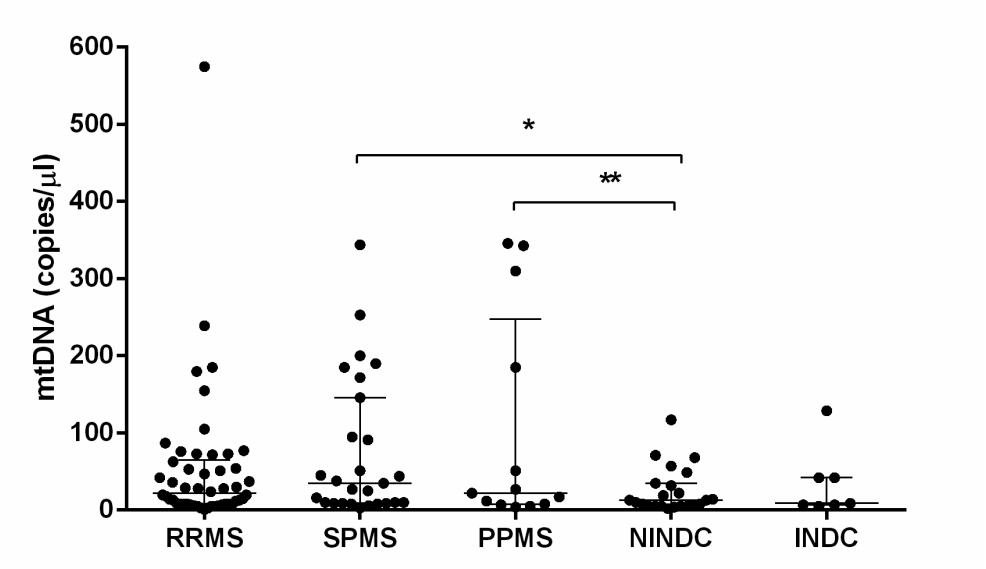
SUPPLEMENTARY figures and tables



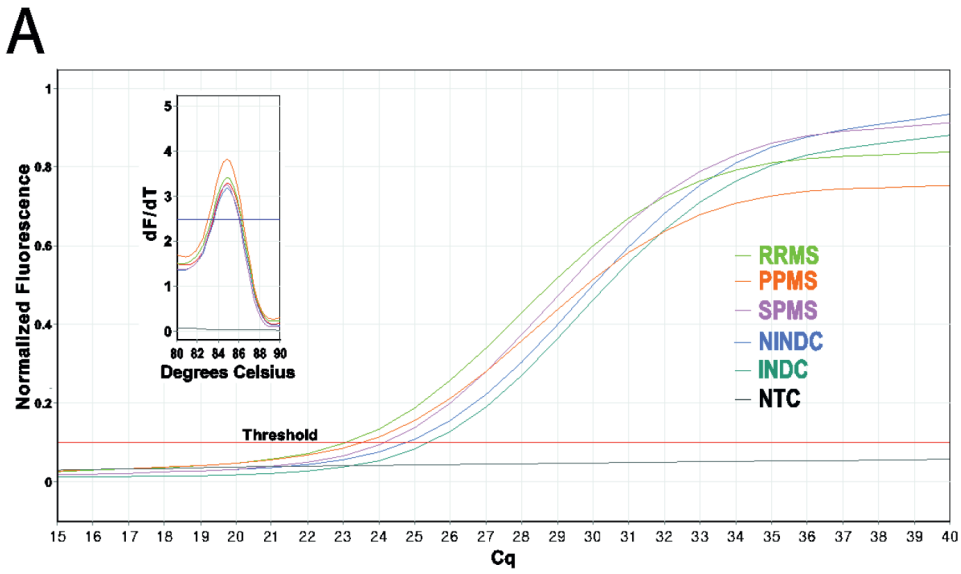
Supplementary Figure 1. Comparison of mtDNA levels between the subtypes **(***Dutch cohort)***.**

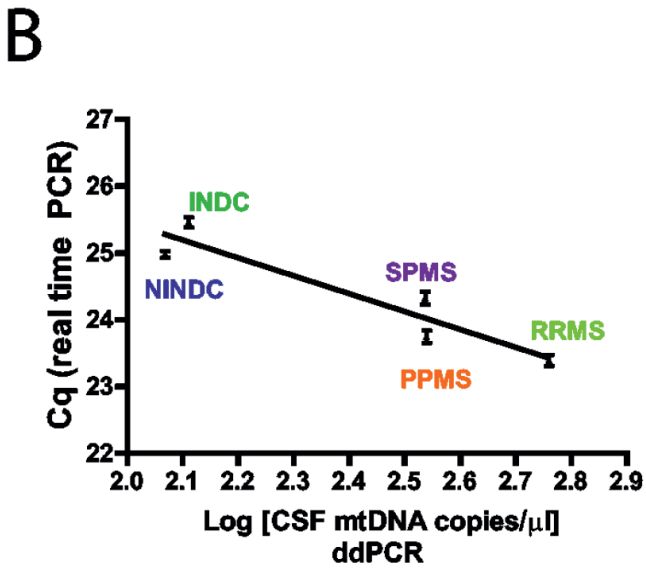
Scatter plot representing mtDNA concentrations for each individual grouped by diagnosis.

The horizontal lines correspond to medians and interquartile range.

*indicates p=0.01 based on linear regression, adjusted for disease duration.

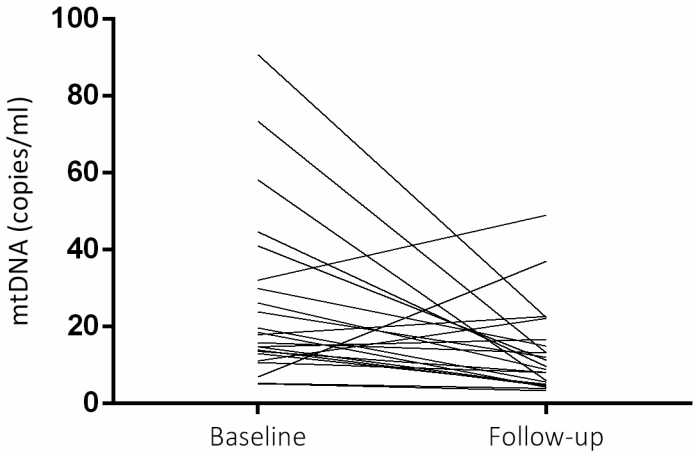
INDC=inflammatory neurologic disease controls, NINDC=non inflammatory neurologic disease controls, PPMS=primary progressive multiple sclerosis, SPMS=secondary progressive multiple sclerosis, RRMS=relapsing remitting multiple sclerosis





Supplementary Figure 2A and 2B:. Relationship between ddPCR and real time PCR for the quantification of mtDNA in CSF in samples from control and MS groups.

A) Amplification plots of mtDNA-85 by real time qPCR. Representative CSF (2ul) samples from each of study groups were analyzed in sextuplicate in 20 ul qPCR reaction using intercalated dye detection. NTC = non-template control: reaction containing all PCR reagents but without CSF sample was analyzed in parallel. Inset: Shows the melting curve of the PCR products and the absence



Supplementary Figure 3. The effect of fingolimod individually on mtDNA concentration levels (*Swedish cohort).*

To compare baseline and follow up mtDNA copies in the fingolimod-relapsing remitting multiple sclerosis group (n=23), a Wilcoxon Signed rank test was performed. The follow-up moment lies after 6-12 months.

Supplementary Table 1. Clinical results/outcomes subtypes (SPMS and PPMS separated) (*Dutch cohort*)

The concentration of mtDNA copies were not normally distributed and therefore a natural logarithm was taken. Using log transformation of the regression coefficients, ratios of the mitochondrial DNA concentrations between the different subgroups were calculated. Patients with SPMS showed an 3.52 times higher CSF concentration of circulating cell-free mtDNA compared to non-inflammatory control patients (p = 0.01).

	ratio	*P value*	95% confidence interval	
			Low bound	Upper bound
INDC to NINDC	1.21	0.73	0.39	3.78
RRMS to NINDC	1.73	0.15	0.80	3.70
SPMS to NINDC	3.52	**0.01**	1.33	9.32
PPMS to NINDC	2.85	0.05	0.99	8.16
RRMS to INDC	1.43	0.54	0.46	4.45
SPMS to INDC	2.90	0.10	0.79	10.65
PPMS to INDC	2.35	0.21	0.61	9.08
SPMS to RRMS	0.49	0.06	0.24	1.02
PPMS to RRMS	1.64	0.24	0.71	3.85

The analyses was adjusted for disease duration. INDC=inflammatory neurologic disease controls, NINDC=non inflammatory neurologic disease controls, PPMS=primary progressive multiple sclerosis, SPMS=secondary progressive multiple sclerosis, RRMS=relapsing remitting multiple sclerosis.

Supplementary Table 2. Clinical results/outcomes per subtypes *(Dutch cohort).*

The concentration of mtDNA copies were not normally distributed and therefore a natural logarithm was taken. Using log transformation of the regression coefficients, ratios of the mitochondrial DNA concentrations between the different subgroups were calculated. Patients with progressive MS (PMS) showed a 3.23 times higher CSF concentration of circulating cell-free mtDNA compared to non-inflammatory control patients (p = 0.01).

	ratio	*P value*	95% confidence interval	
			Low bound	Upper bound
INDC to NINDC	1.21	0.73	0.39	3.78
RRMS to NINDC	1.73	0.15	0.80	3.70
PMS to NINDC	3.23	**0.01**	1.32	7.93
RRMS to INDC	1.48	0.54	0.40	4.41
PMS to INDC	2.66	0.12	0.77	9.17
PMS to RR	1.87	0.05	0.99	3.55

All adjusted for disease duration. INDC=inflammatory neurologic disease controls, NINDC=non inflammatory neurologic disease controls, PMS=progressive multiple sclerosis (SPMS and PPMS combined), RRMS=relapsing remitting multiple sclerosis.

Supplementary table 3. Results EDSS (*Dutch cohort*)

The concentration of mtDNA copies were not normally distributed and therefore a natural logarithm was taken. Based on EDSS scores, patients were divided into three categorical groups (EDSS 0-3.5, 4.0-5.5, 6-10). Using log transformation of the regression coefficients, ratios of the mitochondrial DNA concentrations between the different groups were calculated. There was a trend of increasing median mtDNA copies µl /CSF with increasing EDSS categories.

	ratio	*P* value	95% confidence interval	
			Low bound	Upper bound
EDSS 4.0-5.5 to EDSS 0-3.5	1.64	0.19	0.78	3.42
EDSS 6-10 to EDSS 0-3.5	2.13	0.08	0.91	4.99
EDSS 6-10 to EDSS 4.0-5.5	1.30	0.52	0.58	2.95

The analyses was adjusted for disease duration. EDSS=expanded disability severity scale.

Supplementary table 4. Clinical results/outcomes subtypes (*Swedish cohort).*

The concentration of mtDNA copies were not normally distributed and therefore a natural logarithm was taken. Using log transformation of the regression coefficients, ratios of the mitochondrial DNA concentrations between the different subgroups were calculated.

	Ratio	*p* value	95% confidence interval	
			Low bound	Upper bound
ONDC to RRMS	0.58	0.07	0.32	1.04
HC to RRMS	0.97	0.95	0.40	2.34
ONDC to HC	1.69	0.22	0.73	3.92

The analyses was adjusted for disease duration. HC= healthy controls, ONDC=other neurologic disease controls, RRMS=relapsing remitting multiple sclerosis

Supplementary text – MRI protocol

The protocol consisted of axial pre- and post-contrast T1-weighted (repetition time [TR]=700 ms, echo time [TE]=15 ms, 5.0mm slice thickness, 0.5mm inter-slice gap) images for atrophy measurements and BH assessment and T2-weighted ([TR]=2700 ms, [TE]=90 ms, 5.0mm slice thickness with 0.5mm inter-slice gap) images for lesion quantification.

T1 hypointense or black hole lesion volumes, T2 lesion volumes and presence of gadolinium enhanced lesions were quantified using home-developed semi-automated seed-growing software based on a local thresholding technique.

Post-contrast T1-weighted images were used to assess brain atrophy. We measured normalized brain volumes (NBV), using Structural Image Evaluation, using Normalization of Atrophy Cross-sectionally (SIENAX; part of FSL, see http://fsl.fmrib.ox.ac.uk).

Supplementary text – PCR primers

The sequences of the primers and hydrolysis probe targeting mtDNA are as follows: A) Forward mtDNA-85F (5'-CTCACTCCTTGGCGCCTGCC-3'), B) Reverse mtDNA-85R (5'-GGCGGTTGAGGCGTCTGGTG-3'), C) Hydrolysis probe FAM-mtDNA-85P (6-carboxyfluorescein -5'- CCTCCAAATCACCACAGGACTATTCCTAGCCATGCA-3'-Black Hole Quencher-1). These primers hybridize an mtDNA target sequence not associated with known polymorphisms according to the National Institutes of Health (USA) genetic sequence database GenBank. Basic local alignment search within the GRCh38 human genome reference sequence indicated that these primers do not have a full complementary target in the human genome. The PCR amplicon obtained with these mtDNA primers corresponds to bases 14,848-14,932 of the human mtDNA Cambridge reference sequence NC_012920.1.

PCR assay conditions were optimized to obtain a single amplicon by melting curve analysis and agarose gel electrophoresis. The amplification efficiency observed with these primer combinations was more than 0.95. The amplicon was sequenced and found to correspond to the expected mtDNA sequence.

Chapter 4

Acid sphingomyelinase: no potential as a biomarker for multiple sclerosis

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Multiple sclerosis and related disorders, 2018

Abstract

Background Multiple sclerosis (MS) lacks reliable biomarkers that reflect disease activity. Recent evidence suggests that an altered sphingolipid metabolism is associated with MS pathogenesis.

Objective To explore acid sphingomyelinase (ASM) activity and altered sphingolipid metabolism as potential biomarkers in serum of MS patients, to predict active and progressive disease, and response to disease modifying therapy (DMT).

Methods Levels of serum ASM activity were longitudinally analyzed in 40 clinically isolated syndrome, 64 relapsing remitting (RR) and 10 primary progressive MS patients, and 22 healthy controls (HC). ASM activity and sphingolipid levels were measured in a different sample of 61 RRMS patients using DMT.

Results A significant difference in ASM activity levels was observed between MS patients and HC *(p*<0.001). There was no correlation between ASM activity levels and disease activity, progression or response to DMT. Ceramide (Cer)-$C_{16:0}$, Cer-$C_{24:0}$ and sphingomyelin (SM)-$C_{20:0}$, SM-$C_{22:0}$, SM-$C_{24:0}$ and SM-$C_{24:1}$ showed a significant increase during fingolimod use.

Conclusion Although higher levels in MS patients were found, ASM activity levels do not show potential as a biomarker for predicting disease activity, progression or response to DMT. Two ceramides and four types of sphingomyelin require further investigation as potential markers for treatment response.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disorder of the central nervous system (CNS), characterized by heterogeneity in disease activity, progression, and treatment response. The need for reliable blood based biomarkers is obvious.[1-5]

Recent evidence suggests that alterations in the sphingolipid pathway may reflect disease activity.[6, 7] Due to the activity of enzymes essential in the sphingolipid pathway, such as sphingomyelinases, ceramides of different chain lengths may be produced and participate in different cellular processes such as differentiation, proliferation and programmed cell death.[8, 9] Increased serum ceramide levels have been detected in CSF of patients with MS.[10, 11]

So far, one study demonstrated an altered protein expression of acid sphingomyelinase (ASM) in MS brain samples and identified that reactive astrocytes are the primary source of enzyme activity and subsequent ceramide production.[7] In general, ASM is present in lysosomes and it may be secreted upon different stimuli.[12] Interestingly, both its secretion and activity has been associated with inflammatory processes.[13, 14]

Currently, the only disease modifying treatment (DMT) in MS with a clear role in the sphingolipid pathway is fingolimod, which is a phosphorylated sphingosine-1-phosphate (S1P)-receptor antagonist. [15] The natural substrate for the S1P receptor, S1P, is also part of the sphingolipid metabolism (see figure 1).

We here explore the potential of ASM activity levels in the serum as a candidate biomarker to identify MS patients with an active or progressive disease course. Furthermore, several targets of the sphingolipid metabolism were explored longitudinally in relation to DMT.

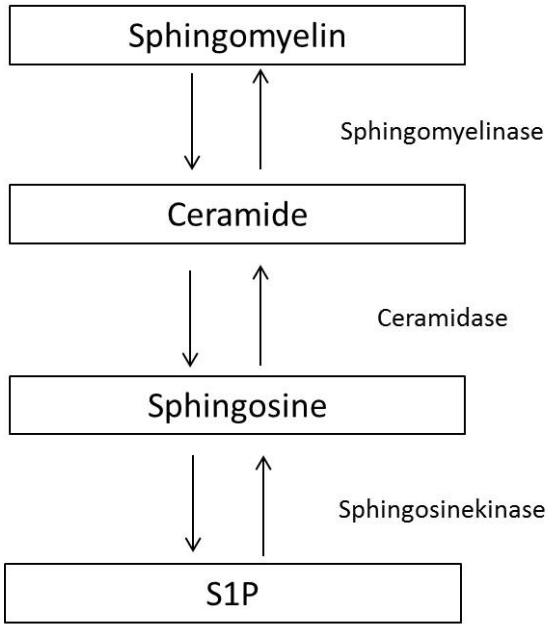

Figure 1. Schematic simplified pathway for the sphingolipid metabolism

Subjects and Methods

2.1 Patients

We included 114 MS patients from the MS Center Amsterdam and 22 healthy controls (HC). All patients were part of a prospective early inception cohort.[16] Patients were followed since diagnosis and had at least six-year of follow-up. MS subtypes included clinically isolated syndrome (CIS, n=40), relapsing remitting MS (RRMS, n=64) and primary progressive MS (PPMS, n=10) according to the 2005 McDonald criteria.[17] A total of 34 CIS patients converted to definite MS during the six-year follow-up.

To study the effect of DMT, we additionally selected 61 well-monitored RRMS patients from our outpatient clinic on second-line treatment, including 47 RRMS patients on fingolimod and 14 RRMS patients on natalizumab. See Table 1A and Table 1B for patient characteristics.

Table 1A and 1B. Patient characteristics.

A	CIS	RRMS	PPMS	HC
Disease progression part				
N=	40	64	10	22
Gender, *n* female (%)	27(67.5)	44(68.8)	5(50)	6(27.3)
Age in years, mean, SD	36.3±7.6	33.8±8.7	43.8±8.0	44.4±9.1
Disease duration in years, mean (SD)	0.6±0.5	1.8±2.2	2.8±2.6	n.a.
EDSS at baseline, median (IQR)	2.0(1.5-2.5)	2.0(2.0-3.0)	3.0(2.5-4.0)	n.a.
Disease modifying treatment at baseline, *n*	0	5[a]	n.a.	n.a.
Disease modifying treatment at follow-up, *n*	0	24[b]	n.a.	n.a.
Disease duration is calculated with the visit date and the date of onset.				
EDSS: Expanded Disability Status Scale; n.a., not applicable; SD: standard deviation				
[a] interferon-β *n*=3, glatiramer acetate *n*=1, natalizumab *n*=1				
[b] interferon-β *n*=19, glatiramer acetate *n*=1, fingolimod *n*=2, natalizumab *n*=3				

B		
DMT part	Fingolimod	Natalizumab
N=	47	14
Gender, *n* female (%)	28(59.6%)	5(35.7)
Age in years, mean, SD	38.4±9.8	34.4±11.5
Disease duration in years, mean (SD)	11.6±7.6	8.9±8.5
Disease duration is calculated with the visit date and the date of onset.		
DMT: disease modifying treatment; n.a., not applicable; SD: standard deviation		

2.2. Clinical outcomes

Relapses were defined as a period of new neurological deficits, existing longer than 24 hours and not attributable to another cause than MS. Disease progression was measured using the Expanded Disability Status Scale (EDSS)[18]. We defined disability worsening as ≥1 point increase in the EDSS score, or ≥0.5 point increase for patients with a baseline score of ≥6.0.[19] EDSS assessment was performed by certified raters. Disease activity was assessed according to the 2013 Lublin criteria.[20]

We determined patients as responders to fingolimod if there was no disease activity at two years of use following the 2013 Lublin criteria.[20]

2.3. MRI acquisition

At baseline, all patients received 1T or 1.5T MRI scans (Siemens Impact), following a standard McDonald protocol including 2D dual-echo PD/T2 and post gadolinium T1.

At year six, patients (n=65) received 3T-MRI scans (GE Signa HDxt), with the protocol including a 3D-T1 weighted fast spoiled gradient-echo sequence, a 2D dual-echo PD/T2 and a 2D spin-echo T1-weighted sequence. Brain volumes were analyzed using SIENAX[21], after lesion filling, providing normalized total brain volume (NBV).

Radiological activity was defined as a new or enlarged (> 50%) lesion on T2-weighted images compared with the MRI scan performed at year six follow up compared to the baseline scan. The delta number of T2 lesions on MRI was used to group patients into four separate categories for the number of lesions were considered: 0, 1–3, 4–9 and 10 or more T2 lesions.[22]

For the DMT part, patients received MRI scans according to the magnetic resonance imaging in MS (MAGNIMS) guidelines on MS diagnosis and patient monitoring.[23, 24]

2.4 Serum collection

Serum samples were collected in evacuated blood collection tubes. The samples were kept at room temperature, processed within 24h, and stored at the VUmc biobank at -80 ^{o}C.

2.5 ASM activity assay

ASM activity was quantified using the Acid Sphingomyelinase Assay Kit (Echelon Biosciences, Salt Lake City, USA) according to manufacturer's instructions supplemented with 0,1mM ZnCl2 and the standard curve using recombinant ASM as reference.

2.6 Lipid extraction

Sphingolipids were extracted as previously described[25, 26] and based on the modified Bligh and Dyer method.[27] Samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MSMS) as reported before.[26]

Nine-point calibration curves were constructed by plotting area under the curve, separately for ceramides, sphingomyelins and S1P, for each calibration standard Cer-$C_{14:0}$, Cer-$C_{16:0}$, Cer-$C_{18:0}$, Cer-$C_{20:0}$, Cer-$C_{22:0}$, Cer-$C_{24:1}$, Cer-$C_{24:0}$, S1P, SM-$C_{16:0}$, SM-$C_{18:0}$, SM-$C_{22:0}$, SM-$C_{24:1}$, SM-$C_{24:0}$ (Avanti polar lipids, Alabaster, AL, USA; $SM_{20:0}$ and $SM_{22:0}$ Matreya LLC, PA, USA) normalized to the respective internal standard. Correlation coefficients (R2) obtained were > 0.99. Sphingolipid concentrations were determined by fitting the identified sphingolipid species to these standard curves based on acyl-chain length. Instrument control and quantitation of spectral data was performed using MultiQuant software (AB Sciex Inc.). Data are expressed as pmol/ml for ceramides, S1P and for sphingomyelins.

2.7 Statistical analysis

For comparing the different ASM activity levels in the different subtypes a one-way ANOVA was performed. When comparing HC and MS patients an independent T-test was used.

In order to asses associations between ASM activity levels and clinical outcomes and MRI variables, logistic and linear regression models were used for binary and linear outcomes respectively. Due to non-linearity of the data, ASM activity levels were divided in subgroups (quartiles). All results were corrected for sex and disease duration.

The four patient subgroups based on the number of new T2 lesions were compared using a one-way ANOVA.

To compare baseline and follow up ASM activity levels in the fingolimod-RRMS group and natalizumab-RRMS group, a paired t test was performed.

To evaluate the effect over time of fingolimod on the levels of ceramide, S1P and sphingomyelinase in fingolimod-RRMS group, a paired t test was performed.

The regression analysis were performed using SPSS version 22.0 (Windows). $P<0.05$ was considered significant ($p<0.0074$ after Bonferroni correction for multiple testing, considering the levels of ceramide, $p<0.0083$ for the sphingomyelins).

2.8 Standard Protocol Approvals, registrations, and patient consent

The study was approved by the institutional ethics review board and and was registered under number 2002.140. All subjects gave written informed consent prior to participation.

Results

3.1 ASM activity and MS clinical subtypes.

When pooling CIS, RRMS and PPMS, higher ASM activity levels were observed in patients than in HC ($p<0.001$)(Figure2A). No significant differences in ASM activity were detected between the different MS subtypes ($p=0.42$)(Figure2B).

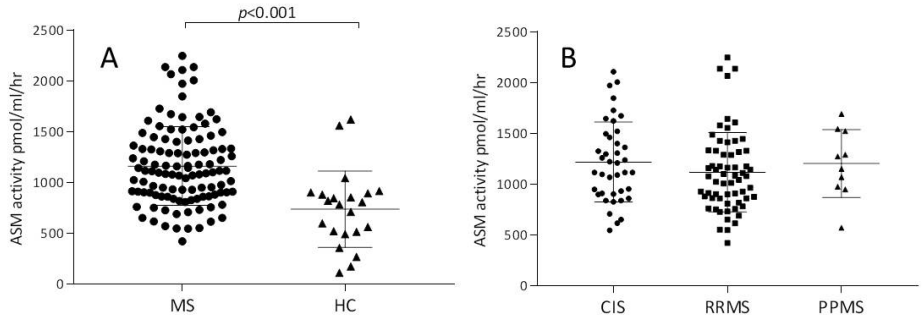

Figure 2A and figure 2B. ASM activity levels in MS subtypes and healthy controls.

To compare the different ASM activity levels in the different subtypes an one-way ANOVA or unpaired T-test was performed.

A: higher ASM activity levels were observed in patients (n=114) compared with healthy controls (n=22)(p<0.001).
B: no significant differences were detected between the different MS subtypes(p=0.42).

3.2 ASM activity and clinical outcomes.

At year 6, 72.3% of RRMS patients had experienced relapses (median annual relapse rate (ARR) of 0.36 (IQR 0.19-0.53)). There was no significant association between the ASM activity (baseline value) and disease activity based on the presence of relapses (yes or no) and ARR.

At year 6, 26.3% showed EDSS progression compared with the baseline EDSS. There was no significant association between ASM activity (baseline value) and EDSS progression.

3.3 ASM activity and baseline MRI scan.

The study population was divided into two groups based on the presence (n=43) or absence (n=66) of gadolinium-enhanced lesions on MRI at baseline (five patient did not receive gadolinium). The presence of enhancing lesions (yes or no) did not correlate with ASM activity levels and there was no significant association between the ASM levels and T2 lesion volumes.

The T1-hypointense lesion volumes were not normally distributed and therefore a natural logarithm was taken (median 183.0 ml, IQR 62.0-342.5). Due to non-linearity of the data, ASM activity levels were divided in subgroups (quartiles). The T1 lesions volumes were 2.83 times higher in group 3 of the ASM activity levels (min 1115 pmol/ml/hr - max 1364 pmol/ml/hr) compared to group 1 ASM activity levels (min 418 pmol/ml/hr – max 880 pmol/ml/hr) (p=0.012). When comparing ASM activity levels of group 1 with group 4 (lowest activity levels vs highest activity levels), no significant association was found with T1 lesions volumes (ratio 1.44, p=0.39).

3.4 ASM activity levels and brain volume measurements on MRI at year 6.

At year 6 of follow-up, the normalized brain volumes (NBV, mean 1496.8±50.6), normalized grey matter volumes (NGMV, mean 815.3±42.2), normalized white matter volumes (NWMV, mean 681.5±30.5), normalized deep grey matter volumes (NDGMV, mean 59.8±3.6) and normalized total lesion volumes (median 6.8, IQR 4.1-13.2) were measured. Baseline ASM activity levels were not associated with any of the assessed volume measures at year 6 (all *p*-values ≥0.05).

3.5 Predictive value of ASM activity levels for MRI activity.

Baseline ASM activity levels did not significantly associate with the four separate categories in delta number of T2 lesions (*p*=0.36). When the number of new T2 lesions was used in a linear regression model as a continuous variable, there was no significant association between ASM activity and the delta T2 lesions (ratio 1.00, *p*=0.18).

3.6 ASM activity levels during the years.

There were no significant differences between ASM activity levels at baseline compared with year 6 (*p*=0.86).

3.7 Effect of fingolimod and natalizumab on ASM activity levels

Next, we analyzed the potential effect of fingolimod (*n* = 47) and natalizumab (*n* =14) on ASM activity levels in additional patients specifically selected based on DMT usage.

There was no significant change in ASM activity levels during follow up with fingolimod (*p*=0.11) or natalizumab (*p*=0.24).

3.8 Effect of fingolimod on different sphingolipids.

A total of 25 out of 47 fingolimod users were randomly selected for sphingolipid analysis. Ceramides with 7 different acyl chain lengths were analyzed: Cer-$C_{14:0,}$ Cer-$C_{16:0}$, Cer-$C_{18:0}$, Cer-$C_{20:0}$, Cer-$C_{22:0}$, Cer-$C_{24:0}$ and Cer-$C_{24:1}$. After Bonferroni correction for multiple testing Cer-$C_{16:0}$ and Cer-$C_{24:0}$ showed a significant increase during fingolimod use (*p*=0.0002 and *p*=0.0005, respectively)(Figure3AB).

Sphingomyelins with 6 different acyl chain lengths were analyzed: SM-$C_{16:0}$, SM-$C_{18:0}$, SM-$C_{20:0}$, SM-$C_{22:0}$, SM-$C_{24:0}$ and SM-$C_{24:1}$. After Bonferroni correction for multiple testing SM-$C_{20:0}$, SM-$C_{22:0}$, SM-$C_{24:0}$ and SM-$C_{24:1}$ showed a significant increase during fingolimod use (Figure 4ABCD).

S1P levels slightly, but non significantly decreased when comparing before and during fingolimod (*p*=0.12).

3.9 Treatment responder vs treatment non-responder.

A total of 30 patients on fingolimod did not show disease activity (no clinical relapse and/or occurrence of contrast-enhancing T1 and/or new or enlarging T2 lesions on brain MRI) after two years of fingolimod use. Ten patients did show disease activity. There was no significant association between the ASM activity levels or sphingolipids at baseline and treatment response at year 2.

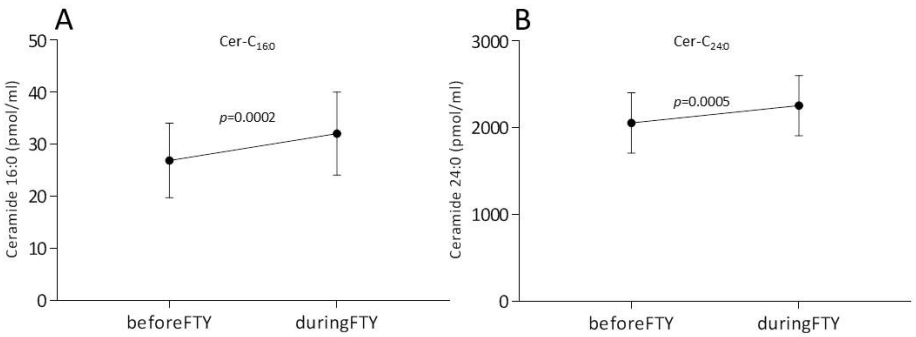

Figure 3A and 3B. The effect of fingolimod on ceramide levels.

After Bonferroni correction for multiple testing Cer-$C_{16:0}$ (4A) and Cer-$C_{24:0}$ (4B) showed a significant increase during fingolimod use (p=0.0002 and p=0.0005, respectively).

Cer: ceramide; FTY: fingolimod

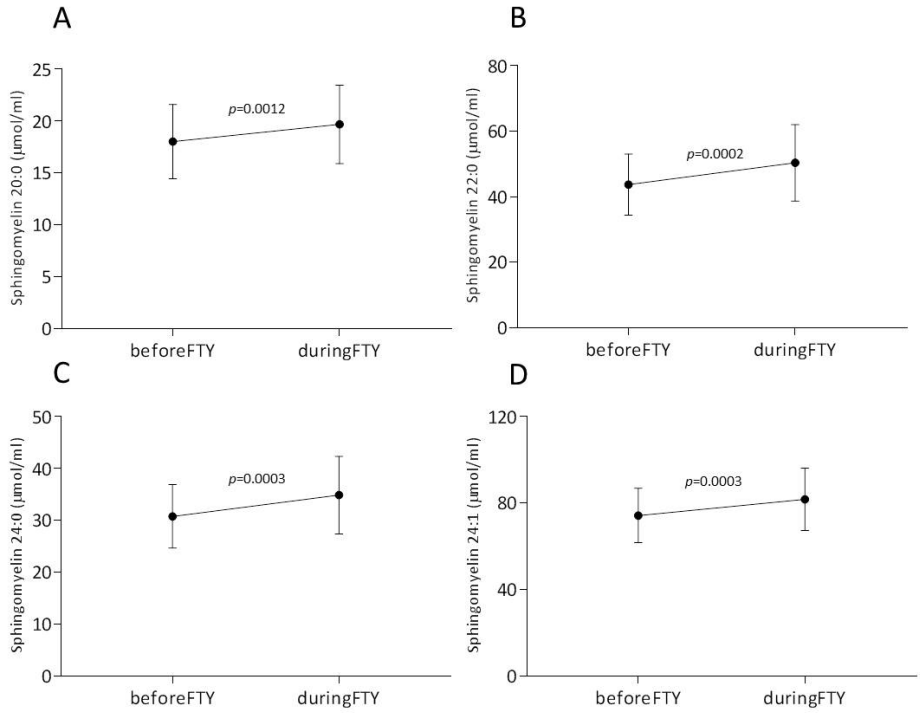

Figure 4ABCD. The effect of fingolimod on sphingomyelin levels.

After Bonferroni correction for multiple testing specific serum levels of different sphingomyelins showed an increase during fingolimod use: SM-$C_{20:0}$ (5A), SM-$C_{22:0}$(5B), SM-$C_{24:0}$(5C) and SM-$C_{24:1}$(5D) (p=0.0012, p=0.0002, p=0.0003 and p=0.0003,respectively).

SM: sphingomyelin; FTY: fingolimod.

Discussion

The present study extensively explored the potential of ASM activity levels in serum to serve as a candidate biomarker in MS. In parallel, levels of several sphingolipids were determined longitudinally in relation to DMT. When pooling all types of MS, a significant higher ASM activity level was observed than in HC. The increased levels of ASM activity in MS patients compared to HC may reflect ongoing inflammatory processes. The activity of ASM allows conversion of sphingomyelin into ceramides. Ceramides may induce neuronal mitochondrial dysfunction and axonal damage by participating in different cellular signaling cascades and processes such as differentiation, proliferation and programmed cell death.[8, 9] So far, ASM activity levels have only been determined in the CSF of MS patients, demonstrating increased levels compared to other neurological diseases (OND).[28] Besides higher levels of ASM activity, the number of exosomes that carry ASM in the CSF was significantly higher in MS patients than in patients with OND and this was correlated to CSF ASM activity.[28]

ASM activity was not significantly different in the serum of patients with RRMS, SPMS and PPMS and we did not find an association between ASM activity and the ARR, disease activity, or MRI variables, including enhancing lesions, which suggests that serum ASM activity is not a marker inflammation in MS. EDSS progression was not related to levels of ASM activity in the current study whereas previous studies have shown that the quantity of ASM (per exosome) in the CSF was correlated to the EDSS of MS patients.[28] Correlations between EDSS and other components in the sphingolipid metabolism such as $HexCer_{16:0}$ and $HexCer_{24:1}$ were also observed (in CSF), [10] suggesting more potential for CSF than serum with respect to suitable MS biomarkers.

In the second part of this study we investigated the association between the sphingolipid metabolism and DMT. ASM activity levels did not reflect treatment response. Our data are in contrast with an earlier study which showed that fingolimod in MS patients reduced the production of pro-inflammatory lipids (such as ceramide) and reduced the mRNA expression of ASM in human astrocytes.[7] However, we did observe a significant increase of two types of ceramides (Cer-$C_{16:0}$ and Cer-$C_{24:0}$) and four types of sphingomyelin (SM-$C_{20:0}$, SM-$C_{22:0}$, SM-$C_{24:0}$ and SM-$C_{24:1}$) during fingolimod use. Only one previous study explored the effect of fingolimod (and other MS therapies) on different sphingolipids in MS patients[29]. They observed that IFN-ß treatment strongly increased plasma levels of Cer-$C_{16:0}$, Cer-$C_{18:0}$, Cer-$C_{20:0}$, and Cer-$C_{24:1}$ compared to healthy controls, untreated patients, or patients receiving fingolimod or natalizumab medication.[30] One hypothesis why increasing levels of ceramides and sphingomyelin were observed in our study is that fingolimod is an S1P mimicking agent and thereby may decrease levels of endogenous S1P, which in turn may lead to increasing levels of ceramides and sphingomyelins due to the sphingolipid rheostat.

In summary, within a relatively small sample size we have shown that there is no association between ASM activity and disease activity, disability progression, or MRI measures of tissue damage in subgroups of MS patients. In addition, treatment response was not reflected by differences in ASM activity levels and S1P. We identified two ceramides (Cer-$C_{16:0}$ and Cer-$C_{24:0}$) and four types of sphingomyelin (SM-$C_{20:0}$, SM-$C_{22:0}$, SM-$C_{24:0}$ and SM-$C_{24:1}$) that increased during fingolimod treatment and the utility of these markers in treatment monitoring studies needs to be further investigated.

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Chapter 5

Tissue Transglutaminase associates with progression of multiple sclerosis

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Neurology: Neuroimmunology & Neuroinflammtion

Abstract

Objective: The clinical course of multiple sclerosis (MS) is variable and largely unpredictable pointing to an urgent need for markers for disease activity and progression. Recent evidence revealed that tissue Transglutaminase (TG2) is altered in patient-derived monocytes. Therefore, we evaluated the potential use of blood cell-derived TG2 mRNA as biomarker in patients with MS.

Methods: In peripheral blood mononuclear cells (PBMCs) from 151 healthy controls and 161 MS patients, TG2 mRNA was measured and correlated to clinical and MRI parameters of disease activity (annualized relapse rate, gadolinium-enhanced lesions and T2-lesion volume) and disease progression (Expanded Disability Status Scale (EDSS), normalized brain volume and hypointense T1-lesion volume).

Results: PBMC-derived TG2 mRNA levels were significantly associated with disease progression; i.e. worsening of the EDSS over 2 years of follow-up (std ß=0.26), normalized brain volume (std ß= -0.18), normalized grey (std ß= -0.15) and white matter volume (std ß= -0.17) in the total MS patient group at baseline. In addition, TG2 expression was significantly associated with worsening of the EDSS scores over 2 years of follow-up in RRMS patients (std ß= 0.26). Similarly, in the PPMS subtype, TG2 mRNA levels were significantly associated with EDSS (std ß= 0.48), normalized brain volume (std ß= -0.56), normalized grey (std ß= -0.43) and white matter volume (std ß= -0.65) at baseline. In addition, TG2 mRNA associated with T1-hypointense lesion volume in PPMS patients at baseline (std ß= 0.48).

Interpretation: PBMC-derived TG2 mRNA levels hold promise as biomarker for disease progression in MS patients.

Introduction

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS).[1, 2] It is characterized by inflammation, demyelination, axonal degeneration paralleled by a complex clinical course.[3, 4] MS is considered the most frequent acquired neurological disease leading to permanent disability in young adults. The severity and clinical course of MS varies among patients and is largely unpreditable[5, 6], resulting in a need for biomarkers to predict the disease course. To date only a few biomarkers have been translated into the clinic of which the majority of the clinically used biomarkers detect either conversion from clinically isolated syndrome (CIS) to MS (e.g. IgG oligoclonal bands) or treatment response (e.g. anti-drug antibodies).[7] In addition, several of the clinically used or proposed biomarkers rely on cerebrospinal fluid (CSF) collection, which is an invasive procedure.

Neurofilaments (Nf) are emerging as the most promising biomarker in MS. In fact, CSF-derived NF light (NfL) chain has been identified as a biomarker for disease activity and for treatment response in MS patients. It is considered to reflect early, acute, inflammatory-mediated axonal damage. However, it correlates less accurately with disease progression.[8] Therefore, there is an unmet need for biomarkers that predict MS progression and can be derived from blood.

Tissue Transglutaminase (TG2 or Transglutaminase 2) is a Ca2+-dependent enzyme that cross-links (among others) molecules belonging to the apoptotic pathway[9,10], membrane trafficking[11, 12] and cytoskeleton reorganization.[13,14] In addition, its expression is regulated by inflammatory mediators.[15-17] Recently, we showed the presence of TG2 in infiltrating macrophages in MS lesions[18] as well as increased TG2 mRNA in MS patient-derived monocytes[19] suggesting a possible role for monocyte/macrophage-derived TG2 in the pathophysiology of MS. Indeed, inhibition of TG2 activity reduced the influx of monocytes into the CNS and concomitant ablation of clinical symptoms has been observed in rats suffering from chronic-relapsing experimental autoimmune encephalomyelitis (cr-EAE).[20] In addition, TG2 has been proposed to contribute to encephalitogenic T cell differentiation in mice suffering from EAE.[21]

The clinical implications of TG2 have already been described for several human diseases. In fact, it has been reported that deregulation of TG2 expression and activity are associated with tumor invasiveness and metastasis formation.[22-24] Moreover, an increase in TG2 can be used as an additional biomarker for all grades of cervical dysplasia.[25] Furthermore, antibodies directed to TG2 are used as a serological marker for the diagnosis of Coeliac disease.[26,27] Nevertheless, the potential application of TG2 as a blood cell-derived biomarker in MS patients has not been studied yet.

Hence, the aim of the current study was to assess whether TG2 expressed by peripheral blood mononuclear cells (PBMC) is altered in patients with MS and whether TG2 mRNA levels associates with measures of disease activity and progression.

Materials and methods

Subjects

From the MS Center Amsterdam (VU University Medical Center), a total of 169 MS patients were included from the GeneMSA cohort in the years 2004 and 2005. Patients were diagnosed with MS according to the McDonald Diagnostic Criteria28 and also further classified either as RR, SP, or PPMS patients.[29] Disability was scored at the baseline visit and after two years using the Expanded Disability Status Scale (EDSS).[30] Inclusion criteria for MS patients were: 1) age between 18 and 65 years; 2) EDSS ranging between 0 and 7.5; 3) at MRI, all patients had been relapse-free and steroid-free for at least 1 month. In addition, a total of 157 healthy control (HC) volunteers were included (blood only). Inclusion criteria for HC were: 1) age between 18 and 65 years; 2) no history or family history of MS. The study was approved by the local ethical committee. Written informed consent was obtained from all participants.

At baseline, in the RRMS group, 37% of the patients (n=39) were treated with disease modifying treatment (DMT; n=9 glatiramer acetate; n=29 interferon β; n=1 mitoxantrone). In the SPMS group, 22.2% of the patients (n=8) were treated with DMT (n=2 glatiramer acetate; n=6 interferon β). In the PPMS group, 9.1% of the patients (n=2) were treated with DMT (n=1 interferon β; n=1 methotrexate).

Isolation of primary human peripheral blood mononuclear cells

At the baseline visit, peripheral blood was drawn by venipuncture, collected into EDTA tubes (Becton Dickinson). PBMCs were isolated from the 169 MS patients and 157 HC by density centrifugation using Ficoll (Ficoll Isopaque PLUS, GE Healtcare) and stored in liquid nitrogen until further processing.

mRNA isolation and cDNA synthesis

Total RNA was isolated from primary human PBMCs using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was further purified using the MicroElute RNA clean up kit (Omega Bio-Tek). The RNA purity was assessed using the NanoDrop 1000 (Thermo Scientific). The quality ratios 260/280 and 260/230 were above 1.9 and 1.7, respectively, for all samples included in the study. Total RNA (200 ng/sample) was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions.

Semi-quantitative real-time PCR (qPCR)

For semi-quantitative real-time PCR (qPCR), the Power SYBR Green Master Mix (Applied Biosystems) was used. The following primer sequences (Eurogentec, Maastricht, the Netherlands) were used: TG2 forward 5'-AGAGGAGCGGCAGGAGTATG-3', TG2 reverse 5'-AGGATCCCATCTTCAAACTGC-3', HPRT1 forward 5'-AGCCCTGGCGTCGTGATTAGT-3', HPRT1 reverse 5'-CGAGCAAGACGTTCAGTCCTGTCC-3', POLR2F forward 5'-GAACTCAAGGCCCGAAAG-3', POLR2F reverse 5'-TGATGATGAGCTCGTCCAC-3'. qPCR was performed in LightCycler® 480 Multiwell Plate 384 (Roche, Basel, Switzerland) on a LightCycler® 480 Real-Time PCR System (Roche). The reaction mixture (10 µl) was composed of 1× Power SYBR Green buffer (Applied Biosystems), 1.86 pmol of each primer and 5 ng cDNA. The thermal cycling conditions were an initial 10 min at 95° C followed by 50 cycles of 15 sec at 95° C and 1 min at 60° C. The specificity of the reaction was checked by melt curve analysis of the individual qPCR reactions. The relative expression level of TG2 was determined by the LinRegPCR software (version 2014.3; website: http://www.hfrc.nl) using the following calculation N0=Nq/ECq (N0=target quantity, Nq=fluorescence threshold value, E=mean PCR efficiency per amplicon, Cq=threshold cycle[31], after which the value was normalized relative to the geometric mean of the mRNA levels hypoxanthine phosphoribosyltransferase 1(HPRT1) and polymerase (RNA) II polypeptide F (POLR2F). HPRT1 and POLR2F were chosen as reference genes as being most stably expressed based on the results of the GeNorm software analysis (version 3.5) in which the stability of six different human housekeeping genes (GAPDH, MRIP, POLR2F, HPRT1, PGK1, SDHA) was assessed in a random selection of MS and HC subject samples.

MRI acquisition and analysis

Of the MS patients, imaging was performed at the baseline visit on a 1.5T MRI scanner (Siemens Vision using the standard circularly polarized head-coil) using a standardized and previously described protocol.[32] The protocol included three-dimensional (3D) T1 weighted images (repetition time [TR]: 9.7–20.8 ms; echo time [TE]: 2–4 ms; inversion time [TI]: 300–400 ms) for brain volume measurements, consisting of 1.0 mm-thick slices and a 1.0×1.0 mm2 in plane resolution. Additionally, dual echo proton density (PD)-T2-weighted images (TR: 2000-4000 ms; TE: 14-20/80-108 ms), with interleaved axial 3.0 mm-thick slices and an in plane resolution of 1.0×1.0 mm^2 and postcontrast T1-weighted spin-echo images (TR: 467–650 ms; TE: 8–17 ms; axial 3.0 mm-thick slices with an in plane resolution of 1.0×1.0 mm2) were obtained for lesion quantification.

Experienced raters manually outlined T2-hyperintense lesions on the PD/T2 images and T1-hypointense lesions on the T1 weighted spin echo images. Subsequently, lesion volumes for T2 lesions (T2LV) and T1-hypointense lesions (T1LV) were calculated. The number of gadolinium enhancing brain lesions on postcontrast T1-weighted spin-echo images was scored.

Lesion filling was then applied to the 3D T1-weighted images to prevent an effect of hypointense lesions on brain volumetry.[33] Whole brain volumes were than calculated using SIENAX (part of FSL 5.0.4) and the recommended settings for brain extraction.[34] The whole brain volume was then normalized for differences in head-size, resulting in normalized total brain volume (NBV), normalized total gray matter volume (NGMV) and normalized white matter volume (NWMV) at baseline.

Statistical analysis

According to the interquartile range (IQR) rule[35], 6 HC and 8 RRMS patients were excluded from all the analysis based on extreme TG2 mRNA levels (e.g. > 1.5 IQR). All outcomes were evaluated for normal distribution. Due to the non-normal distribution of TG2 mRNA levels, group comparisons were performed using the logarithmic transformation of TG2 mRNA levels as dependent variable in linear regression analyses (correcting for gender and age differences).

Linear regression analysis (MS patients as one group or stratified for the different groups) with either EDSS, change in EDSS over two years follow-up (normalized to baseline measurement), disease duration, NBV, NGMV, NWMV, T1LV or T2LV as dependent variables and TG2 mRNA levels as independent variable were performed while correcting for age and gender in order to determine the association between TG2 mRNA levels and clinical parameters. Due to the non-normal distribution and to the presence of 0 values in T1LV and relative change in EDSS, the association analyses were performed adding +1 to the variables prior to logarithmic transformation. Due to the non-normal distribution of T2LV, the association between TG2 mRNA level and T2LV was performed using the logarithmic transformation of T2LV.

We additionally investigated the effect of DMTs on TG2 expression, the differences in TG2 mRNA levels between patients with active/inactive disease (based on presence/absence of gadolinium enhancing lesions) and the association between TG2 mRNA and annualized relapse rate (ARR) using linear regression analysis corrected for gender and age. In those three analysis, the logarithmic transformation of TG2 mRNA was used as dependent variable. $P<0.05$ was considered statistically significant. All the analyses were performed using SPSS version 22.0 (IBM Corp, NY, USA).

Results

TG2 mRNA levels in PBMCs of MS patients

A total of 151 HC and 161 MS patients were included in the study. The demographic and clinical characteristics of both groups are described in Table 1.

First, we investigated the levels of TG2 mRNA in PBMCs derived from MS patients and HC subjects. The linear regression analysis showed that TG2 mRNA levels were comparable between the two groups (median (IQR): HC: 0.41 (0.24–0.65); MS: 0.37 (0.22-0.61); p=0.27). In addition, RR MS patients-derived PBMCs showed significantly lower levels of TG2 mRNA compared to that of HC subjects (median (IQR): HC: 0.41 (0.24-0.65); RR: 0.35 (0.17-0.57); p=0.013), while PPMS patients tended to show higher TG2 mRNA levels than HC subjects (median (IQR): HC: 0.41 (0.24-0.65); PP: 0.46 (0.28-1.13); p=0.079). Interestingly, SPMS and PPMS patients showed significantly higher TG2 mRNA levels compared to RRMS patients (median (IQR): SP: 0.43 (0.26-0.73); RR: 0.35 (0.17-0.57); *p*=0.01 and PP: 0.46 (0.28-1.13); RR: 0.35 (0.17-0.57); *p*<0.01) (Table 2).

Table 1. Demographic and clinical information of study cohort.

	HC	MS	RRMS	SPMS	PPMS
N	151	161	103	36	22
Male/Female	57/94	68/93	38/65	16/20	14/08
Age at BL: Mean ± SD in years	41.9±11.5	45±10	42.7±10	51.4±7.2	51.6±7.9
Disease duration at BL in years, as mean ± SD	NA	10.6±7.5	8.7±6.7	17.2±7.1	9.1±5.4
ARR (2 years): Mean ± SD	NA	0.10±0.35	0.16±0.43	0.014±0.08	NA
EDSS at BL: Mean (Range)	NA	3.9 (0-7.5)	3 (0-6.5)	5.6 (2-7.5)	5.1 (2-7.5)
EDSS at 24 months: Mean (Range)	NA	4.1 (1-8)	3.3 (1-6.5)	6.0 (3-8)	5.5 (1.5-8)
NBV at BL: Mean (ml) ± SD	NA	1500±98.4	1151±104	1465±88	1500±75.6
NGMV at BL: Mean (ml) ± SD	NA	743±60.8	754±61.9	715±54.4	730±51.1
NWMV at BL: Mean (ml) ± SD	NA	757±50.4	757±52.9	750±52.4	769±32.2
T2LV at BL: Median (25-75 percentile)	NA	3.4 (1.5-9.7)	3.56 (1.6-10.2)	3.8 (2.2-13.7)	2.03 (0.8-4.5)
T1LV at BL: Median (25-75 percentile)	NA	0.61 (0.1-2.9)	0.54 (0.1-2.6)	1.36 (0.4-5.7)	0.22 (0.1- 1.6)
Relapse during 2 years follow up (yes/no)	NA	16/145	15/88	NA	NA
Gd+ at BL (yes/no/unknown)	NA	39/120/2	29/72/2	8/28	2/20
DMT at BL (Untreated/Treated)*	NA	112/49	64/39	28/8	20/2

HC=healthy controls; MS=whole MS group; RR=relapsing remitting; SP=secondary progressive; PP=primary progressive; BL= baseline; ARR= Annualized relapse rate; EDSS= Expanded Disability Status Scale; NBV=Normalized Brain Volume; NGMV=Normalized Grey Matter

Volume; NWMV= Normalized White Matter Volume; T2LV= T2 Lesion Volume; T1LV= T1 lesion; DMT at BL=disease modifying treatment at baseline; * treatment description to be found in materials and methods section; Gd+ at BL=Gadolinium enhancing lesions at baseline; NA=Not Applicable; SD = standard deviation

Table 2. Linear regression analyses of TG2 mRNA levels between groups.

			95% confident interval	
	***p* value**	**Ratio**	**Lowerbound**	**Upperbound**
HC vs MS	**.27**	**.91**	**.78**	**1.07**
HC vs RRMS	**.01**	**.80**	**.67**	**.95**
HC vs SPMS	**.32**	**1.14**	**.88**	**1.49**
HC vs PPMS	**.08**	**1.33**	**.97**	**1.86**
RRMS vs SPMS	**.01**	**1.43**	**1.08**	**1.88**
RRMS vs PPMS	**<0.01**	**1.67**	**1.20**	**2.34**
PPMS vs SPMS	**.41**	**0.85**	**.59**	**1.25**

HC: M 0.41 (0.24 - 0.65); MS: M 0.37 (0.22 - 0.61); RR: M 0.35 (0.17 - 0.57); SP: M 0.43 (0.26 - 0.73); PP: M 0.46 (0.28 - 1.13); HC=healthy controls; MS=whole MS group; RR=relapsing remitting; SP=secondary progressive; PP=primary progressive; Ratio = Exp(B), exponentiation of the B coefficient; TG2 mRNA levels were not normally distributed and therefore the natural logarithm was taken. TG2 mRNA distribution in the different groups is expressed as median (IQR)

Association of TG2 mRNA with measures of disease activity

Next, we determined whether PBMC-derived TG2 expression has any potential relevance as read out for disease activity. We observed no significant association between PBMC-derived TG2 mRNA levels and measures of disease activity. In fact, TG2 mRNA levels did not significantly associate with (1) the presence or absence of gadolinium-enhanced lesions, (2) presence of relapses during follow-up (3) use of DMTs and (4) annualized relapse rate (ARR) either in MS patients or any MS subtype. In addition, no significant association was present between TG2 mRNA levels and T2LV (Table 3).

Table 3. Association of Tg2 mRNA with measures of disease activity.

		Median of relative TG2 mRNA			95% confident interval		
Type		**yes**	**no**	**p value**	**Ratio**	**Lower bound**	**Upper bound**
MS	Gd+ at BL	.30	.39	.16	.83	.63	1.08
	Relapse during FU	.41	.36	.73	1.07	.71	1.61
	DMT at BL	.35	.38	.64	.94	.73	1.21
RRMS	Gd+ at BL	.35	.38	.08	.75	.55	1.03
	Relapse during FU	.39	.33	.65	1.10	.72	1.69
	DMT at BL	.30	.35	.92	.98	.73	1.32

	ARR	
	P value	Std β
MS	.60	.04
RRMS	.48	.07
SPMS	NA	NA
PPMS	NA	NA

	T2LV	
	P value	Std β
MS	.98	- .002
RRMS	.77	.03
SPMS	.09	- .33
PPMS	.07	.43

MS= all groups combined; RR=relapsing remitting; SP=Secondary Progressive; PP= Primary Progressive; Gd+ =Gadolinium enhancing lesions; DMT= disease modifying treatment; BL= baseline, ARR= Annualized Relapse Rate; T2LV= T2 lesion Volume; Ratio = Exp(B), exponentiation of the B coefficient; NA=Not Applicable; std β= correlation coefficient

Table 4. Association of TG2 with measures of disease progression.

	MS	*p* value	RRMS	*p* value	SPMS	*p* value	PPMS	*p* value
EDSS(std β)	.26	<0.001*	.05	.61	.37	.03*	.48	.03*
EDSSΔ(std β)	.16	.05	.26	.02*	-.19	.30	.04	.87
NBV(std β)	-.18	.02*	-.15	.09	-.18	.37	-.57	<0.01*
NGMV(std β)	-.15	.03*	.12	.16	-.05	.80	-.43	.02*
NWMV(std β)	-.17	.03*	-.15	.18	-.25	.21	-.65	<0.01*
T1LV (std β)	.05	.54	.01	.93	-.04	.81	.48	.046*

MS= all groups combined; RR=Relapsing Remitting; SP=Secondary Progressive; PP= Primary Progressive; EDSS= Expanded Disability Status Scale; NBV=Normalized Brain Volume; NGMV=Normalized Grey Matter Volume; NWMV= Normalized White Matter Volume; T1LV= T1 Lesion Volume; NA=Not Applicable; std β= correlation coefficient; * =significant effect Δ EDSS Change in 2 years.

Association of TG2 mRNA with measures of disease progression

In addition to disease activity, we determined whether PBMC-derived TG2 mRNA is potentially relevant as a read-out for disease progression (Table 4). Indeed, TG2 mRNA was positively associated with clinical disability, i.e. EDSS score at baseline in MS patients (std. ß=0.26; p<0.001) (Fig 1A). Moreover, TG2 mRNA levels associated positively with EDSS scores at baseline in SPMS patients (std ß=0.37; *p*=0.03) and PPMS patients (std ß=0.48; *p*=0.03) (Fig 1G). Interestingly, in MS patients and in particular in RRMS patients, TG2 mRNA levels were associated with worsening of disability, as measured by the change in EDSS score, over a 2 year follow-up (MS: std. ß=0.16; *p*=0.05 and RR: ß=0.26; *p*=0.02) (Fig 1B and F, respectively).

Furthermore, in MS patients, a significant negative association of TG2 mRNA with both NBV (std ß=-0.18; *p*=0.02), NGMV (std. ß=-0.15; *p*=0.03) and NWMV (std. ß=-0.17; *p*=0.03) at baseline was observed (Fig 1C-E, respectively). Similarly, in PPMS patients, a significant negative association between TG2 mRNA levels and NBV (std ß=-0.56; p=0.01), NGMV (std ß=-0.43; *p*=0.02;) and NWMV (std ß=-0.65; *p*<0.01) at baseline was observed (Fig 1H-J, respectively).

Interestingly, in PPMS patients also T1LV at baseline showed a significant positive association with TG2 expression levels (std ß=0.48; *p*=0.04) (Fig 1K).

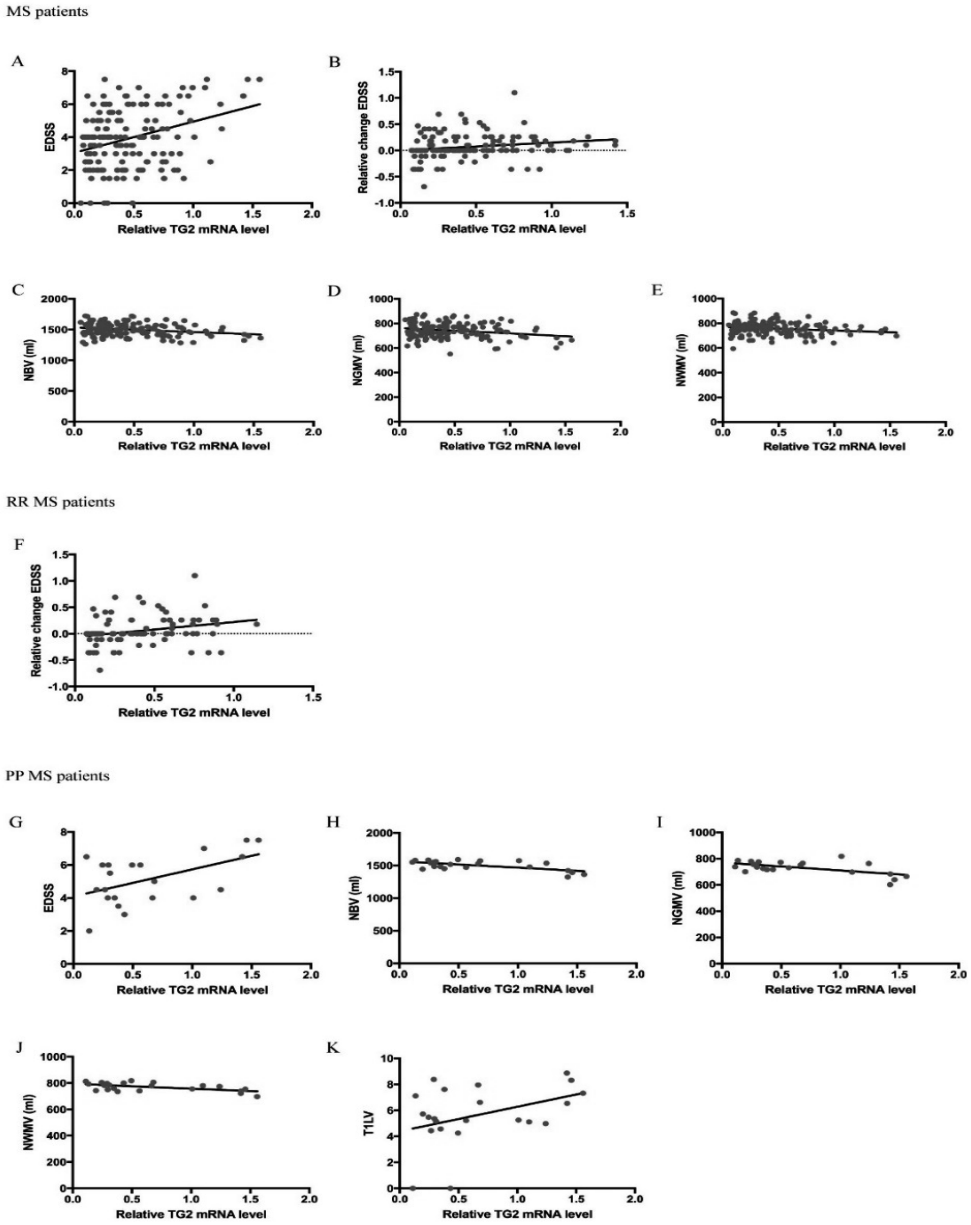

Figure 1. Association of TG2 with measures of disease progression in (subtypes of) MS patients.

Association of PBMCs-derived TG2 mRNA levels with A) Expanded Disability Status Scale (EDSS); B) relative change of EDSS over 2 years follow-up; C) normalized brain volume (NBV); D) normalized gray matter volume (NGMV) and E) normalized white matter volume (NWMV) in MS patients. Association of PBMCs-derived TG2 mRNA levels with F) relative change of EDSS over 2 years follow-up in RR MS patients. Association of PBMCs-derived TG2 mRNA levels with G) Expanded Disability Status Scale (EDSS); H) normalized brain volume (NBV); I) normalized gray matter volume (NGMV); J) normalized white matter volume (NWMV); K) T1 lesions volume (T1LV). Linear regression analysis was performed controlling for gender and age. Relative change of EDSS was calculated as Log (1+Relative change EDSS). T1LV was calculated ad Log(1+T1LV).

Discussion

During the last decades, there has been an increasing interest in TG2 as a potential prognostic marker or therapy target in various human pathologies.[36-38] Previous research has highlighted the possible role for TG2 in the pathogenesis of MS[19,39] as well as in its animal model experimental autoimmune encephalomyelitis (EAE). [20,21,40] In search for potential blood-derived biomarkers, this study is the first to report PBMC-derived TG2 mRNA levels in MS patients, and in its clinical subtypes, in relation to markers of disease activity and progression.

First, we found no difference in TG2 mRNA levels between MS patients as a group and HC subjects, probably due to high variability in expression levels in the group of MS patients. Next, we observed that in PBMCs derived from RRMS patients significantly lower levels of TG2 mRNA were measured compared HC subjects, whereas in PBMCs of SPMS and PPMS patients more TG2 mRNA was found. In a previous study, we observed clear enhanced levels of TG2 mRNA in blood derived monocytes from MS patients.[19] The current study presents a different TG2 expression pattern in MS patient-derived PBMCs. Thus far, regulation of TG2 expression in the whole PBMC population has not been studied, but it is known that TG2 expression is induced in monocytes and macrophages by the anti-inflammatory cytokine interleukin-419.[41] Lymphocytes, in particular T-cells, have also been described to produce TG2.[42,43] Thus, our data may indicate that TG2 expression is regulated by inflammation within several cell subtypes of the PBMC population. Nevertheless, PBMC-derived TG2 mRNA levels do not differ between MS patients and HC subjects and, therefore as such, it cannot clearly discriminate between the two groups.

Regarding the association between TG2 and measures of disease activity (i.e. presence of a relapse, annualized relapse rate, Gd+ lesions and T2-lesion volume), no significant associations were found, suggesting that the presence of active disease is not associated with the level of TG2 mRNA in PBMCs. Moreover, TG2 mRNA levels were not associated with the use of DMTs, which target inflammatory processes ongoing during MS. Although we cannot draw conclusions on individual MS patients, this finding suggests that the inflammatory status of the disease does not affect TG2 expression in PBMC's.

In addition, association between TG2 expression and measures for disease progression was evaluated. Interestingly, we observed that TG2 mRNA levels significantly associated with clinical and radiological measures of disease progression; In fact, TG2 expression showed significant association with patients disability (EDSS) as well as NBV, NGMV and NWMV at baseline in both the total group of MS patients and in PPMS patients. Furthermore, in PPMS patients, TG2 mRNA was also significantly associated with T1LV. Together, these data indicate that in particularly in PPMS patients, PBMC-derived TG2 mRNA levels are

associated with progression of the disease and axonal damage. Although the described associations were detected cross-sectionally, TG2 mRNA also showed power to associate with disease progression longitudinally; in fact, in both the total group of MS patients and in the RRMS subtype, TG2 mRNA levels at baseline were associated with worsening of the clinical symptoms (change in EDSS) over a 2 year follow-up. Thus, we are the first to point to PBMC-derived TG2 mRNA as biomarker for disease progression in MS patients as group, and more specifically in the PPMS or RRMS subgroups of MS patients. Subsequent prospective, longitudinal studies in MS patients are required to evaluate individual PBMC-derived TG2 mRNA levels over the course of the disease in association with measures of disease activity and in particular, disease progression. This may eventually result in a patient predictive blood-derived biomarker for disease progression in RRMS and PPMS patients for which there is an unmet need.

To date, many of the proposed candidate biomarkers for MS disease activity and progression are measured in the CSF which can only be collected through an invasive procedure, thus making a CSF-derived biomarker less patient friendly over prolonged time. In addition, there is an urgent need for biomarkers that can predict disease progression and reflect ongoing axonal damage specifically in progressive patients. To date, NfL seems to be most promising as a marker of MS disease activity and response to DMT[44-47] being also validated in serum samples.[48,49] Although some recent papers also suggest correlations with disease progression in MS[8,50], this has to be confirmed. In addition, serum level of glia fibrillary acid protein (GFAP) has been proposed as biomarker for disease severity in PPMS patients.[51] However, no association with MRI parameters were found.[51]

In conclusion, the novel findings reported in this study indicate that PBMCs-derived TG2 mRNA levels associate with clinical and radiological measurements of MS disease progression and therefore holds promise as biomarker for disease progression in MS and especially in PPMS patients.

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Part 3

Treatment monitoring biomarkers

Chapter 6

Serum neurofilament light levels do not reflect disability progression in natalizumab-treated relapsing remitting multiple sclerosis patients

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In preparation

Abstract

Objective: To investigate the potential of serum NfL (sNfL) as a biomarker of disability progression during natalizumab (NTZ) treatment.

Methods: We measured sNfL longitudinally in a cohort of 89 NTZ-treated RRMS patients followed clinicallyfor a median of 5.2 (range 3-10) years. We examined whether sNfL at baseline (BL) and after 12 months of treatment predicted disability (EDSS) progression in the following 2 years, and whether the longitudinal trajectories of sNfL levels differed between progressors and non-progressors.

Results: We observed a significant reduction in sNfL levels at 3 months (median sNfL at BL 14.8 pg/ml, median sNfL at 3 month 11.1 pg/ml), which reached its nadir of close to 50% of BL levels at 12 months (median SNfL at 12 months 7.9 pg/ml) after NTZ initiation. Between the year 1 follow-up visit and year 3, 35/89 (39.3%) individuals showed EDSS progression. We found no difference in the longitudinal dynamics of sNfL in progressors versus non-progressors. sNfL levels at BL and at 12 months did not predict EDSS progression in the following 2 years.

Conclusion: Our findings confirm the potential of sNfL to monitor the reduction in focal inflammatory damage that accompanies NTZ introduction, but sNfL fails to capture longer-term EDSS worsening during NTZ treatment.

Introduction

Multiple Sclerosis (MS) is a chronic inflammatory and degenerative disease of the central nervous system (CNS). In a majority of patients, the initial clinical course of the disease is characterized by the occurrence of relapses followed by remissions (relapsing remitting MS, RRMS). With time, the relapse rate wanes, and patients transition progressively into a clinical phase characterized by gradual accumulation of disability (secondary progressive MS, SPMS), which builds up independently of the occurrence of relapses. In the last two decades, the number of treatments to significantly reduce relapse rates in RRMS has steadily risen.[1] Of these treatments, natalizumab (NTZ), a humanized monoclonal antibody against cell adhesion molecule alpha4-integrin, is one of the most effective.[2] The molecular mechanisms driving long-term disability progression in MS are unclear.[3] Although most prominent in SPMS, disability progression occurs insidiously in early RRMS patients, at least partially independently of relapses and/or new T2 or Gadolinium-enhancing (GE) lesions.[4] Treatments that significantly hamper the rate of disability progression are scarce, indicating progression involves mechanisms that differ from relapse-related neuroaxonal damage. In order to evaluate the potential of novel therapies to reduce progression rate and disability accumulation, biomarkers allowing to quantify and/or predict this process are needed.

Neurofilament light (NfL) is a cross disease axonal damage biomarker [5]which increases in cerebrospinal fluid (CSF) and serum of RRMS patients during relapses and concomitantly to the appearance of new T2 and/or GE lesions, returning to baseline within a couple of months of the acute event.[6, 7] In addition, NfL levels decrease in individual RRMS patients after disease modifying therapy (DMT) initiation.[8] This finding was reported for all DMTs, including injectable DMTs, fingolimod, natazimab, rituximab and alemtuzumab.[9-12] The magnitude of the decrease in NfL levels correlates with the efficiency of the DMT in reducing relapse rate, and NfL was reported to further decrease in individuals switching from first-line to second-line DMTs[10]. Together, these data indicate NfL is a valid marker reflecting acute neuroaxonal damage, and holds high potential to monitor inflammatory disease activity in individual MS patients in the clinical setting.

Recent studies suggests that there is an association between serum NfL (sNfL) levels and disability progression.[7, 13, 14] However, a recent study found no association of baseline NfL with year 10 EDSS.[15] It remains unclear whether NfL may predict or reflect disability progression in the absence of acute focal inflammation-related neuroaxonal damage. In view of the anti-inflammatory effectiveness of NTZ, studying NfL levels during NTZ treatment provides the opportunity to study the relation of NfL with disease progression independent of inflammatory mechanisms. Therefore, the main aim of this study was to investigate the potential of sNfL as a biomarker of disability progression with minimal contribution of relapses and new T2 or GE MRI lesion formation, collectively referred to as focal inflammatory activity.

Methods

Cohort

Patients were selected from an ongoing prospective observational cohort study which was initiated in 2006 at the VU University Medical Center, Amsterdam, The Netherlands. RRMS patients were included in this natalzimab cohort study if they were 18 years or older at time of NTZ initiation.[16] Patients were monitored clinically and radiologically at yearly intervals. We selected 89 patients from this cohort, if a minimum follow-up time of 3 years from NTZ initiation was achieved. Clinical disease severity was assessed using neurological examination and quantified using the Expanded Disability Status Scale (EDSS). The cohort was retrospectively divided into 2 outcome groups, according to the presence of disability progression. Clinical progression was defined in two ways. First, as an increase in EDSS of 1.5, 1 or 0.5 in case of a reference EDSS of 0, 1–5 or ≥ 5.5, respectively between year 1 and year 3 (Figure 1).[17] Next, we applied the EDSSplus, where disability progression was defined as progression on one of three assessments (EDSS, 25-FWT, 9-HPT).[16, 18] Progression on the timed 25-foot walk test (25-FWT) and 9-hole peg test (9-HPT) were defined as a minimum of 20% worsening on both the 25-FWT or the 9-HPT, confirmed after at least 6 months. Progression on EDSS, 25-FWT, 9-HPT or MRI during the first year of treatment was excluded, in order to exclude the impact of relapse recovery shortly after start of treatment in patients showing improvement.[16, 19]

The local medical ethical committee consented to this study, and all patients gave written informed consent for the collection and use of medical data and biological fluids for research purposes. This study was in accordance with the ethical principles of the Declaration of Helsinki.

Serum NfL measurement

Blood was collected at the start of treatment, after 3 months, 1 year, 2 years, and at last follow-up (Figure 1) via standard vena puncture and centrifuged at 1800 *g* for 10 min at room temperature. The serum was aliquoted and stored at −80°C until analysis. sNfL levels were quantified using an in-house developed Simoa assay, as previously described.[20] The assay was validated prior to use according to standardized international protocols.[21] The samples of each individual patient were analyzed within one run, and the personnel performing the analyses was blinded for the clinical data.

Magnetic resonance imaging

MRI protocols included proton-density (PD)/T2-weighted and postcontrast T1-weighted images as earlier described in the paper of Dekker et al.[16] MRI scans were obtained at baseline and yearly for John Cunningham (JC) virus negative patients, and 3-monthly for

patients with a JC virus antibody positive status, according to the progressive multifocal leukoencephalopathy (PML) pharmacovigilance protocol. For this study the yearly scans were checked for new disease activity.

Statistical analyses

Statistical data analysis was performed using SPSS for windows, version 22. Graphs were constructed using GraphPad Prism version 7.02. A probability level of $p<0.05$ was considered statistically significant. Chi-square tests, Mann Whitney U tests and Kruskal-Wallis tests with post-hoc Bonferroni corrections were used to compare baseline demographics and clinical characteristics. Neurofilament measures were compared using age and gender-corrected[5] univariate analyses of variance (log-transformed data). Binary logistic regression (backward selection) was used to identify predictors for EDSS progression and EDSSplus progression.

Results

Assessment of disability progression

In total, 89 RRMS patients treated with NTZ followed clinically for at least 3 years were included in this study (median total follow-up time of 5.2 years, IQR 4.3-6.7). At baseline, mean age was 36.9 years (SD +/- 8.5), 74.2% patients were female and median disease duration was 7.4 years (IQR 3.8-12.1) (Table 1). Median EDSS after 1 year follow-up was 3.5 (IQR 2.5-4.5), and median EDSS after 3 years follow-up was 3.5 (IQR 3.0-4.5) (Table 1). During the first 3 years of follow-up excluding the first 3 months, 16.9 % patients experienced a relapse, and between year 1 and year 3, 6.7% showed new T2 or Gadolinium enhancing (GE) lesion(s) on follow-up brain MRI (Table 1). Between the year 1 follow-up visit and the year 3 follow-up visit, the time-window to define progression in this study, 35/89 patients (39.3%) showed EDSS progression (classified as progressors), and 54/89 (60.7%) did not (classified as non-progressors) (Table 1). Median disease duration at BL was longer in progressors versus non progressors (Table 1, p=0.03). The proportion of individuals with relapses after start treatment was low (9 patients and respectively 6 patients) did not differ between progressors and non-progressors (Table 1). The percentage of individuals with new T2 or GE lesion(s) on follow-up brain MRIs was significantly higher in the progressors versus non progressors (p=0.02).

Additionally, the group was divided based on EDSSplus. From the 89 patients, 77 patients could be included for the analyses (12 patients had missing data), 33 patients (42.9%) showed EDSSplus progression and 44 (57.1%) did not.

Table 1. Demographics of the cohort.

	Total cohort	Non-progressors	Progressors	p-value
N (% of whole cohort)	89 (100)	54 (60.7)	35 (39.3)	-
% females	74.2	75.9	71.4	*ns*
Mean age at BL (SD) in years	36.9 (8.5)	35.9 (8.4)	39.3 (8.0)	*ns*
Median disease duration at BL (IQR) in years	7.4 (3.8-12.1)	6.4 (3.0-10.4)	9.4 (5.7-15.7)	***0.03***
Median time from BL to last FU (IQR) in years	5.2 (4.3-6.7)	5.0 (4.0-6.3)	5.4 (5.0-7.2)	*ns*
% individuals with relapse during the first year of FU, excluding the first 3 months	6.7	5.6	8.6	*ns*
% individuals with relapse during the second year of FU	4.5	3.7	5.7	*ns*
% individuals with relapse during the third year of FU	5.6	7.4	2.9	*ns*
Cumulative % individuals with relapse during first 3 years of NTZ treatment, excluding the first 3 months	16.9	11.1	25.7	*ns*
% individuals with new T2 or GE lesions during the second year of FU	4.5	-	11.4	-
% individuals with new T2 or GE lesions during the third year of FU	2.3	1.9	2.9	*ns*
Cumulative % individuals with new T2 or GE lesions during first 3 years of NTZ treatment	6.7	1.9	14.3	***0.02***
Median EDSS at 12mo FU (IQR)	3.5 (2.5-4.5)	3.5 (2.5-4.0)	3.5 (2.5-5.0)	*ns*
Median EDSS at 24mo FU (IQR)	3.5 (2.5-5.0)	3.0 (2.5-4.0)	4.0 (2.9-5.6)	*ns*
Median EDSS at 36mo FU (IQR)	3.5 (3.0-4.5)	3.0 (2.5-4.0)	4.0 (3.0-5.5)	*ns*
Median EDSS at last FU (IQR)	4.0 (3.0-5.75)	3.5 (2.0-4.5)	5.0 (3.5-6.0)	*ns*

Longitudinal dynamics of serum neurofilament light levels in response to natalizumab treatment

NfL was measured in serum on the day of NTZ initiation before the infusion (BL), 3 months, 1 year, and 2 years after BL, and on the last follow-up visit (Figure 1). In the total cohort, median sNfL decreased significantly from 14.8 pg/ml at BL to 11.1 pg/ml at 3 months, and reached its nadir of 7.9 pg/ml at 1 year. Baseline levels of sNfL did not differ between progressors and non-progressors (Table 1). In addition, the levels still did not differ between EDSSplus progressors and EDSSplus non-progressors (p=0.83)

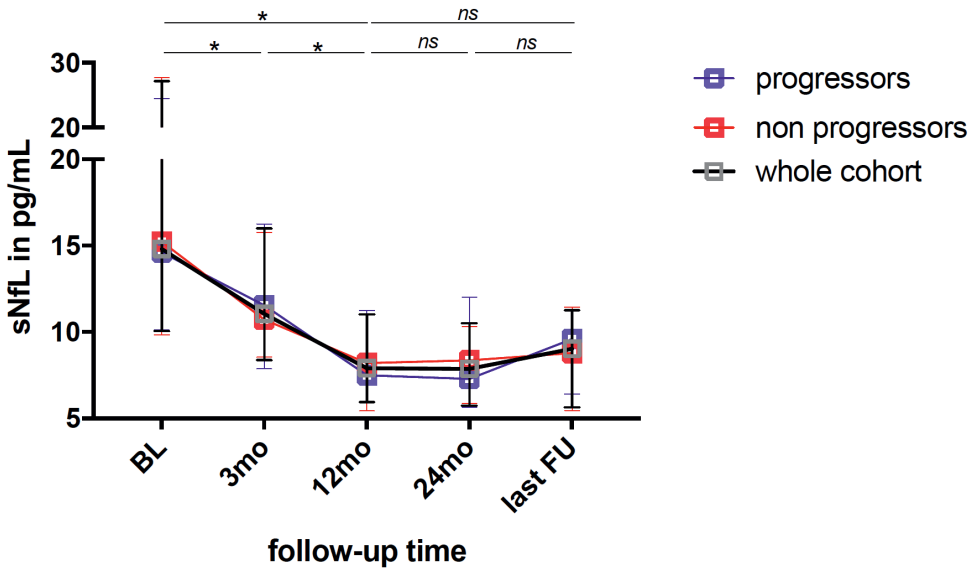

Figure 1.

Median and interquartile range of serum neurofilament light (sNfL) in picogram per milliliter (pg/mL) at baseline (BL), 3 months (3 mo), 12 months (12 mo), 24 months (24 mo) and at last follow-up (FU = median total follow-up of 5.2 years , IQR 4.3-6.7) in the whole cohort (N=89), in EDSS progressors (N=35), and in non-progressors (N=54). *= p<0.05; ns=non-significant.

Serum neurofilament light as a predictor of future disability progression

We investigated predictors of EDSS progression between the year 1 and the year 3 visits, using a logistic regression model. sNfL at BL or at year 1 did not predict EDSS progression, neither did age at NTZ onset, gender and EDSS at year 1. The only significant predictor of EDSS progression was disease duration at BL (p=0.03, OR 1.10 95% CI 1.00-1.19).

As an additional analysis we investigated predictors of EDSSplus progression between the year 1 and the year 3 visits. sNfL at BL or at year 1 did not predict EDSSplus progression, neither did age at NTZ onset, gender, disease duration at BL and EDSS at year 1 (data not shown).

Discussion

In this study, we aimed to define the potential of sNfL to reflect and predict disability progression occurring independently of relapses or new T2/GE MRI lesion formation, collectively referred to as focal inflammatory activity. We took advantage of a cohort of 89 RRMS patients treated with NTZ, a DMT which is very effective in reducing relapse rates and novel T2 or GE lesions, but has no effect on disability progression as assessed by EDSS.[22] Indeed, no clinical or radiological signs of focal inflammatory activity were present in over 80% of our patients during the follow-up period of a median of 5.2 years, showing the effectiveness of the treatment. Nevertheless, 39.3% of the patients showed clinical disability progression during a 2-year follow-up period (between year 1 and year 3 visits) after NTZ initiation. Thus, this cohort allowed us to examine disease progression largely uncoupled from focal inflammatory activity.

We observed a reduction in sNfL levels of almost 50% in the total group, that reached their nadir within 1 year after NTZ initiation, in accordance with other studies.[8, 10] Further, we found that the levels of sNfL remained low for the entire follow-up period, which extended to up to 10 years. However, we observed no difference in the longitudinal dynamics of sNfL levels in progressors versus non-progressors, suggesting sNfL does not capture neurodegenerative processes underlying disability progression when uncoupled from focal inflammatory activity. From a clinical perspective, this indicates that sNfL shows to be only useful to monitor the decrease in focal inflammatory activity that occurs in response to NTZ, and not to predict disability progression. In clinical trial settings, these results suggest sNfL may not be a sensitive end-point to quantify disability progression and its modification in response to DMTs. Recent data showing that sNfL correlates with residual inflammatory activity, as monitored by CSF inflammation markers, after NTZ treatment, further support the concept that sNfL primarily reflects inflammatory activity.[23]

Our results of median baseline values of 15 pg/ml are at the lower side of the range of results reported by others[7, 24] using the Simoa platform. For absolute levels to be comparable between studies, more standardization is needed, and the development of reference material to calibrate methods against.

Recently, sNfL was reported to predict EDSS worsening in the following year, [14] regardless of DMT status at time of measurement. In our NTZ cohort, sNfL levels at baseline, and at 12 months did not predict EDSS progression in the following 2 years and sNfL levels at BL and 12 months did not differ significantly between both groups. In the study by Barro et al[14], DMT status was heterogeneous at time of sNfL measurement, with some patients treated, others not and none of the patients were on NTZ treatment. Moreover, in the present study,

all patients were treated during the assessment of progression, uncoupling damage arising from focal inflammatory activity from neurodegeneration.

Our results are supported by the result of Chitnis et al[15] and Siller et al.[25], similarly, showing no correlation between EDSS progression and baseline sNfL levels. In contrast to the lack of a relation with clinical progression, at sNfL levels were correlated with 10 year MRI brain lesions and brain atrophy in MS patients in the study of Chitnis. The study by Siller et al. similarly demonstrated that brain parenchymal volume decreased more rapidly in patients with higher baseline sNfL. In addition, cross sectional relations of NfL, measured either in CSF or serum, with EDSS were less strong than with atrophy, as an objective measure of pathological progression. Since in our study, atrophy measurements were not available, the question whether sNfL can predict pathological progression independent of focus disease activity is still open.

Clinical progression in RRMS individuals was recently characterized and the term “silent progression” as suggested by Cree et al.[4] Whether the mechanisms underlying silent progression involve inflammatory processes that are not targeted by currently available DMTs, or by mechanisms independent of inflammation, remains to be determined.

The strength of this study is the prospective nature of the detailed longitudinal clinical and radiological data and longitudinal sample collection. However, the study also has limitations. The data would have benefited from a larger sample size and longer follow-up duration . Nevertheless, the lack of even a trend towards a difference in longitudinal sNfL measurements between progressors and non-progressors strengthens the truthfulness of our findings.

In conclusion, our findings confirm the potential of sNfL to monitor the reduction in focal inflammatory damage that accompanies NTZ introduction, but fails to capture longer-term EDSS worsening (“silent progression”) that is largely independent of relapse activity.

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Chapter 7

Switching natalizumab to fingolimod within six weeks reduces recurrence of disease activity in MS patients

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Abstract

Background Natalizumab is an effective treatment in relapsing-remitting multiple sclerosis. Mainly because of the risk of progressive multifocal leukoencephalopathy (PML), a substantial proportion of John Cunningham (JC) virus positive patients switch to fingolimod. Previous reports show a clear benefit when the duration of a wash-out (WO) period of natalizumab is 0-3 months in comparison to longer WO periods. However, there is no consensus regarding the optimal duration of a WO period under 3 months.

Objective We compared MS disease activity after different WO periods. In addition we investigated several factors that possibly influence recurrence of disease activity, including serum natalizumab concentration and lymphocyte counts.

Methods From a prospective observational cohort study of natalizumab treated patients we selected 52 patients who switched to fingolimod. We divided the patients in three groups (<6 weeks, 6-8 weeks, >8 weeks WO). Serum natalizumab concentration and lymphocyte count were assessed during and after natalizumab treatment.

Results Patients with a WO period of >8 weeks had a significant higher recurrence of disease activity (OR 6.8, 95% CI 1.4–32.8) compared to patients with a WO period of <6 weeks. Serum natalizumab concentration and lymphocyte count did not predict recurrence of disease activity.

Interpretation A short WO period decreases the risk of recurrence of disease activity. The possible impact of a short WO period on the risk of carry-over PML in JC virus positive patients remains uncertain.

Introduction

Natalizumab, a humanized monoclonal antibody targeting the α-4-integrin receptor, is an effective treatment in relapsing-remitting multiple sclerosis (RRMS).1 Unfortunately, the risk of progressive multifocal (PML), an opportunistic brain infection caused by the John Cunningham (JC) virus, complicates the long-term use of natalizumab in JC virus positive patients.2 Because of the risk of PML, patients often switch to alternative disease modifying therapy like fingolimod, a sphingosine 1-phosphatereceptor modulator. Natalizumab limits lymphocyte transport over the blood brain barrier, whereas fingolimod prevents recirculation of lymphocytes from the lymph nodes. The different immunosuppressive pathways and the fact that natalizumab may be detectable in the serum up to 6 months after discontinuation, may theoretically increase the risk of opportunistic infections like PML.[3] Therefore, it has been suggested to consider a wash-out (WO) period between natalizumab discontinuation and fingolimod initiation. However, the length of the WO period remains a point of discussion as the increase of disease activity after natalizumab discontinuation is a well-known and clinically relevant issue.[4, 5] Recent publications have reported an increase of disease activity with longer WO periods, resulting in an advised WO period shorter than 3 months.[6, 7] However, even though current evidence is pointing towards the superiority of very short WO periods,[8, 9] uniformity in how short the WO period should be within the timeframe of 2-3 months, is still lacking.

Apart from the WO period, disease breakthrough after discontinuation of natalizumab may also depend on natalizumab serum concentration which may vary largely between patients and are shown to correlate with disease activity.[10, 11] Another important factor may be the extent and swiftness by which fingolimod succeeds to reduce the number of circulating lymphocytes. Whereas natalizumab is associated with a stable increase of peripheral lymphocytes[12-14], sphingosine 1-phosphatereceptor modulation caused by fingolimod aims to lower amounts of circulating lymphocytes.[15, 16] The interplay between drug-induced increased and subsequently decreased lymphocyte count may have impact on the recurrence of disease activity.

The aim of our study was to investigate the correlation between the recurrence of disease activity within six months of natalizumab discontinuation with different WO periods (<6 weeks, 6-8 weeks and >8 weeks) and the influence of serum natalizumab concentration and lymphocyte count during treatment in RRMS patients switching from natalizumab to fingolimod.

Methods

Patients

This study is part of an ongoing prospective observational cohort study conducted at the VU University Medical Center including all RRMS patients treated with natalizumab. Patients in this cohort are at least annually subjected to a brain MRI and clinical testing including Expanded Disability Status Scale (EDSS).[17]

We included all patients who switched from natalizumab to fingolimod between February 2013 and August 2016. We excluded patients who did not start fingolimod within six months after discontinuation of natalizumab, or without a brain MRI within six months. All patients in the observational cohort gave written informed consent for the use of the clinical, laboratory and imaging data for research and teaching purposes. A waiver from our local institutional review board stating that the requirements of the Medical Research Involving Human Subjects Act did not apply and that official IRB approval was not mandatory for the use of these data, was obtained.

Measurement of clinical disease activity

The main outcome is disease activity within six months of natalizumab discontinuation.

We assessed disease activity according to the 2013 Lublin criteria.[18] Following these criteria, disease activity was defined as 'active' when the patient had a clinical relapse and/or the occurrence of contrast-enhancing T1 and/or new or enlarging T2 lesions on brain MRI.

We collected data of the patients' clinical files regarding relapses one year before, during, and one year after natalizumab use. A relapse was defined as a period of new neurological deficit, existing longer than 24 hours, and not attributable to another cause than MS.

Clinical disability was defined by the Expanded Disability Status Scale (EDSS). We collected the EDSS scores at time of natalizumab initiation and at time of switch (initiation of fingolimod) from the clinical database of the observational cohort. The EDSS was assessed by certified raters.

MRI acquisition

The MRI acquisition and the scan interval was performed according to the MAGNIMS guidelines on MS diagnosis and patient monitoring.[19, 20] MRI examination was performed annually during natalizumab usage in patients with a negative JC virus serostatus. In JC virus seropositive patients (all patients described in this study), the frequency of brain MRI increased to every 3 months, which is our current pharmacovigilance protocol suggested by different international panel guidelines.[20, 21] Following natalizumab discontinuation, a follow-

up scan was performed after 3 and 6 months because of the risk of carry-over PML. The scan performed within the first three months of fingolimod treatment, was used as the baseline scan of fingolimod treatment. MRI scanning was performed at a 1.5 or 3 Tesla scanner (Siemens Magnetom Impact, Erlangen, Germany) including 3D fluid attenuated inversion recovery and axial PD/T2 weighted sequences. We assessed brain MRI for active T2 lesions (new of enlarging) and contrast enhancing lesions in case gadolinium was administered.

Blood sampling

Blood samples were routinely obtained every three months before natalizumab infusion.

Blood samples during fingolimod treatment were also routinely obtained following our protocol: at baseline, month 1 and 3-monthly from month 3 during the first year. Hematological count, including lymphocytes were measured in these samples in the clinical chemistry laboratory at the VU University medical center. Serum was subsequently stored at -80°C at the biobank at the VU University medical center. In order to assess clearance of natalizumab, we measured natalizumab concentration before discontinuation of natalizumab and within six months after discontinuation. Natalizumab concentration before discontinuation was measured right before a new infusion (trough concentration). Intra-individual natalizumab trough concentration are stable in a four week interval regimen.[22] Sera were sent to Sanquin Laboratory to measure natalizumab concentration using a cross-linking assay using polyclonal rabbit anti-natalizumab F(ab)2 fragments for capture and a mouse anti-IgG4 monoclonal antibody for detection. This method is described in more detail elsewhere.[11] Serum JC virus status and indexes were obtained every six months via Unilabs, Copenhagen, Denmark.

Statistical analysis

In order to assess the association between disease activity and WO period, we divided the WO period into three groups; a WO period <6 weeks, 6-8 weeks and >8 weeks. Differences between the distinct groups were analyzed using a logistic regression model and potential confounders (sex and age) were analyzed via assessing the change of the regression coefficient of the risk factor of interest, before and after adjusting for the potential confounder. If the regression coefficient changed more than 10%, the confounder was considered relevant and will be adjusted for. No confounders were found and the statistical results did not change when comparing adjusted regression models with unadjusted regression models.

The association between disease activity and concentration of natalizumab was studied using a multivariable logistic regression model. Results are displayed as an odds ratio (OR) with the confidence interval (CI) per unit decrease, adjusted for WO-period.

For comparing mean natalizumab concentration at certain time points we used the independent sample T test, as concentrations were normally distributed. For correlation analysis between lymphocyte count and disease activity, a logistic regression model was used. Potential confounders were analyzed, but not found. In this analysis, the mean lymphocyte count over the last year natalizumab treatment was used as continuous variable. Calculations were performed using SPSS version 22.0 (Windows). A *p*-value <0.05 was considered significant for main effects.

Results

Demographic characteristics

In our observational cohort, 60 patients switched from natalizumab to fingolimod because of the risk of developing PML. Six patients were excluded because of a WO period longer than 6 months. Two patients were excluded because of incomplete imaging during follow-up.

Of the remaining 52 patients, baseline demographics are described in table 1.

Table 1. Demographic characteristics.

	Total *n* = 52	WOP<6 weeks *n* = 16	WOP 6–8 weeks *n* = 18	WOP>8 weeks *n* = 18
Age (years) at time of natalizumab discontinuation, mean (SD)	40.5 (8.9)	43.7 (8.6)	38.4 (10.1)	39.7 (7.5)
Washout period (weeks), median (IQR)	6.7 (5.6–9.7)	4.4 (4.3–5.4)	6.6 (6.3–7.0)	10.9 (9.1–12.3)
Gender, *n* female (%)	28 (53.8)	11 (68.8)	6 (33.3)	11 (61.1)
Duration (years) of natalizumab treatment, mean (SD)	4.3 (2.3)	4.9 (2.4)	4.1 (2.4)	4.0 (2.3)
EDSS at baseline natalizumab, median (IQR)	3.5 (3.0–5.0)	5.0 (3.0–6.0)	3.5 (2.5–4.4)	3.3 (2.1–4.0)
EDSS at baseline fingolimod, median (IQR)	4.0 (2.8–5.5)	4.3 (3.0–6.0)	4.3 (2.5–4.9)	4.0 (2.3–5.3)
JC virus index before natalizumab discontinuation, mean (SD)	2.2 (1.4)	2.4 (1.1)	1.6 (1.4)	2.0 (1.7)

EDSS: Expanded Disability Status Scale; IQR: interquartile range; SD: standard deviation; WOP: washout period.

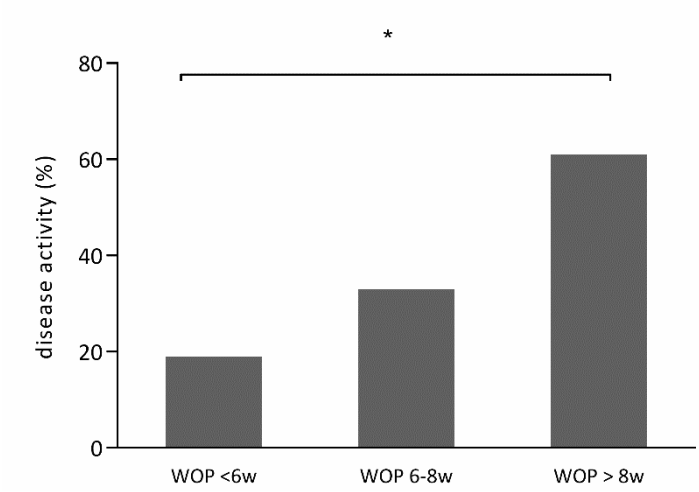

Figure 1. Disease activity and washout period.

Percentage (%) of patients with disease activity (based on MRI and/or relapse according to 2013 Lublin criteria18) in each WO period group. Disease activity increases comparing patients with longer WO periods compared to shorter periods. *Indicates an OR 6.8 with 95% CI 1.4–32.8, p = 0.02. WOP: washout period.

Disease activity

Twenty patients (38.5%) experienced disease activity within 6 months of natalizumab withdrawal. Seventeen patients (32.7%) had activity on brain MRI (either new T2 lesions and or gadolinium enhancing lesions) and six patients (11.5%) experienced a clinical relapse within six months of natalizumab discontinuation. No more than one relapse was reported per patient. All relapses occurred after at least three months of natalizumab discontinuation with a median delay of 3.9 months (IQR 3.7-4.6). None of the patients developed PML.

Disease activity increased with longer WO periods compared to shorter periods (see figure 1 and table 2). The patients with a WO period > 8 weeks showed a significant increase in disease activity with an OR of 6.8 (95% CI 1.4–32.8, *p*=0.02) when compared with the group of patients with a <6 weeks WO period. Comparing the patients with a WO period >8 weeks with a WO period 6-8 weeks, there was no significant difference in disease activity with an OR of 3.1 (95% CI 0.8–12.5, *p*=0.1).

Table 2. Disease activity and washout period.

	n	OR	95% CI	*p*-value
WO period				
<6 weeks	16	1.0		
6–8 weeks	18	2.2	0.4–10.7	0.34
>8 weeks	18	6.8	1.4–32.8	0.02

CI: confidence interval; OR: odds ratio; WO: washout. Patients with a WO period >8 weeks showed a significant increase in disease activity when compared with a <6 weeks WO period.

In the group with a WO period >8 weeks (n=18), four patients had a WO of 12 to 24 weeks. When comparing <6 weeks WO with a WO of 8-12 weeks (n=14), disease activity remained significantly correlated with a longer WO period (OR 7.8, 95% CI 1.5–41.2, *p*=0.02).

Subgroup analysis comparing disease activity in the group with the shortest WO (<4 weeks) with 8-12 weeks WO, shows comparable results in favor of the shortest wash-out (OR 7.2, 95% CI 1.1-47.9, p=0.04).

Of the six patients experiencing a relapse, 3 patients had a WO-period >8 weeks, 1 patient had a WO-period of 6-8 weeks and 2 had a WO-period of <6 weeks. Seventeen patients experienced radiological activity. The percentage of patients with radiological disease activity increased with longer wash-out periods, i.e. 11.1% in WO-period <6 weeks, 33.3% in WO-period 6-8 weeks and 50.0% in WO-period of >8 weeks. Comparable with disease activity, radiological activity only appeared statistically significant when comparing the group with a WO of <6 weeks with a WO of >8 weeks (OR 7.0, 95% CI 1.2–40.1, p=0.03). In the 17 patients experiencing radiological activity, the median number of new T2 lesions was 3 (range 1 to 24). Seven patients had a T2 increase of ≥5, five of these patients (71.4%) had a wash-out period of >8 weeks. The remaining two had a WO of <6 weeks and 6-8 weeks.

Before natalizumab treatment, the annual relapse rate (ARR) was 1.5±1.0, whereas the ARR under natalizumab treatment was 0.05±0.1 and for the year after natalizumab discontinuation 0.20±0.5. The ARR before natalizumab treatment did not correlate with disease activity after six months of natalizumab discontinuation (OR per unit increase in ARR 1.1; 95% CI 0.6–1.8, *p*=0.9). No correlation was found between the duration of natalizumab use and disease activity (OR per additional year of natalizumab use 1.0; 95% CI 1.0–1.0, *p*=0.1).

Natalizumab concentration

The mean natalizumab trough serum concentration during natalizumab treatment was 21.6±12.1 µg/ml. Natalizumab concentration was not associated with disease activity within six months after discontinuation (OR 1.0 after adjustment for the duration of the WO period, 95% CI 1.0–1.1, *p*=0.9). The 20 patients who experienced disease activity had similar natalizumab concentration during natalizumab treatment compared to patients who did not experience disease activity (mean concentration of respectively 21.1±10.4 µg/ml and 21.9±13.2 µg/ml, see figure 2).

Of 42 patients (80.8%), a follow-up natalizumab concentration 4 weeks to 6 months after the last natalizumab infusion was available. When the mean concentration was assessed longitudinally, we observed an exponential decline of natalizumab concentration as expected. On average, three months after the last natalizumab infusion, the natalizumab concentration drops below therapeutic levels of 1 µg/ml.[23] In the six patients of whom we had a natalizumab concentration available 5-6 months after discontinuation, natalizumab was still measureable, although at very low values.

From 16 out of 20 patients who experienced disease activity, follow-up data on natalizumab concentration was available. Three months after natalizumab discontinuation, the patients with disease activity had a mean concentration of 0.9 µg/ml (*n*=6) versus 4.2 µg/ml in the patients (*n*=18) without disease activity (*p*=0.3, figure 3).

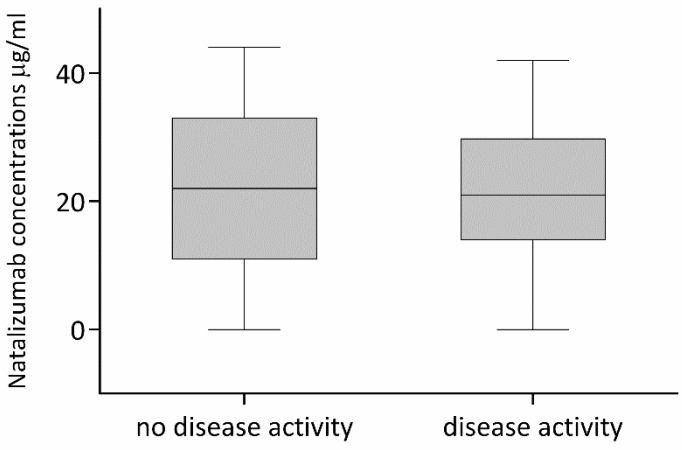

Figure 2. Natalizumab concentration and disease activity.

Disease activity (either active T2 lesions and/or gadoliniumenhancing lesions on MRI or a clinical relapse) within 6 months of natalizumab discontinuation. Natalizumab concentration was not associated with disease activity within 6 months after discontinuation (OR: 1.0, 95% CI: 1.0–1.1, p = 0.9), after correction for the WO period.

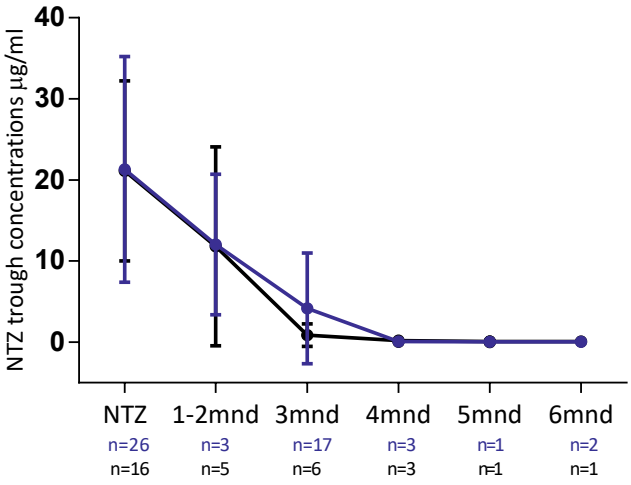

Figure 3. Longitudinal natalizumab concentration in patients with disease activity versus patients without disease activity.

Follow-up mean natalizumab concentration in patients with disease activity versus patients without disease activity (blue line) 6 months after natalizumab discontinuation. At 3 months of natalizumab discontinuation the lines divide, this was not statistically significant (p = 0.3). NTZ: natalizumab.

Lymphocyte count and disease activity

Based on the last measured lymphocyte count during natalizumab, the patients were divided into two groups, one group with normal lymphocyte count (1.0-3.5 x 10E9/L) and the other with increased lymphocyte count (>3,5 x 10E9/L). Correcting for the WO period, we found no significant correlation for disease activity (OR 1.0, 95% CI 0.3–3.2, *p*=0.9).

All patients showed an expected decrease in lymphocyte count at the moment of three months fingolimod use (mean difference of 2.8±0.7 x 10E9/L, *p*=<0.01). In the two groups (normal and increased lymphocyte count), we investigated the relative difference of lymphocyte count between the last measured lymphocyte count under natalizumab and the lymphocyte count at approximately 3 months of fingolimod use. We found no significant difference in the relative change of lymphocyte count between the two groups (β=0.03, *p*=0.1, figure 4A and 4B).

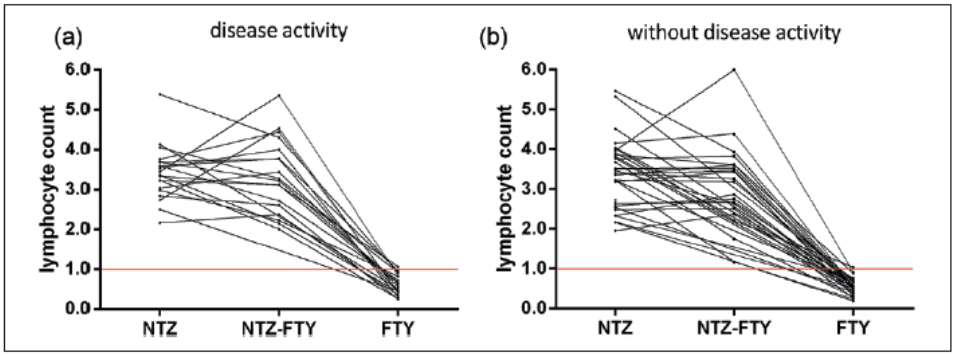

Figure 4. Lymphocyte count in patients (a) with disease activity versus (b) without disease activity.

Follow-up mean lymphocyte count during three different moments in time. NTZ: mean lymphocyte count during last infusion of natalizumab; NTZ-FTY: lymphocyte count during the washout period (in between natalizumab and fingolimod); FTY: lymphocyte count at the moment of 3 months fingolimod use. The red line is the cut off value of lymphocytopenia <1.0 × 10E9/L. There was no significant difference between the two groups in lymphocyte count (β = 0.03, p = 0.1). NTZ: natalizumab FTY: fingolimod.

Discussion

The main finding of this study is that switching patients from natalizumab to fingolimod with a WO period of more than 8 weeks increases the likelihood of recurrent disease activity almost seven-fold compared to patients with a WO period of less than 6 weeks.

Our results confirm earlier studies that report an increase of disease activity after 2-4 months of WO period in comparison to shorter WO periods.[5-9, 24] Ideally, to limit the risk of recurrence of disease activity, the WO period should be short enough to allow fingolimod to be clinically effective before natalizumab concentration drops under therapeutic levels. The downside of such a short WO period is the ongoing risk of PML in JC virus positive patients after discontinuation of natalizumab and how fingolimod could possibly increase the risk of PML and negatively influence the course of this serious complication.

PML after discontinuation of natalizumab (carry-over PML) is rare with 15 reported cases to date.[25] PML under fingolimod monotherapy is also described but even rarer, with nine reported cases so far (Novartis safety update October 2016). All current literature regarding WO periods when switching from natalizumab to fingolimod recommend a delay of less than three months because of the risk of recurrence of disease activity.[5-9, 25] We estimate that the possible increased risk of PML with a decrease of 1-2 months of WO period does not outweigh the significant reduction of disease recurrence. Therefore, we recommend a WO period of less than 6 weeks when switching from natalizumab to fingolimod. Extra precautions regarding the risk of PML in JC virus positive patients may be taken, such as a

baseline MRI and repeated scans 3 and 6 months after cessation of natalizumab and timely testing of JC virus DNA in the cerebrospinal fluid in case of suspicious MR activity.[25]

Although intra-individual natalizumab trough concentration during natalizumab treatment are stable, concentrations vary widely between patients, presumably because of the difference in body mass index and metabolism.[26] In agreement with an earlier report, we found a large inter-individual variability of natalizumab trough concentration under natalizumab treatment.[26] We hypothesized that lower concentration would predispose to disease recurrence after natalizumab discontinuation. However, we found no significant difference in concentration under natalizumab treatment when comparing patients with disease activity and patients without disease activity, which is in agreement with a recently published study of 12 patients switching from natalizumab to fingolimod.[27] In our study, the possible explanation could be that, the clinical disease activity appeared after three months of natalizumab discontinuation when concentration of the drug had already decreased under therapeutic levels.[5] When comparing longitudinal natalizumab concentration in patients with and without disease activity, the mean concentration at three months was lower in patients who did not experience disease activity (0.8 versus 4.2 μg/ml). However, no definite conclusions can be made because of limited sample size of the longitudinal concentrations. The α-4-integrin receptor on which natalizumab binds, desaturates when natalizumab concentration decrease under 1 μg/ml.[23] Our longitudinal results show that on average, natalizumab concentration decrease under the therapeutic level 3 to 4 months after the last infusion.

Natalizumab inhibits the adhesion of lymphocytes to the endothelium and is associated with a stable increase of peripheral lymphocytes.[12] Yet increasing evidence shows that natalizumab also plays a role in attachment of hematopoietic precursor cells to bone marrow stromal cells and on that account facilitates the release of lymphocytes from the bone marrow.[28, 29] The return of disease activity after natalizumab discontinuation most likely reflects resumption of lymphocyte migration across endothelial membranes of the blood brain barrier as natalizumab is cleared from the circulation.[30] Fingolimod is probably mainly clinically effective because it induces a systemic lymphopenia. When switching from natalizumab to fingolimod, we hypothesized that clinical effect of fingolimod might be delayed due to the relative lymphocytosis natalizumab causes in a proportion of patients. However, this study shows no correlation between lymphocyte count and disease activity. T cell subsets are unknown in this study, so describing the fingolimod-mediated changes on CD4+ and CD8+ T cells was not possible. The lack of correlation between an overall lymphocyte count and disease activity will not rule out the probability that the changes in subpopulations of lymphocytes (including CD4+ / CD8+ ratio) may have impact on the recurrence of disease activity.

Our relatively small sample size warrants cautious interpretation of our data. To determine the optimal WO period in case of switching from natalizumab to fingolimod, larger randomized trials are needed, preferably comparing different WO periods, including one arm starting fingolimod immediately after the final natalizumab infusion. However, in future studies the influence of the WO period on the risk of PML will still be difficult to establish given the rarity of this serious complication.

In conclusion we found that a WO period of less than 6 weeks is associated with a reduced recurrence of disease activity when compared to a WO-period of 8 weeks. Natalizumab concentration and lymphocyte count during natalizumab treatment did not predict disease activity after natalizumab discontinuation.

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Chapter 8

The majority of NTZ treated MS patients have high NTZ concentrations at time of re-dosing

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Multiple Sclerosis, 2018

Abstract

Background Natalizumab is efficacious in the treatment of relapsing-remitting multiple sclerosis. All patients receive the same treatment regimen of 300mg every four weeks, despite differences in pharmacokinetics between individual patients.

Objective To give neurologists insight in natalizumab concentrations at time of re-dosing, we investigated longitudinal natalizumab concentrations in 80 patients in relation to disease activity, with possible influencing factors.

Methods In a prospective observational cohort study, natalizumab trough serum concentrations were measured in 80 patients. Data on demographics, duration of treatment, Expanded Disability Status Scale, clinical exacerbations, brain MRI and body weight were collected.

Results We measured high (≥10μg/ml) natalizumab trough concentrations in 94% of patients. Intra-individual concentrations were stable. The spread in concentrations was substantial and did not correlate with disease activity. We found a negative association between natalizumab concentration and body weight (β=-0.30, p=0.010).

Interpretation The majority of patients showed high natalizumab serum concentrations at time of re-dosing. Alternative treatment regimens could lead to more efficient use of natalizumab, but caution is warranted regarding the possibility of recurrence of disease activity. Prospective clinical trials are needed to establish the safety of extended dose intervals in natalizumab treatment.

Introduction

Natalizumab (NTZ), targeting the α-4 integrin receptor, is an efficacious treatment for relapsing-remitting multiple sclerosis (RRMS).[1] In a phase I trial, NTZ stayed detectable in the serum for 3-8 weeks after infusion with dosing of 1-3 mg/kg.[2] Based on the different therapeutic dosages of 3-6 mg/kg in phase II trials, a fixed dose of 300mg once in four weeks was chosen for phase III trials so the majority of patients (with weights ranging between 50 and 100kg) would fall between a dose of 3 to 6 mg/kg.[3] Nowadays, a dose of 300mg every four weeks has been approved by the EMA/FDA for the treatment of RRMS. In this treatment regimen, NTZ concentrations may stay detectable in serum in up to 200 days after cessation of therapy.[4]

Serum NTZ concentration corresponds with the percentage of α-4 integrin receptor saturation.[5] Desaturation of the α-4 integrin receptor occurs when the serum NTZ concentration falls under 1-2 µg/ml.[5] Above this threshold of 2 µg/ml, NTZ receptor saturation will roughly fall between 70-100%.[5] An adequate receptor saturation is estimated as ≥70-80% saturation, although prospective data confirming this assumption are lacking.[6, 7] Based on a model with results from a large phase II trial, approximately 90% of patients showed NTZ trough concentrations largely exceeding 2.5 µg/ml. Levels exceeding 2.5 µg/ml could indicate that the approved treatment regimen of NTZ for RRMS results in a relative over-treatment; i.e. the patient receives more NTZ than necessary for optimal drug efficacy.[3, 8] Furthermore, it is suggested that higher NTZ receptor saturation could increase the risk of progressive multifocal encephalopathy (PML), the feared complication of NTZ treatment.[9] This unconfirmed hypothesis leads to clinicians extending dose intervals in NTZ treatment with the aim to reduce the PML risk by decreasing NTZ exposure.[9-11]

The aim of our study was to measure NTZ serum trough concentrations and correlate concentrations with disease activity and possible influencing factors.

Methods

Patients

In 2006, we initiated a prospective observational cohort study to monitor different aspects of NTZ treatment at the MS Centre of the VU University Medical Centre in Amsterdam the Netherlands. All patients (nearly 220) starting NTZ have been included in this observational cohort. Patients in this cohort are annually subjected to a brain MRI and clinical testing including the Expanded Disability Status Scale (EDSS). For this present study we included all patients of the cohort who are currently treated with NTZ. Because NTZ concentration can fluctuate in the first year, mainly because of transient NTZ antibodies, we excluded patients with a NTZ treatment duration less than 12 months.[12] All the patients included in this study received a strict treatment regimen of 300mg natalizumab every four weeks.

Measurement of NTZ concentration

Of all participants, blood samples were routinely obtained every three months before NTZ infusion. Serum was subsequently stored at -80°C at the biobank of the VU Medical Centre. For this study we cross-sectionally measured NTZ concentrations of selected samples, using a cross-linking assay using polyclonal rabbit anti-NTZ F(ab)2 fragments for capture and a mouse anti-IgG4 monoclonal antibody for detection. This method, performed at Sanquin Laboratory, has recently been described in more detail.[13] The detection limit of the assay is approximately 0.01 µg/ml.[13] To investigate the stability of trough concentrations we tested a second sample, with an interval of 3 to 7 months. Receptor desaturation can occur with serum concentrations of ≤2 µg/ml, which is our appointed cut-off point for an inadequate concentration.[5] Taking into account the differences of individual pharmacodynamics, we assumed that serum concentrations of 2 to 10 µg/ml result in adequate receptor saturation. Concentrations exceeding 10 µg/ml do not result in higher receptor saturation and is therefore labeled as the cut-off for high trough concentration.[5]

Data collection

Demographic data, number of NTZ infusions, annual relapse rate before NTZ treatment, number of gadolinium enhancing lesions on the baseline scan, EDSS at start of NTZ treatment, JCV status at time of the measured concentration, clinical exacerbations during NTZ treatment and body weight were assessed. The body weight was measured within three months of the blood sampling date. A clinical exacerbation was defined as new neurological symptoms lasting for more than 24 hours and accompanied by new neurological signs found by a neurologist at the examination. All patients were subjected to a yearly MRI scan of the brain, including 3D fluid-attenuated inversion recovery, axial PD/T2-weighted sequences and gadolinium enhanced T1-weighted sequences. Those patients at higher risk for PML (JCV positive and > 12 months on NTZ) were subjected to 3-monthly scans (without gadolinium except for the annual MRI scan) as is current recommended protocol.[14, 15] All MRI-scans

were evaluated by an experienced neuro-radiologist. The 2013 criteria of Lublin et al were used when referred to 'active MS'.[16] According to these criteria, when referring to active MS, the patient experiences clinical relapses and/or occurrence of contrast-enhancing T1 or new or enlarging T2 lesions on brain MRI. In the present study we assessed MS activity starting 12 months after start of NTZ treatment. If the patient experienced any clinical and/or radiological disease activity in the follow-up period they were classified as 'active MS'.

The local institutional review board approved the observational study and written informed consent was obtained from all participants.

Statistics

Continuous variables are expressed as mean and standard deviation if normally distributed or as median and interquartile range if not normally distributed. NTZ concentrations were normally distributed. For associating NTZ concentrations with different variables, we used the mean of the intra-individual longitudinal NTZ concentrations, except for the association with the number of NTZ infusions, in which we used the first measured NTZ concentration. For calculating the influence of different variables (body weight, NTZ infusions, age and gender) on NTZ concentration we used a linear regression model. For calculating the influence of NTZ concentration on disease activity we used a logistic regression model. Additional adjustments were made for confounding factors such as body weight, NTZ infusions, age and gender.

All reported *p* values are based on statistic tests, with a significance level set at <0.05. The statistical analyses were performed using the Statistical Package for Social Sciences (SPSS) version 22.0 (IBM Corp., Armonk, NY, USA).

Results

Approximately 220 patients have started NTZ treatment at the VU Medical Centre. At time of the start of this study, 101 patients were treated with NTZ. The main reason for discontinuing NTZ was the risk of PML, other reasons were pregnancy related reasons, allergic reactions and progression to the secondary progressive phase. Of the 101 currently treated patients, 21 were excluded because of a treatment duration of less than 12 months. In the remaining 80 patients, 155 blood samples were tested for NTZ trough concentrations. Age of the patients ranged from 20 to 60 years. Patient characteristics are described in table 1.

Table 1. Demographic characteristics.

	Total, $n=80$
Age, mean (SD)	40.6 (10.1)
Gender, female, *n* (%)	55 (68.6)
Number of NTZ infusions, mean (SD)[a]	64.7 (32.2)
JCV positive, *n* (%)	29 (36.3)
EDSS at baseline NTZ, median (IQR)	3.3 (2.5)
ARR before NTZ start, mean (SD)	1.4 (0.9)
Number of gadolinium enhancing lesions at baseline, median (IQR)	1.5 (4)

SD: standard deviation; NTZ: natalizumab; EDSS: Expanded Disability Status Scale; IQR: interquartile range; ARR: annual relapse rate; JCV: John Cunningham virus.
[a]Number of NTZ infusions at the time of the first measured concentration.

NTZ serum trough concentrations ranged from 0.1 µg/ml to 80.0 µg/ml with a mean of 26.1 ±14.1 µg/ml. One patient (1.3%) had an inadequate concentration (< 2 µg/ml), 4 patients (5%) had an adequate concentration (2 to 10 µg/ml) and 75 patients (93.8%) had a high concentration (≥10 µg/ml) at time of re-dosing. The patient showing the lowest concentration (0.1 µg/ml at two measurements) appeared to have persistent high (>9000 AE/ml) NTZ antibodies.

Of 75 patients (93.8%) we measured a follow-up trough concentration. The mean concentration of all the cross sectional samples did not differ (both 26.1 µg/ml) at the two different time points, i.e. at group level no rise or fall in concentration was observed. Longitudinal concentrations per patient fluctuated with a median difference of 3.0 µg/ml. In 9 patients the two samples differed more than 10 µg/ml, all these patients showed very high concentrations above 30 µg/ml.

The body weight of the patients ranged from 49.1 to 109.0 kg with a mean weight of 75.1 ±13.9 kg. An inverse association was found between body weight and NTZ concentration (see figure 1, β = -0.30, 95% C.I.-0.52 to -0.07; p = 0.010; r^2 = 0.084). We corrected body weight for potential confounders, but none of these variables appeared to be relevant. Patients weighing up to 75kg showed a mean concentration of 29.2 ±15.6 µg/ml, whereas the mean concentration of patients weighing 75kg or more was 22.7 ±11.9 µg/ml (β = -6.6, 95% C.I. -12.8 to -0.34; p = 0.039).

There was no association found between the number of NTZ infusions and trough NTZ serum concentrations, with a substantial spread in concentrations regardless duration of treatment (see figure 2, β = 0.022, 95% C.I. -0.092 to 0.113; p = 0.84).

The NTZ trough concentration was comparable between males and females, with a mean concentration of 25.0 ±13.4 and 26.9 ±14.1 µg/ml respectively (β = -1.46, 95% C.I. -8.27 to 5.35, *p* = 0.67). The concentration was not significantly associated with age (β = - 0.011, 95% C.I. -0.37 to 0.30; *p* = 0.94).

Mean duration of NTZ treatment was 5.0 ±2.5 years. 15 patients (17.7%) had active disease under NTZ treatment (11 patients with new T2 lesions, 5 patients with a clinical exacerbation). Patients who had active disease under treatment received a median treatment duration of 5.1 years, patients with non-active disease received a median treatment duration of 4.9 years. Mean concentrations were similar between patients with active and non-active disease with a mean concentration of 26.4 µg/ml and 24.7 µg/ml respectively (see figure 3). When adjusting for body weight, concentrations were not statistically different for the active disease group versus the non-active disease group (OR 0.98, 95% C.I. 0.94 to 1.03; *p* = 0.41).

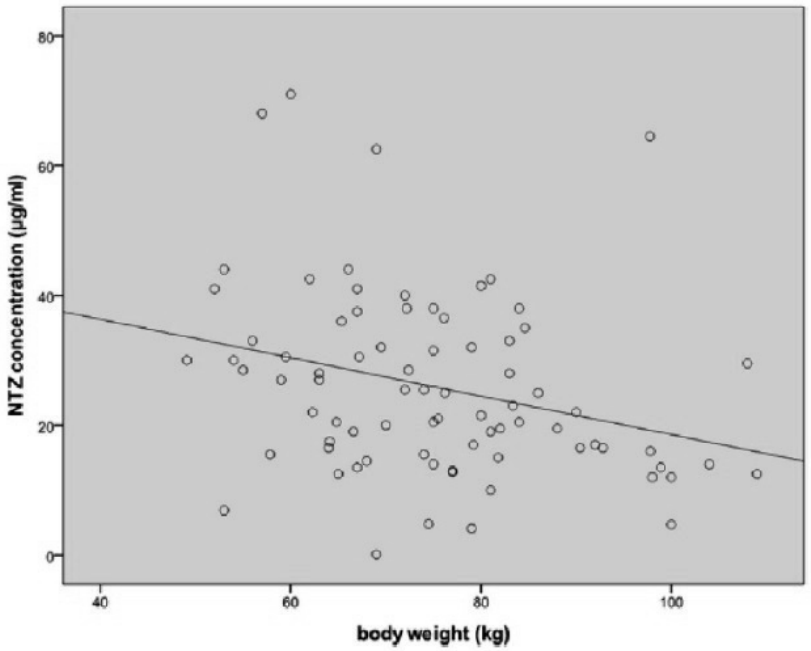

Figure 1. Body weight and NTZ trough concentration plot.

An inverse association is found (β = –0.30, 95% CI = –0.52 to –0.07; $p = 0.010$; $r^2 = 0.084$).

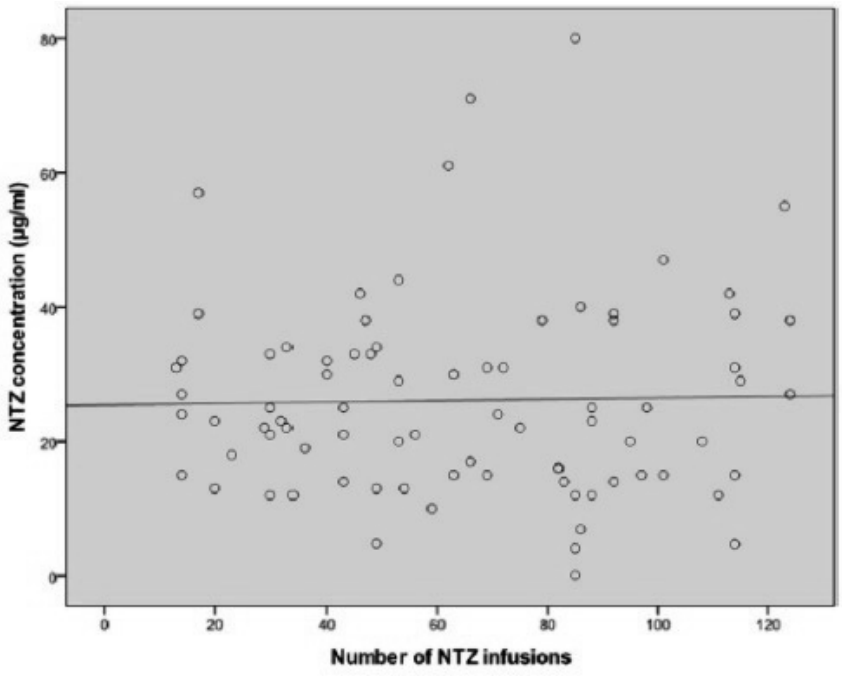

Figure 2. Number of NTZ infusions (duration of treatment) and NTZ trough concentration plot.

NTZ concentration of the first measured sample is displayed with associated number of NTZ infusions. No association is found (β = 0.022, 95% CI = –0.092 to 0.113; $p = 0.84$). We do not see a rise in concentrations in long-term (5–10 years) NTZ users.

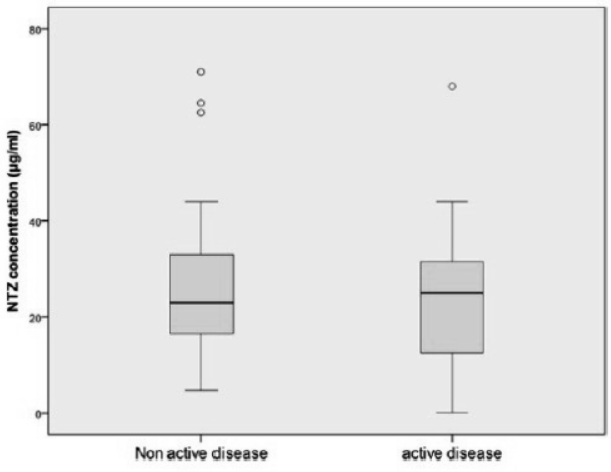

Figure 3. Active disease ($n = 15$) versus non-active disease ($n = 65$) (according to the 2013 Lublin criteria) and NTZ trough concentrations (OR = 0.98; 95% CI = 0.94 to 1.03; $p = 0.41$).

Discussion

NTZ is proven to be efficacious in the treatment of RRMS in a dosing schedule of 300mg every four weeks. Despite large variations in patient pharmacokinetics, all natalizumab treated patients receive the same treatment regimen, where a personalized approach to the treatment schedule might be more appropriate.[1] Some neurologists are exploring extended dose intervals in order of reducing the risk of PML, although it is not confirmed that higher NTZ concentrations increase the risk of PML.[9, 11] Obviously, modified treatment schedules should not interfere with drug efficacy. Our study addresses two important questions: 1. What is the proportion of high NTZ trough concentration in long-term treated MS patients? and 2. Can we explain individual differences of NTZ concentrations?

In our study of 80 patients, 99% of patients showed adequate to high trough NTZ concentrations (≥2 µg/ml), with 94% having high (≥10 µg/ml) NTZ concentrations. The mean trough NTZ serum concentration in our cohort was above 20 µg/ml which is in agreement with recently presented data.[17] The mean concentration was not lower in patients with active versus non active disease, which suggests that high concentrations do not result in an increase of treatment efficacy in comparison to lower but still adequate concentrations. Considering this and the large proportion of high NTZ concentrations, NTZ could perhaps be administered less frequently (or with a lower dose) to reach NTZ concentrations that are lower but still cause adequate receptor saturation and consequently, optimal drug efficacy.[5] Caution is advised though, because of a large spread in concentrations and the well-established rebound effect which occurs after cessation of NTZ treatment.[18]

In the RESTORE trial, 19% of patients (*n*=23) stopping NTZ, of whom the majority switched to another therapy, experienced a relapse within 28 weeks.[19] The rebound effect showed an increase over time, although 8% of relapses occurred within 4-8 weeks of NTZ withdrawal. A large retrospective study however, showed no increase in disease activity with extending intervals up to 8 weeks and five days.[9] In our study, 6.25% of patients showed either inadequate or adequate NTZ concentrations at time of re-dosing. For this group, extending intervals might result in rapidly falling concentrations under the therapeutic level and consequently an early rebound effect.

The large spread in NTZ trough concentrations could be explained by the variation in patient pharmacokinetics and characteristics. We associated age, sex, body weight, duration of treatment and disease activity with NTZ concentrations. The only factor associated with NTZ concentration was body weight. This is in agreement with earlier reports, where some studies suggested a dose modification based on patients body weight.[20, 21] Our results indeed confirmed an inverse association but this correlation was weak, only accounting for

less than 10% of the variability in NTZ concentration. Therefore, body weight is an unreliable predictor for NTZ concentration.

NTZ can be found in serum up to six months after cessation of therapy.[4] It has been suggested that NTZ concentrations increase over time in individual patients.[20] In our study, in 75 patients with a second measurement of NTZ trough concentration, the intra-individual concentrations were stable. Although we do not present long-term longitudinal follow-up trough NTZ serum concentrations, we did not find a correlation between duration of treatment and NTZ concentration. This data highlights that we should not expect very high concentrations in long-term NTZ treated patients (> 5 years).

A limitation of the present study is that measurements of saturation of the α-4 integrin receptor are lacking. Previous studies show that NTZ concentration is correlated with the α-4 integrin receptor saturation, however above a certain concentration threshold the receptor will be fully saturated (75-100%). Based on available literature we estimated this threshold to be 10 µg/ml NTZ serum concentration, but this cut-off point needs to be confirmed in larger trials. Furthermore, if measuring natalizumab levels, it is of importance to realize that the drug is a wild-type IgG4 antibody that becomes monovalent in vivo via 'Fab arm exchange'. This will affect concentration measurements to various degrees.[13, 22] Comparative studies between assays will be necessary to eliminate potential discrepancies between studies.

In conclusion, the large majority (94%) of NTZ patients have high NTZ trough concentrations which could be an indication that most patients receive a 'relative over-treatment'. Extended dose intervals could help reduce costs of medication and increase quality of life for the patient with fewer hospital visits, but further studies are needed to establish the safety of alternative treatment regimens. We are now conducting a prospective clinical trial with concentration based extended dose intervals in completely stable NTZ treated patients, to assess whether concentration based extended treatment regimens do not result in recurrence of disease activity. Results of such trials will hopefully give a decisive answer to the question if extending dose intervals in natalizumab treatment is feasible without losing drug efficacy.

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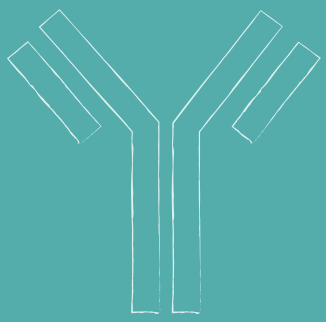
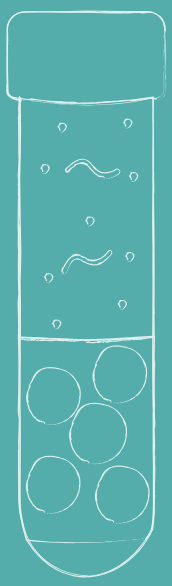
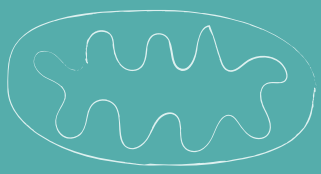
Summary and general discussion



General objective

In MS, there are only very few body fluid biomarkers that are accepted as reliable and practicable parameters so far. Biomarkers play a role in determining MS (e.g. cerebrospinal fluid-specific oligoclonal bands (OCB)), in differential diagnosis (e.g. anti-aquaporine-4 and anti-MOG serology in NMO-SD), in monitoring disease modifying treatment (DMT) (e.g. anti-interferon antibodies and anti-natalizumab antibodies) and predicting adverse effects (screening anti-JCV antibodies in natalizumab treatment for the risk of PML).

The problem of diagnosing MS is largely solved by using MRI, clinical presentation and neurological assessment which led to the formulation of the revised diagnostic criteria.[1]

However, making a timely diagnosis can still be complicated in a minority of patients. This may occasionally result in an undesirable late diagnosis and subsequently delayed treatment. To date, validated and discriminative body fluid biomarkers reflecting and predicting natural disease progression and disease activity are lacking.[2, 3] Such biomarkers are crucial for personalized medicine and may provide guidance in the rapidly evolving treatment armamentarium in MS.

The main aim of this thesis was to explore novel biomarkers that are associated with disease progression and to explore biomarkers in monitoring treatment and treatment safety. With this aim different markers reflecting potential roles in neuroinflammation, demyelination, and axonal damage were analyzed. For monitoring DMT and DMT safety natalizumab concentrations and neurofilament light were explored.

During this search, two biomarkers with a more diagnostic purpose were also worthy to analyze namely kappa free light chains and mRNA-sequencing of blood platelets. After all, biomarkers useful in determining MS can likewise be potential biomarkers in predicting disease progression and treatment monitoring.

The following section summarizes the main results of our biomarker studies in MS. In addition, main findings are discussed in relation to the existing literature, and clinical implications and future perspectives will be addressed.

PART 1 Diagnostic biomarkers

Chapter 1.
Kappa free light chains, a valid tool in the diagnostics of MS.

In the latest 2017 revisions of the McDonalds criteria the OCB have a prominent role in patients with clinically isolated syndrome (CIS). However, the assessment of OCB is labor intensive, requires trained personnel and is in some cases examiner- and method-dependent, which may affect its reliability. The question raised: 'Is there a biomarker that can be measured with an automated and quantifiable procedure?'

Since the late 1970s, multiple studies have reported increased CSF levels of kappa free light chains (KFLC) in MS[4-14] and due to the development of the more sensitive nephelometric and turbidimetric FLCs assays[4-8] KFLC and lambda free light chains (LFLC) can be easily detected. However, until our study described in **chapter 1**, a large multicenter study to validate this biomarker was lacking.

In **chapter 1** we validated KFLC and LFLC indices as a diagnostic biomarker in MS compared with OCB in a large multicenter study including samples from eighteen MS-centers across Europe (219 controls and 526 CIS/MS patients) with a known OCB status. We measured KFLC and LFLC in paired CSF and serum samples. We defined cut-offs for abnormal FLC indices and based on the defined cut-off, subjects were classified as positive or negative for kappa or lambda FLC as binary result. The sensitivity, specificity and accuracy between the two different diagnostic tools (OCB and FLC) were compared.

We found a cut-off for the KFLC index of 6.6, and for the LFLC index a cut-off of 6.9. Compared to OCB the KFLC-index is more sensitive (.88 vs .82) at the cost of a lower specificity (.83 vs .92). This resulted in a higher negative predictive value for the KFLC-index compared to OCB, but a lower positive predicted value. This suggest that the KFLC-index is a valid test for diagnosing MS. In addition, our results indicated that the LFLC-index is not a valid test for diagnosing MS.

Discussion and future perspectives

Validating FLCs in a large multicenter study is necessary assuming it can be a valid tool as a potential cost-effective replacement of the OCB. The sensitivity and specificity found in **chapter 1** for the KFLC index in CIS/MS were lower compared to other smaller studies.[13-17] However, in these previous studies, there was a less heterogeneous control group (except in the study described by Senel et al. 2019). For example, similar as for the OCBs, KFLCs can be elevated in inflammatory controls[18], and thus specificity will be lower when included. The

large number of patients and the large heterogeneous control group in our study gave us a reflection of the real-life clinical situation thus avoiding spectrum bias and allowed us to give a more representative sensitivity and specificity for OCB, KFLC and LFLC indices.

It is challenging to establish cut –off values for biomarkers. The cut-off in this study was calculated using a data-driven Gaussian mixture modeling approach. This is a different approach compared to other studies. We reasoned that the cut-off should be defined by biological levels and not based on clinical diagnosis, since the latter contains clinical uncertainty due to lack of a golden standard. The cut-off found in **chapter 1** for KFLC is in line with results from previous multi-center studies showing a KFLC-index cut-off of 5.9 and 7.0.[8, 13] This almost comparable cut-off for the KFLC index in two multi-center studies supports its robustness and implies that it may be used as an universal cut-off.

The last two decades multiple revisions were published of the McDonald MS criteria. This resulted in that not all included patients were diagnosed based on the same MS criteria in our study, which may have influenced the diagnosis of CIS patients particularly. We addressed this problem by pooling all CIS and MS patients. We performed several sensitivity analyses in CIS or MS patients separately, and by reclassifying and excluding specific clinical groups. No relevant differences were seen when comparing MS to control group.

We concluded that for clinical practice, the KFLC index is more accurate in excluding CIS/MS compared to OCB but for ruling in a diagnosis of CIS/MS, analysis of OCB appears to be more accurate. By replacing OCB with KFLC in diagnostic practice there is a slightly higher chance that a patient with a diagnosis different from MS will get the diagnosis of MS and maybe unnecessarily exposed to potential negative side effects of early treatment. Since the KFLC-index is more sensitive at the cost of a lower specificity, we should stress that replacement of OCB by the KFLC-index is not optimal to arrive at high diagnostic certainty.

An advantage of the higher sensitivity of KFLC, is that an earlier diagnosis and subsequent treatment start may be considered. It may be an option to start treatment based on the KFLC result and clinical/MRI findings according to the novel McDonald criteria, or to use KFLC results in patients on treatment when a switch may be considered while on DMT. This could be considered as a subject for future studies.

One important note is that the best set up for a future study would be to include a test population of suspected MS cases instead of already diagnosed cases. The use of the KFLC values as a predictor for CS conversion to MS is demonstrated in several studies.[19-21] However, a recent study showed that FLC concentrations at CIS diagnosis were not significantly higher in CIS-converters.[13]

Since 1995 different papers have been published exploring KFLC values as a disease progression marker. Different studies found that KFCL predicted subsequent physical deterioration in MS patients.[22-24] Nonetheless, one study did not find evidence for a relation of KFCL with disease progression.[25] The role of KFLC in predicting disease activity or disease progression is interesting for future studies using large cohorts of patients with long-term follow up.

Chapter 2.
mRNA-sequencing of blood platelets as novel diagnostic biomarker in MS.

So far, no blood biomarker has been convincingly confirmed as a useful tool in the diagnostic work-up of MS. Blood-based approaches harbor obvious advantages and recently, the long-neglected blood platelets, being the second most abundant cell type in peripheral blood, have shown emerging potential as a new source for biomarker discovery in several diseases, but not yet for MS.[26, 27]

During the final stages of platelet production, platelets are loaded with pre-messengerRNAs before developing from the megakaryocyte.[28, 29] As a result, platelets contain a rich messengerRNA (mRNA) repertoire that can change during megakaryocyte development but also during platelet formation and platelet circulation. Especially the change of RNA transcripts during circulation, possibly achieved by specific splicing queues, is of relevance in this chapter.

Platelets are known to respond to activating signals from their environment with specific splicing of their pre-mRNAs and uptake of RNA from different cell types, leading to a unique and dynamic RNA repertoire [30-35]. The diagnostic potential of this specific splicing of mRNAs in platelets has already been demonstrated, as recent findings have shown that mRNA sequencing from tumor educated blood platelets distinguishes healthy controls (HC) from cancer patients with an accuracy of 95% [36].

Platelets seem to not just be involved in inflammatory and immune responses but may also contribute to the pathogenesis of MS.[37-39] As a result of their involvement in immune response and apparent causal role in the progression and development of the disease, we hypothesized in **chapter 2** that blood platelets contain a disease specific mRNA signature, thereby investigated their potential as diagnostic biomarker for MS. In this chapter, we isolated and sequenced platelet RNA of blood samples from 57 MS patients and 66 age- and gender-matched healthy controls (HCs). 60% of the matched samples were employed to develop a particle swarm-optimized (PSO) support vector machine classification algorithm.

The remaining 40% of the samples served as an independent validation series. In total, 1249 RNAs with differential spliced junction expression levels were identified between platelets of MS patients as compared to HCs. The spliced platelet RNA was subsequently used as input for the development of a diagnostic MS classifier capable of detecting MS with >80% accuracy in the independent validation series (n=50, AUC: 0.87, 95%-CI: 0.77-0.97, p<0.001).

Discussion and future perspectives

The blood-based approach presented in this chapter could assist in the diagnosis of MS without being as invasive as CSF collection, therefore allowing for easy disease determination in case of a diagnostic challenge. Ultimately, it would be favorable if blood platelets mRNA could replace CSF OCB to substitute the requirement of fulfilling dissemination in time on MRI according the 2017 revisions of the McDonald criteria.[1]

To our knowledge, our study was the first utilizing mRNA found in circulating platelets as a blood-based biomarker for distinguishing MS patients from asymptomatic individuals. Recently, the protocol determining mRNA in platelets was published, enabling the MS community to test platelet RNA for diagnostic algorithm development.[40]

Obviously, there are limitations that need to be discussed concerning our data. First, aside use of age- and gender-matched asymptomatic controls, no individuals were included with other auto-immune or (neuro-) inflammatory disease potentially reducing the diagnostic accuracy. Second, all MS patients have been diagnosed with the disease for a minimum of ten years. Third, the sample size analyzed is still relatively small, potentially resulting in algorithm overfitting. To reach true clinical relevance, additional studies should also focus on early-stage MS cases and CIS patients to assess the early-detection potential. Despite above mentioned problems, we provided evidence for the clinical potential of circulating blood platelet derived mRNA as liquid biomarker for RRMS. More studies are needed, however, to assess the performance of this diagnostic tool in various presentations of MS. Also, platelet derived mRNA may have potential as progression marker in MS and as a response marker in DMT users. We are currently expanding our measurements to our treatment cohorts.

PART 2 Disease course biomarkers

Chapter 3.
Exploring cerebrospinal fluid mtDNA concentration as a biomarker in MS disease progression and activity.

In this part of the thesis we explored markers reflecting disease course, with a focus on disease progression.

In recent years, impaired mitochondrial function is increasingly recognized as a key pathological hallmark of MS.[41, 42] Demyelination leads to an increase in energy demand in order to maintain an appropriate intra-axonal ion balance and could thereby affect the number, transport and activity of mitochondria.[43-46] The number of mitochondria is highly increased in chronically demyelinated axons as well as in reactive astrocytes[43, 45] and extensive neuronal mtDNA deletions have been observed in MS cortical brain samples.[47] Based on the observations that mitochondrial dysfunction plays a crucial role in MS pathology and the possible role of mitochondrial dysfunction in clinical disease progression, we explored in **chapter 3** the potential of mtDNA levels in the CSF as a candidate biomarker of identifying patients with progressive disease in a Dutch cohort (50 RRMS patients, 40 PMS patients, 23 non-inflammatory and 7 inflammatory controls).

The main conclusion of this chapter was that concentrations of free circulating mtDNA copies are increased in CSF of patients with progressive MS compared with non-inflammatory control patients. Also, there was a trend ($p=0.08$) for a modest positive correlation with Expanded Disability Status Scale (EDSS) specifically in progressive MS patients. In addition, we showed that patients with a high T2 lesion volume displayed higher mtDNA concentrations compared to patients with a relative low T2 lesion volume. The group with lower normalized brain volumes showed higher mtDNA concentrations compared to patients with higher normalized brain volumes, suggesting a positive correlation between the concentration of free circulating mtDNA copies and brain atrophy. Altogether, our data may suggest that increased concentrations of cell free mtDNA are associated with MS disease activity and progressive disease.

Furthermore, we explored the effect of disease modifying treatment on free mtDNA levels in longitudinally obtained CSF samples in a Swedish cohort (42 RRMS patients, 20 other neurological disease controls and HC). We showed that patients treated with fingolimod had significantly decreasing (almost 50%) mtDNA copy levels at follow-up compared to baseline.

Discussion and future perspectives

Previous studies showed reduced levels of mtDNA in both Alzheimer[48, 49] and Parkinson[50] disease cases and it has been speculated that a decrease in mtDNA might be a common phenomenon observed in neurodegenerative diseases. In contrast to this, elevated mtDNA levels as we found in PMS, have also been detected in CSF samples from children with traumatic brain injury and were highly predictive of a poor outcome.[51] This might suggest that high values of free circulating mtDNA in CSF can be seen as a potential biomarker of acute cellular and mitochondrial stress. It is nowadays widely accepted that neurodegeneration and concomitant brain atrophy are common pathological features of MS, particularly in the progressive phase of the disease. Demyelination leads to an increase in axonal energy demand, which may superimpose effects of neurodegeneration in MS, which is a possible explanation for the higher concentrations of mtDNA in progressive MS in this chapter. However, a recent study analyzing mtDNA in PMS, showed that when mtDNA was analyzed in ventricular CSF (post mortem) at the end-stage of progressive multiple sclerosis mtDNA levels were depressed.[52] This study used a small sample size and comparing post mortem with samples taken from living patients warrants cautious interpretation of the data.

The cellular origin of enhanced mtDNA levels in the CSF of progressive MS patients found in our study is yet unknown, however it is conceivable that mtDNA is released upon neuro-axonal injury or oligodendrocyte damage, as these are prominent features of progressive MS. Alternatively, mtDNA might be secreted into the extracellular compartment by extracellular vesicles derived from distinct CNS cells, such as reactive astrocytes, particularly in lesions that are packed with mitochondria.

In line with our results, Varhaug et al. showed increased levels of cell free mtDNA in CSF of MS patients compared with controls.[53] Further, they found an inverse correlation between the duration of the specific symptoms and levels of mtDNA, concluding that increased mtDNA concentration may reflect early, active inflammatory activity. Increased mtDNA levels in the CSF might also contribute to the disease process by activating an immune response. Mitochondrial DNA is a damage-associated molecular pattern (DAMP), which can bind to glial Toll-like receptor-9 and trigger an inflammatory response.[54] Hence, it is conceivable that enhanced mtDNA levels in the CSF might elicit a glial immune response. Fissolo et al. further explored the potential role as a diagnostic and disease activity biomarker in MS by measuring CSF cell-free mtDNA levels in a large cohort of individuals with relapse-onset and progressive clinical forms of MS, patients with CIS and control subjects.[55] No significant differences were observed between MS patients, CIS patients and neurologic disease controls. Within the CIS group, mtDNA levels did not significantly differ between CIS patients who converted to MS and those who continued as CIS. Similar to our results, no significant differences were observed between relapsing and progressive forms of MS. Within the MS group, they showed that mtDNA levels were similar between patients in relapse and

remission, patients with and without gadolinium enhancing lesions, and patients with and without progression on the EDSS score during follow-up.

All the above mentioned results signify that additional research is needed to explore the functional effects and use of mtDNA concentrations in the CSF of MS patients.

A possible role of mtDNA as a biomarker of fingolimod treatment response can also be the subject of future studies. It is likely that fingolimod reduces inflammation-mediated cellular damage and subsequent release of mtDNA based on the significant decrease of mtDNA after initiating fingolimod. However the precise mechanism underlying reduced mtDNA CSF levels upon fingolimod treatment warrants further investigations and CSF as source for monitoring treatment effects has its obvious drawbacks. Also, further comparisons with other disease modulatory treatments are needed in order to understand if this effect is specific for fingolimod or a generic response to reduced inflammation and concomitant CNS cell injury.

Chapter 4.
Acid sphingomyelinase, no potential marker for disease progression.

Recent evidence suggests that alterations in the sphingolipid pathway may reflect disease activity in MS.[56, 57] Due to the activity of enzymes essential in the sphingolipid pathway, such as sphingomyelinases, ceramides of different chain lengths may be produced and participate in different cellular processes such as differentiation, proliferation and programmed cell death.[58, 59] Increased ceramide levels have been detected in CSF of patients with MS.[60, 61]

An altered protein expression of acid sphingomyelinase (ASM) in MS brain tissue samples (obtained at rapid autopsy and immediately frozen in liquid nitrogen or fixed in formalin) has been demonstrated in a previous study. They identified that reactive astrocytes are the primary source of enzyme activity and subsequent ceramide production.[57]

The study in **chapter 4** explored the potential of acid sphingomyelinase (ASM) activity levels in the serum as a candidate biomarker to identify MS patients with an active or progressive disease course. Furthermore, we explored several targets of the sphingolipid metabolism in relation to DMT.

Levels of serum ASM activity were longitudinally analyzed in 40 CIS, 64 RRMS and 10 primary progressive MS patients (PPMS), and 22 HC. ASM activity and sphingolipid levels were measured in a different sample of 61 RRMS patients using DMT. Analyses of ASM activity

levels showed that when pooling all types of MS, a significant higher ASM activity level was observed than in HC. The levels did not significantly differ in the serum between patients with RRMS, SPMS and PPMS and we did not find an association between ASM activity and the annualized relapse rate, disease activity, MRI variables or EDSS progression.

In the second part of this chapter we investigated the association between the sphingolipid metabolism and DMT. Despites ASM activity levels did not reflect treatment response, we did observe a significant increase of two types of ceramides (Cer-$C_{16:0}$ and Cer-$C_{24:0}$) and four types of sphingomyelin (SM-$C_{20:0}$, SM-$C_{22:0}$, SM-$C_{24:0}$ and SM-$C_{24:1}$) during fingolimod use.

Discussion and future perspectives

The activity of ASM allows conversion of sphingomyelin into ceramides. Ceramides may induce neuronal mitochondrial dysfunction and axonal damage by participating in different cellular signaling cascades and processes such as differentiation, proliferation and programmed cell death.[58, 59] So far, ASM activity levels have only been determined in the CSF of MS patients, demonstrating increased levels compared to other neurological diseases (OND).[62] Besides higher levels of ASM activity, the number of exosomes that carry ASM in the CSF was significantly higher in MS patients than in patients with OND and this was correlated to CSF ASM activity.[62]

In contrast with other studies[60, 62], we did not observe correlations between EDSS and ASM or other components in the sphingolipid metabolism. In these previous studies the markers were measured in CSF, suggesting more potential for CSF than serum with respect to suitable MS biomarkers.

Regarding the possible effect of fingolimod on reducing the production of pro-inflammatory lipids (such as ceramide), we could not confirm this within **chapter 3**. Only one previous study explored the effect of fingolimod (and other MS therapies) on different sphingolipids in MS patients[63]. They observed that IFN-ß treatment strongly increased plasma levels of Cer-$C_{16:0}$, Cer-$C_{18:0}$, Cer-$C_{20:0}$, and Cer-$C_{24:1}$ compared to healthy controls, untreated patients, or patients receiving fingolimod or natalizumab medication.[64]

One hypothesis why increasing levels of ceramides and sphingomyelin were observed, in 25 treated MS patients in our study, is that fingolimod is an S1P mimicking agent and thereby may decrease levels of endogenous S1P, which in turn may lead to increasing levels of ceramides and sphingomyelins due to the sphingolipid rheostat.

Although higher levels in MS patients were found, we concluded that ASM activity levels did not show potential as a biomarker for predicting disease activity, progression or response

to DMT. Two ceramides and four types of sphingomyelin require further investigation as potential markers for treatment response.

Chapter 5.
Serum tissue transglutaminase associates with disease progression.

Tissue Transglutaminase (TG2) is a Ca2+-dependent crosslinking enzyme, regulated by inflammatory mediators.[65-67] Recently, the presence of TG2 in infiltrating MHC-II positive cells in MS lesions was demonstrated [68] as well as increased TG2 mRNA in MS patient-derived monocytes[69] suggesting a possible role for TG2 in the pathophysiology of MS. The clinical implications of TG2 have already been described for several human diseases. [70-72] Nevertheless, the clinical implication of TG2 in MS patients has not been studied yet. The aim of **chapter 5** was to assess whether TG2 expressed by peripheral blood mononuclear cells (PBMC) is altered in patients with MS and whether this correlates with measures of disease activity and progression.

In this chapter, peripheral blood mononuclear cells (PBMCs) were isolated from 151 HC and 103 RRMS patients, 36 secondary-progressive MS patients (SPMS) and 22 PPMS patients. We observed that TG2 mRNA levels were differentially expressed in healthy controls compared to RRMS patients and that the mRNA levels were associated with disease progression measured as either high EDSS score (std ß=0.26; *p*=0.02), normalized brain volume (NBV, std ß=-0.18; *p*=0.02), normalized white (NWMV, std ß=-0.17; *p*=0.03) and grey matter volume (NGMV, std ß=-0.15; *p*=0.03) in MS patients. In addition, in PPMS patients, TG2 mRNA levels were also associated with T1-hypointense lesion volume and T2-lesion volume. Our results suggest that PBMCs-derived TG2 mRNA levels can be used as a biomarker for multiple sclerosis progression, especially for PPMS.

Discussion and future perspectives

We observed no differences in TG2 mRNA levels between MS patients and HC subjects. Nevertheless, TG2 showed significant associations with EDSS as well as NBV, NGMV and NWMV at baseline thus suggesting that TG2 mRNA levels could represent a quantitative measure of neuronal loss. No significant evidences were found for a contribution of TG2 in disease activity measured as both relapse or annualized relapse rate (ARR) in RRMS patients. Those data suggest that alterations in TG2 mRNA levels are not attributed to the inflammatory phase of the disease. In addition, a trend toward expressing lower TG2 mRNA levels was observed in RR patients with active disease (presence of gadolinium enhancing lesions at the baseline) who represent the most inflammatory group of MS patients. In line with those findings, TG2 mRNA levels were not affected by the applied DMT, which target

infiltration of immune cells in the CNS and/or the production of inflammatory cytokines. Those results were unexpected as TG2 expression is known to be modulated by inflammation and inflammatory mediators.[65-67, 73] An association profile similar to what was observed in the whole MS patients group was also observed in PPMS patients, where TG2 correlates also with T1-hypointense lesion volume and T2-lesion volume. Those data could suggest that in particularly in PPMS patients, alteration in PBMC-derived TG2 mRNA expression are associated with progression of the disease and ongoing axonal damage.

To characterize TG2 expression in PPMS patients, the expression profile of full-length TG2 and TG2 splice variants in PBMCs of PPMS patients were compared to those of HC subjects in an additional study[74] carried out by our own researchers at the MS center Amsterdam. The TG2 variant V4b was significantly higher expressed, and both V4a and V4b variants were relatively more expressed in relation to full-length TG2. These observations open new avenues to unravel the importance of TG2 alternative splicing in the pathophysiology of PPMS.

Furthermore, when pooling RRMS with SPMS patients we observed that TG2 mRNA was significantly associated with clinical disability (EDSS) and MRI measurements (normalized brain volume and normalized white matter volume). Interestingly, in this pooled group, TG2 association with NGMV was not significant. Remarkably, in both RRMS and RRMS/SPMS patients, TG2 mRNA levels showed predictive power for the course of the disease. In fact, TG2 mRNA was associated with worsening of the clinical symptoms (change of EDSS) over a 2 year follow-up. Future studies in an independent cohort are needed to validate our findings. In addition, a prospective study with repeated and long-term measurement of PBMC-derived TG2 mRNA would help to evaluated the stability of TG2 mRNA in time and the robustness of the assay.

PART 3 Treatment monitoring biomarkers

Chapter 6.
Serum neurofilament light in natalizumab treated MS patients.

As already mentioned, biomarkers reflecting and predicting disease activity and progression are crucial for personalized medicine and may provide guidance in the rapidly evolving treatment armamentarium in MS.

Natalizumab (NTZ)(Tysabri, Biogen Inc, Cambridge, MA, USA) is a humanized monoclonal antibody constraining the migration of leukocytes over the blood–brain barrier and it is

well known the be very effective in MS treatment.[75] Several studies showed a decrease in the annualized relapse rate (ARR) and stabilization[75-77] or even improvement in physical disability. [78, 79] In contrast, a recent study showed that when taking into account early inflammation and the impact of natalizumab on disease activity during the initial treatment phase, a higher than expected proportion of patients treated with NTZ showed disability progression.[80]

Neuroaxonal injury may be found in several neurological disorders and is accompanied by release of neuron-specific neurofilament (NF) proteins into extracellular space. These proteins can leak into CSF and into blood and can reach abnormal levels as a result of axonal damage in neurodegenerative, inflammatory, vascular and traumatic diseases.[81, 82]

Neurofilament light (NfL) has increases in CSF and serum of RRMS patients during relapses, returning to baseline within a couple of months of the acute event.[83-86] There are findings that serum NfL (sNfL) levels are also associated with EDDS progression. [84, 87] It remains unclear, however, whether sNfL may, in addition, predict or reflect disability progression in the absence of relapse-related neuroaxonal damage. In **chapter 6** we investigated the potential of sNfL as a biomarker of disability progression in the almost complete absence of, or with limited contribution of acute focal inflammation in a cohort of 89 NTZ-treated RRMS patients. We examined whether sNfL at initiation and after 12 months of treatment predicted disability progression in the following 2 years and whether the longitudinal trajectories of sNfL levels differed in individuals with disability progression from individuals without. In this chapter we observed a significant reduction in sNfL levels at 3 months (almost 50%) and reached its nadir within 12 months after NTZ initiation. We found no difference in the longitudinal dynamics of sNfL levels in patients with or without progression based on EDSS or EDSSplus. sNfL levels at initiation and at 12 months did not predict EDSS or EDSSplus progression in the following 2 years.

Discussion and future perspectives

The significant reduction in sNfL that we observed in **chapter 6** after initiation of NTZ is similar to other studies[84, 87, 85] However, the focus on long-term disease progression in a population without remaining disease activity has been a rather new approach. Recently, in a cohort of MS patients, sNfL was reported to predict EDSS worsening in the following year, [87] regardless of DMT status at time of measurement. In our NTZ cohort, sNfL levels at BL, and at 12 months did not predict EDSS progression in the following 2 years and sNfL levels at BL and 12 months did not differ significantly between both groups. In the study by Barro et al[87], DMT status was heterogeneous at time of sNfL measurement, with some patients treated, and others not. In our cohort, all patients were untreated for at least 2 months prior to sNfL assessment at BL. Moreover, in the present study, all patients were treated during the assessment of progression, uncoupling damage arising from focal inflammatory activity

from neurodegeneration. Our results are supported by the other studies[88, 89] They showed that no significant correlations were measured concerning EDSS progression and baseline sNfL levels.

For future plans, atrophy measurements need to be taken into consideration as it seems that brain atrophy and sNfL levels are correlated.[88, 89] In our study, atrophy measurements were not available yet.

The strength of this study is the prospective nature of the detailed longitudinal clinical and radiological data and longitudinal sample collection. However, the study also has possible limitations. The data would have benefited from a larger sample size and longer duration of follow up. Nevertheless, the complete lack of a trend towards a difference in longitudinal sNfL measurements between progressors- and non-progressors strengthens the truthfulness of our findings. In conclusion, our findings confirm the potential of sNfL to monitor the reduction in focal inflammatory damage that accompanies NTZ introduction, but fails to capture longer-term EDSS worsening ("silent progression") that is largely independent of relapse activity.

Chapter 7.
Treatment response markers: serum natalizumab concentration and lymphocyte count during treatment in RRMS patients switching from natalizumab to fingolimod.

Natalizumab is an effective treatment in relapsing-remitting multiple sclerosis, but is associated with an increased risk to develop progressive multifocal leukoencephalopathy (PML). PML is a severe, potential lethal disease, caused by the John Cunningham (JC) virus. Mainly because of the risk of PML, a substantial proportion of JC virus positive patients switch to fingolimod. The main question in switching from natalizumab to fingolimod is, what the optimal wash-out (WO) period is between the two treatments. Previous reports show a clear benefit when the duration of a WO period of natalizumab is 0-3 months in comparison to longer WO periods. However, there is no consensus regarding the optimal duration of a WO period under 3 months. In **chapter 7** we compared MS disease activity after different WO periods. In addition, we investigated several factors that possibly influence recurrence of disease activity, including serum natalizumab concentration and lymphocyte counts.

From a prospective observational cohort study of natalizumab treated patients we selected 52 patients who switched to fingolimod. We divided the patients in three groups (<6 weeks n=16, 6-8 weeks n=18, >8 weeks n=18 WO). Serum natalizumab concentration and lymphocyte count were assessed during and after natalizumab treatment.

Patients with a WO period of >8 weeks had a significant higher recurrence of disease activity (OR 6.8, 95% CI 1.4–32.8) compared to patients with a WO period of <6 weeks. Serum natalizumab concentration and lymphocyte count did not predict recurrence of disease activity.

Discussion and future perspectives

The results we observed in **chapter 7** confirm earlier studies that report an increase of disease activity after 2-4 months of WO period in comparison to shorter WO periods.[90-95] Ideally, to limit the risk of recurrence of disease activity, the WO period should be short enough to allow fingolimod to be clinically effective before natalizumab concentration drops under therapeutic levels. The downside of such a short WO period is the ongoing risk of PML in JC virus positive patients after discontinuation of natalizumab and how fingolimod could possibly increase the risk of PML and negatively influence the course of this serious complication.

All current literature regarding WO periods when switching from natalizumab to fingolimod recommend a delay of less than three months because of the risk of recurrence of disease activity.[90-94, 96] We estimated that the possible increased risk of PML with a decrease of 1-2 months of WO period does not outweigh the significant reduction of disease recurrence. Therefore, we recommend a WO period of less than 6 weeks when switching from natalizumab to fingolimod. Extra precautions regarding the risk of PML in JC virus positive patients may be taken, such as a baseline MRI and repeated scans 3 and 6 months after cessation of natalizumab and timely testing of JC virus DNA in the cerebrospinal fluid in case of suspicious MR activity.[96]

We hypothesized that lower concentration of NTZ would predispose to disease recurrence after NTZ discontinuation. However, we found no significant difference in concentration under natalizumab treatment when comparing patients with disease activity and patients without disease activity, which is in agreement with a recently published study of 12 patients switching from natalizumab to fingolimod.[97] In our study, the possible explanation could be that, the clinical disease activity reappeared after three months of natalizumab discontinuation because the concentration of the drug had already decreased under therapeutic levels.[92] When comparing longitudinal natalizumab concentration in patients with and without disease activity, the mean concentration at three months was lower in patients who did not experience disease activity (0.8 versus 4.2 μg/ml).

When switching from natalizumab to fingolimod, we hypothesized that clinical effect of fingolimod might be delayed due to the relative lymphocytosis natalizumab causes in a proportion of patients.[98] However, this study showed no correlation between lymphocyte count and disease activity. T cell subsets are unknown in this study, so describing the

fingolimod-mediated changes on CD4+ and CD8+ T cells was not possible. The lack of correlation between an overall lymphocyte count and disease activity will not rule out the probability that the changes in subpopulations of lymphocytes (including CD4+ / CD8+ ratio) may have impact on the recurrence of disease activity.

To determine the optimal WO period in case of switching from natalizumab to fingolimod, larger randomized trials are needed, preferably comparing different WO periods, including one arm starting fingolimod immediately after the final natalizumab infusion. However, in future studies the influence of the WO period on the risk of PML will still be difficult to establish given the rarity of this serious complication. Future biomarker studies in switchers should include serum NfL as this biomarker may capture both the recurrence of MS disease activity as well as damage due to early (carry-over) PML.

Chapter 8.
Natalizumab concentrations

As mentioned in chapter 7, natalizumab is an effective treatment in relapsing-remitting multiple sclerosis. All patients receive the same treatment regimen of 300mg every four weeks, despite differences in pharmacokinetics between individual patients. In this treatment regimen, natalizumab concentrations may stay detectable in serum in up to 200 days after cessation of therapy.[99] Serum natalizumab concentration corresponds with the percentage of α-4 integrin receptor saturation.[100] Desaturation of the α-4 integrin receptor occurs when the serum NTZ concentration falls under 1-2 µg/ml.[100] Above this threshold of 2 µg/ml, natalizumab receptor saturation will roughly fall between 70-100%.[100] An adequate receptor saturation is estimated as ≥70-80% saturation, although prospective data confirming this assumption are lacking.[101, 102] Based on a model with results from a large phase II trial, approximately 90% of patients showed natalizumab trough concentrations largely exceeding 2.5 µg/ml. Levels exceeding 2.5 µg/ml could indicate that the approved treatment regimen of natalizumab for RRMS results in patients receiving more natalizumab than necessary for optimal drug efficacy.[103, 104] Furthermore, it is suggested that higher natalizumab receptor saturation could increase the risk of PML.[105] This unconfirmed hypothesis leads to clinicians extending dose intervals in natalizumab treatment with the aim to reduce the PML risk by decreasing natalizumab exposure.[105-107]

The aim of the study in **chapter 8** was to measure natalizumab serum concentrations and correlate concentrations with disease activity and possible influencing factors. We explored if natalizumab concentration has potential as a marker of treatment response.

In **chapter 8**, natalizumab serum concentrations were measured in serum of 80 patients from a prospective observational cohort study. Data on demographics, duration of treatment, EDSS, clinical exacerbations, brain MRI and body weight were collected.

We measured high (≥10µg/ml) natalizumab concentrations in 94% of patients. Intra-individual concentrations were stable. The spread in concentrations between patients was substantial and did not correlate with disease activity. We found a negative association between natalizumab concentration and body weight (β=-0.30, p=0.010).

Discussion and future perspectives

In part 3.3 of this thesis natalizumab concentrations were explored as potential treatment monitoring markers.

The variation in natalizumab concentrations was substantial. However it did not correlate with disease activity (**chapter 8**). The mean natalizumab serum concentration in our cohort was above 20 µg/ml which is in agreement with recently presented data.[108] The mean concentration was the same in patients with active versus non-active disease, which suggests that high concentrations do not result in an increase of treatment efficacy in comparison to lower but still adequate concentrations. Considering this and the large proportion of high natalizumab concentrations, natalizumab could perhaps be administered less frequently (or with a lower dose) to reach natalizumab concentrations that are lower but still cause adequate receptor saturation and consequently, optimal drug efficacy.[100] Caution is advised though, because of a large spread in concentrations and the well-established rebound effect which occurs after cessation of natalizumab treatment.[109] Therefore, monitoring natalizumab serum concentrations during treatment should be essential.

Extended dose intervals could help reduce costs of medication and increase quality of life for the patient with fewer hospital visits, but further studies are needed to establish the safety of alternative treatment regimens. At this moment a prospective multicenter single-arm trial with one-year follow-up and an extension phase of one year (ClinicalTrials.gov; NCT03516526) is completed at the MS Center Amsterdam (Amsterdam University Medical Center, Vrije Universiteit), with the aim to evaluate if natalizumab efficacy is maintained when switching to personalized extended interval dosing based on individual natalizumab trough concentrations in stable RRMS patients (reference: Neurology in press).

Results of such trials will hopefully give a decisive answer to the question if extending dose intervals in natalizumab treatment is feasible without losing drug efficacy.

Key issues

- For clinical practice, the KFLC index is more accurate in excluding CIS/MS compared to OCB but for ruling in a diagnosis of CIS/MS, analysis of OCB appears to be more accurate.
- mRNA derived from blood platelets could assist in the diagnosis of MS without being as invasive as CSF collection.
- The functional effects of mtDNA concentrations in the CSF of MS patients should be explored in additional research.
- Analyses of ASM activity levels showed that when pooling all types of MS, a significant higher ASM activity level was observed than in healthy controls. The levels did not significantly different in the serum of patients with RRMS, SPMS and PPMS and we did not find an association between ASM activity and the annualized relapse rate, disease activity, MRI variables or EDSS progression.
- Despites ASM activity levels did not reflect treatment response, we observed a significant increase of two types of ceramides (Cer-$C_{16:0}$ and Cer-$C_{24:0}$) and four types of sphingomyelin (SM-$C_{20:0}$, SM-$C_{22:0}$, SM-$C_{24:0}$ and SM-$C_{24:1}$) during fingolimod use.
- When pooling RRMS with SPMS patients TG2 mRNA was significantly associated with clinical disability (EDSS) and several MRI measurements, including those reflecting neurodegeneration (NBV).
- In both RRMS and relapse-onset (RRMS/SPMS) patients, TG2 mRNA levels showed predictive power for the course of the disease.
- There is no difference in the longitudinal dynamics of serum neurofilament light levels in patients with or without progression based on EDSS or EDSSplus during natalizumab treatment.
- Serum neurofilament light levels at initiation of natalizumab and at 12 months did not predict EDSS or EDSSplus progression in the following 2 years.
- Patients with a WO period of >8 weeks had a significant higher recurrence of disease activity compared to patients with a WO period of <6 weeks.
- No significant difference in natalizumab concentration when comparing patients with and without MS disease activity.

The road forward

Making progress on the road to clinical application of MS biomarkers will require time and thorough research. The design of future studies should most optimally be adjusted to the intended purpose of the study i.e. the research question. Although this seems rather obvious, in biomarker research this is not always the case and sometimes cannot even be achieved. Using existing biobanks and datasets may inflate biomarker sensitivity and increase bias. However, this may also hold for prospective studies or studies labelled as

such.[110] When used appropriately and interpreted carefully, the use of existing biobanks and datasets may speed up biomarker discovery and its clinical use. For body fluid biomarker research ideally repeatedly collected longitudinal samples are available together with clinical and radiological data from large cohorts that include the full clinical spectrum of disease phenotypes and disease duration and followed for a sufficiently long time to capture crucial milestones. Depending on the focus of the biomarker research specific (additional) requirements can be defined with respect to the dataset.

Early detection and diagnostic certainty in MS may be important for the long term outcome of individual patients. Since prevention of disability is within reach when immunomodulatory treatment is started early, a certain diagnosis soon after the onset of clinical symptoms is crucial. Apart from clinical judgement and radiological signs of dissociation in space and time, biomarkers may add to diagnosis certainty. This is already the case for CSF analyses, but if more easily collected body fluids could avoid a lumbar puncture, this would be a step forward. Regarding early detection, the dataset should include a sufficient number of early cases, subsequently proven to be MS. In addition, control groups have to be more heterogeneous than we applied in our studies, to reflect the real world and need to be supplemented with suspected MS cases and more inflammatory neurological disease controls.

The issue of early treatment can also be approached in at least two different ways in which biomarkers could play a role. First prediction of the disease course. If biomarkers could help in predicting the course of the disease this would greatly help in selecting patients who would mostly need early intervention. And the other way around, if biomarkers could predict a benign disease course, early treatment is not necessary and potential side effects of treatment could be avoided. A pure approach to study the natural course of the disease from onset would be to analyze biomarkers in large groups of untreated patients with variable disease courses. It is questionable whether these datasets including body fluids are available. Collecting prospective data in this way i.e. while not treating patients, is neither feasible nor ethical. This seriously hampers this approach.

Second the prediction of a treatment response. If biomarkers can reliably predict the response to a treatment in an individual patient, early treatment would change form a ‘trial and error’ approach to a real personalized choice with reasonable certainty of success. In case of monitoring treatment and predicting treatment response large cohorts should be followed for many years, requiring repetitive systematic bio banking and data collection of many patients before and after initiation of treatment. This should not only be analyzed regarding the effect of treatment, but also for treatment safety (to detect side effects such as PML in the earliest possible stage). This is essential for a well-balanced timely treatment strategy in an individual MS patient.

Another area in which body fluid biomarkers may be very promising is the distinction of the underlying pathophysiological process responsible for clinical deterioration. In MS it is assumed that both inflammation and neurodegeneration can cause disability. However, the possibilities for modulating these two mechanisms using available therapeutic interventions are very different. Understanding and detecting the underlying process may help in drug discovery and also in tailored treatment. Blood based biomarkers offer the potential for the development of a low-cost, non-invasive and more time efficient tool compared to both CSF markers and neuroimaging. Blood is much easier to obtain and can be sampled repeatedly over time without much burden for the patient. The results for NfL presented in this thesis show great promiss that blood based biomarkers will be developed for these purposes in the near future. Indisputable, when a potential biomarker is detected, validation for the intended purpose, at both technical and clinical levels, is an important next step. International collaboration and the help of real-world and registry data is warranted.

This thesis focusses on specific single body fluid biomarkers. Although no single blood biomarker is yet able to detect MS, combinations of biomarkers (also called panels or profiles) can be a promising focus for future perspectives. For example, combining sNfL with serum tissue transglutaminase could result in a more sensitive marker for predicting disease progression. Realizing a panel with reduced number of markers will have significant impact on the complexity and cost of diagnosis, treatment monitoring and predicting disease progression.

New (combinations of) biomarkers can also be discovered using an "-omics studies" approach. The general term "-omics" refers to a group of rapidly emerging novel technologies offering the opportunity of large-scale analysis and identification of candidate biomarkers in multiple levels of cell biology (DNA, RNA, proteins, lipids and metabolites). For example, a novel proteomics technology is the SOMAscan, which is based on aptamers that are able to bind conformational protein epitopes with high specificity and sensitivity.[111] The assessment of blood proteomics for MS would aid in gaining insight into the proteins and pathways involved in MS progression. This technique is already used in our center and revealed eight novel biomarkers related to clinical and radiological progression in MS.[111] Another novel array based technology is the Olink Proximity Ligation Assay, which combines complementary antibody based analysis with PCR-based reporting. The technology thereby has a high analytical specificity and sensitivity, and allows analysis of >1500 proteins simultaneously. This novel technology is currently used in multiple biomarker initiatives in our center.

Besides "omic studies", non-coding RNA molecules (microRNA, equal to miRNA) is of interest in ongoing and upcoming studies. miRNA analysis is more manageable and requires fewer resources than microarray or proteomics analysis. Another advantage is durability because miRNAs are more resistant to ribonucleases than messenger-RNA. Lastly, miRNAs

can be detected in a large number of samples, including tissue biopsies, whole blood, blood cells, serum, plasma and urine. Several miRNA profiling platforms, from microarray to deep sequencing, are currently available to prospectively identify miRNAs.[112]

Although the focus in this thesis is on *body* fluid biomarkers, in the future a more comprehensive dataset including multimodal outcomes may offer a more complete representation of the disease phenotype. In addition to body fluid markers, clinical observations and imaging data also digital biomarkers using sensors and algorithms across an abundance of available connected hardware and software tools could be included. At the moment a study performed at our MS center Amsterdam is monitoring MS symptoms with digital questionnaires using an app in combination with a Fitbit device to predict relapses.

The amount of data that will become available will grow exponentially. There will be a need for new ways to discover the golden needle in the haystack. Artificial intelligence (AI) is the study of methods to imitate intelligent human behavior. Machine learning (ML) is a subset of AI that focuses on the study of algorithms that enable a computer to perform specific tasks without specific instructions, but instead inferring patterns from big data. Using ML, it is possible to extract patterns within patient data, and exploit these patterns to predict patient outcomes for improved clinical management. Instead of analyzing potential biomarkers that are hypothesized by human intelligence and biological processes (also based on human intelligence), ML could discover potential (new) biomarkers from big data.

The road forward is challenging, but with all the new technologies it will be a road full of interesting side roads. It may be long, but it will certainly be exciting. Hopefully leading to better possibilities to guide patients with MS on their journey.

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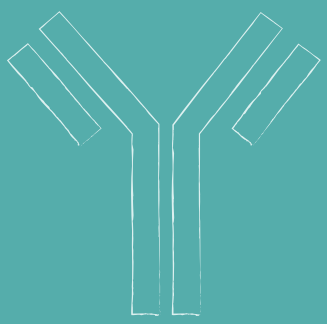
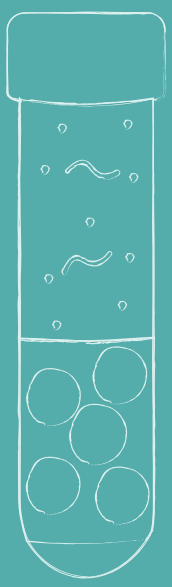
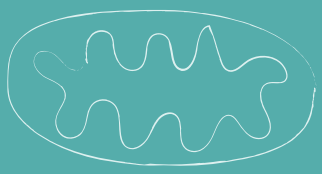
Appendices

Nederlandse samenvatting

Achtergrond

In dit proefschrift zijn verschillende biomarkers onderzocht die te maken hebben met de ziekte Multiple Sclerosis. In deze samenvatting wordt uitgelegd wat de ziekte Multiple Sclerosis inhoudt en waarom er zogenaamde biomarkers nodig zijn om de ziekte beter te begrijpen. In elk hoofdstuk wordt een andere biomarker onderzocht die bij het bespreken van de resultaten steeds kort wordt geïntroduceerd.

Wat is Multiple Sclerosis?

Multiple sclerose (MS) is een ziekte van het centrale zenuwstelsel (hersenen en ruggenmerg) en wordt gekenmerkt door ontstekingen. In Nederland treft MS ongeveer 17.000 mensen, wereldwijd zijn dat er meer dan 2 miljoen. De ziekte komt vooral voor bij jong volwassenen (20 tot 40 jaar) en uit zich twee keer zo vaak bij vrouwen als bij mannen.

Op weefselniveau zie je bij MS dat het *myeline*, het witte vetachtige omhulsel dat de uitlopers van zenuwcellen bedekt als een soort isolatie laagje, wordt afgebroken. Het eigen afweersysteem valt het gezonde weefsel aan middels ontstekingsreacties. Waarom het afweersysteem zo overactief reageert is onbekend. Door de afbraak van myeline, *demyelinisatie*, neemt de werking van zenuwen af en kunnen patiënten neurologische klachten ervaren. Indien de ontstekingsreacties opvlammen en neurologische klachten geven spreken we van *exacerbaties*.

Op MRI-scans kunnen we de ontstekingsgebieden*, laesies,* herkennen aan de typische kenmerken en lokalisatie. De klachten van de patiënt, de neurologische beoordeling en de MRI-beelden worden alle gebruikt om te bepalen of iemand MS heeft. Toch kan het stellen van een tijdige diagnose bij een klein deel van de patiënten ingewikkeld zijn. Daarnaast is het lastig te voorspellen of de patiënt veel of juist weinig ontstekingen, *ziekteactiviteit*, zal krijgen en in welke mate de patiënt lichamelijk geïnvalideerd, *ziekteprogressie*, zal raken. Er is dus een dringende behoefte aan biologische markers, *biomakers*, uit lichaamsvloeistoffen die de ziekteactiviteit en de ziekteprogressie weerspiegelen en voorspellen. Dergelijke biomarkers zijn cruciaal om geneeskunde op maat te kunnen leveren.

Doel van het proefschrift

Het doel van dit proefschrift is het vinden van biomarkers gerelateerd aan 1) de vroegtijdige diagnose van MS, 2) de ziekteactiviteit en ziekteprogressie en 3) het monitoren van de behandeling.

Resultaten

Deel 1. Diagnostische biomarkers

Zoals hierboven beschreven is het in sommige gevallen lastig om de diagnose MS te stellen. In de eerste twee hoofdstukken ligt de aandacht bij twee biomarkers die hier mogelijk bij kunnen helpen: kappa free light chains en messengerRNA. Tot op heden zijn de oligoclonale banden (OCB) de enige lichaamseigen biomarkers die in de praktijk gebruikt worden voor het stellen van de diagnose MS. Deze bandjes, gevormd door immunoglobulinen (antilichamen) zijn in grote mate aanwezig in het hersenvocht van MS patiënten. Bij gezonde mensen worden deze specifieke banden niet aangetoond in het hersenvocht. De beoordeling van OCB in het laboratorium is echter arbeidsintensief, vereist geschoold personeel en de juiste beoordeling is sterk afhankelijk van de examinator en de methode. Dit deed de volgende vraag opkomen: 'Is er een biomarker die kan worden gemeten met een geautomatiseerde en kwantificeerbare procedure?'

Immunoglobulinen bestaan uit twee ketens, light chains en heavy chains. Light chains zijn weer opgebouwd uit kappa- en lambdaketens. Light chains kunnen ook in ongebonden vorm vrij rond circuleren in het hersenvocht en het bloed. In **hoofdstuk 1** hebben we kappa free light chains (KFLC) gevalideerd als diagnostische biomarker in MS en vergeleken met de OCB.

Voor hoofdstuk 1 hebben we van 745 patiënten bloed en hersenvocht verzameld. Dit deden we in samenwerking met 18 andere MS centra in Europa. In deze samples hebben we KFLC en OCB gemeten. We definieerden cut-offs voor abnormale KFLC waarden en op basis van deze cut-offs werden de patiënten geclassificeerd als positief of negatief. Vervolgens hebben we gekeken welke test, OCB versus KFLC beter is in het diagnosticeren van de ziekte MS. We concludeerden dat KFLC beter zijn in het excluderen van de diagnose. De OCB blijven beter in het bevestigen van de ziekte MS.

In **hoofdstuk 2** is de potentie van messengerRNA (mRNA) van bloedplaatjes onderzocht als diagnostische marker. Tot dusver is geen enkele biomarker uit bloed overtuigend bevestigd als nuttig hulpmiddel bij het diagnostisch onderzoek van MS. Op bloed gebaseerde markers bieden duidelijke voordelen en recentelijk hebben studies laten zien dat bloedplaatjes als nieuwe bron voor het ontdekken van biomarkers bij verschillende ziekten zeer veel potentie hebben. De reden dat bloedplaatjes interessant zijn komt omdat ze een rijk mRNA repertoire bevatten. RNA is een transcript van DNA waarmee genetische informatie tot expressie wordt gebracht en gereguleerd. Het mRNA repertoire van bloedplaatjes is veranderlijk en biedt waardevolle informatie.

Bloedplaatjes reageren op activerende signalen uit hun omgeving waardoor het mRNA zich kan splitsen. Daarnaast kunnen bloedplaatjes ook RNA opnemen uit verschillende celtypen, wat leidt tot een nog unieker RNA repertoire. Onze hypothese in **hoofdstuk 2** is dat bloedplaatjes een MS specifieke mRNA signatuur bevatten. Dit unieke mRNA signatuur

zou daarmee potentie hebben als diagnostische biomarker in MS. In bloedsamples van 57 MS patiënten uit het Amsterdam cohort ontdekten we 645 unieke mRNA signaturen die verhoogd aanwezig waren bij MS patiënten in vergelijking met bloedsamples van gezonde mensen. Deze mRNA's werden vervolgens gebruikt als input voor de ontwikkeling van een diagnostische MS classificator die MS kon detecteren met meer dan 80% nauwkeurigheid.

Deel 2 Ziektebeloop biomarkers

In dit deel van het proefschrift hebben we biomarkers onderzocht die het ziektebeloop weerspiegelen, met daarbij de nadruk op de ziekteprogressie.

In **hoofdstuk 3** onderzochten we de potentie van vrij circulerend mitochondrieel DNA (mtDNA) in het hersenvocht als biomarker voor het identificeren van patiënten met progressieve MS.

Demyelinisatie leidt tot een toename van de energievraag in cellen. Bij een verhoogde energievraag kan de activiteit van mitochondriën, de organellen van de cel die energie produceren, in zenuwcellen toenemen. In tegenstelling tot andere organellen hebben mitochondriën hun eigen DNA (mitochondrieel DNA, afgekort: mtDNA). Mogelijk komt dit specifieke mtDNA vrij doordat de mitochondriën stress ervaren door de MS ontstekingen en is dit meetbaar in het hersenvocht van MS patiënten. In dit hoofdstuk hebben we vrij circulerend mtDNA gemeten in twee cohorten uit het Amsterdam cohort (120 MS patiënten) en uit een Zweeds cohort (42 MS patiënten). De belangrijkste conclusie van dit hoofdstuk is dat onze gegevens laten zien dat een verhoogde concentratie vrij circulerende mtDNA verband houdt met de ziekteactiviteit van MS en progressieve ziekte.

In **hoofdstuk 4** onderzochten we het enzym acid sfingomyelinase (ASM). De hersenen bestaan voor een groot deel uit vetten, ook wel lipiden. Een specifiek soort, de sfingolipiden, hebben mogelijk een rol in de ziekteactiviteit van MS. Vanwege de activiteit van bepaalde enzymen die essentieel zijn bij het aanmaken van sfingolipiden kunnen ceramiden worden geproduceerd. Ceramiden hebben vervolgens weer een rol in allerlei processen van de cel. Een belangrijk enzym dat betrokken is bij dit proces is acid sfingomyelinase. De levels van ASM activiteit hebben we gemeten in het bloed van MS patiënten. De ASM activiteit verschilde niet tussen progressieve en niet progressieve MS patiënten. We vonden ook geen verband tussen ASM activiteit en het aantal exacerbaties op jaarbasis, ziekteactiviteit, MRI variabelen of lichamelijke invaliditeit.

In **hoofdstuk 5** staat de Tissue Transglutaminase (TG2) centraal. Dit enzym heeft een belangrijke rol in gereguleerde celdood, bij membraantransport en bij het verstevigen van het celskelet. Er werd beoordeeld of de expressie van dit enzym veranderd is bij MS patiënten en of dit overeenkomt met de mate van de ziekteactiviteit en de ziekteprogressie.

We observeerden dat TG2 levels niet geassocieerd waren met de ziekteactiviteit maar wel met de ziekteprogressie.

Deel 3 Ziektebehandeling biomarkers

In dit laatste deel kijken we naar biomarkers die kunnen helpen bij het monitoren van de behandeling van MS. Er zijn medicijnen op de markt die het ziekteproces gedeeltelijk kunnen remmen. Deze medicijnen hebben verschillende bijwerkingen en kunnen zelfs ernstige complicaties geven. Het zijn namelijk medicijnen die ingrijpen op het immuunsysteem.

Het werkzame effect van het medicijn kan men meten op basis van nieuwe laesies op de MRI en/of er nieuwe neurologische klachten ontstaan. Natalizumab is het eerste MS medicijn met een hoge effectiviteit waarbij exacerbaties en MRI afwijkingen grotendeels worden voorkomen. Dit middel wordt maandelijks toegediend via een infuus en voorkomt dat de witte bloedcellen, *lymfocyten*, zich naar het centrale zenuwstelsel verplaatsen. Het is zeer effectief en wordt goed getolereerd door patiënten. Het aantal aanvallen op de myeline neemt af door dit medicijn maar we zien bij een groot deel van de MS patiënten dat er toch mettertijd lichamelijke achteruitgang optreedt.

In **hoofdstuk 6** hebben we onderzocht of er in het bloed van patiënten die natalizumab gebruiken en die op het moment van afname vrijwel geen ziekteactiviteit hebben, een biomarker aanwezig is die kan voorspellen of lichamelijke achteruitgang zal optreden. De hiervoor onderzochte biomarkers zijn neurofilamenten. Dit zijn afbraakproducten van zenuwuitlopers die bij schade aan de hersenen aanwezig kunnen zijn in het hersenvocht en het bloed. In een groep van 89 met natalizumab behandelde MS patiënten onderzochten we deze neurofilamenten in het bloed. Na drie maanden van de behandeling met natalizumab vonden we een afname van bijna 50%. Na twaalf maanden was de afname van neurofilamenten maximaal.

We vonden geen verschil in concentraties neurofilamenten bij patiënten met en zonder lichamelijke achteruitgang. Tevens voorspelde de concentratie van neurofilamenten bij het starten van natalizumab niet of een patiënt later meer lichamelijke invaliditeit ontwikkelt.

Natalizumab wordt goed getolereerd door MS patiënten. Er is echter wel een potentieel dodelijke complicatie: progressieve multifocale leukoencefalopathie (PML). Dit is een virale hersenontsteking veroorzaakt door het John Cunningham virus (JCV). Vanwege het risico op PML schakelt een aanzienlijk deel van de JCV positieve patiënten over op fingolimod. Fingolimod is een MS medicijn dat patiënten elke dag innemen in tabletvorm. Fingolimod zorgt ervoor dat lymfocyten niet uit de lymfeklieren kunnen treden en daardoor niet richting het centraal zenuwstelsel verplaatsten. De belangrijkste vraag bij het switchen van natalizumab naar fingolimod is: hoe lang moet je wachten tussen de twee behandelingen?

Eerdere studies laten een duidelijk voordeel zien wanneer de duur van deze periode, *washout periode*, niet langer dan 3 maanden is. Te lang wachten kan zorgen voor opvlamming van ziekteactiviteit maar te snel switchen brengt mogelijk ook complicaties met zich mee omdat er dan twee middelen in het lichaam zitten die beide effect hebben op het afweersysteem. Er bestaat geen duidelijkheid wat de optimale duur is van deze periode onder de 3 maanden. In **hoofdstuk 7** vergeleken we de ziekteactiviteit van MS na verschillende washout perioden bij 52 patiënten die van natalizumab switchten naar fingolimod. Daarnaast hebben we verschillende factoren onderzocht die mogelijk van invloed zijn op het terugkeren van de ziekteactiviteit, waaronder de natalizumabconcentraties en het aantal lymfocyten in het bloed. Patiënten met een washout periode van langer dan 8 weken hadden een hogere kans op terugkomst van ziekteactiviteit vergeleken met patiënten met een washout periode van minder dan 6 weken. De natalizumabconcentraties en het aantal lymfocyten voorspelden geen terugkomst van ziekteactiviteit.

Wat opvallend is bij natalizumab is dat alle patiënten elke vier weken hetzelfde behandelschema van 300 mg krijgen, ondanks de verschillen in farmacokinetiek tussen individuele patiënten. Met deze dosering kunnen natalizumabconcentraties in het bloed tot 200 dagen na stopzetting van de therapie detecteerbaar blijven. Het zou erop kunnen wijzen dat dit ertoe leidt dat patiënten meer natalizumab krijgen dan nodig is. Het doel van de studie in **hoofdstuk 8** was om de concentraties te meten en deze te correleren met ziekteactiviteit en mogelijke andere beïnvloedende factoren. We onderzochten of de natalizumabconcentratie potentie heeft als marker voor de behandelingsrespons. De serumconcentraties werden gemeten in het bloed van 80 MS patiënten onder behandeling met natalizumab. We maten hoge natalizumabconcentraties bij 94% van de patiënten. De spreiding in concentraties tussen patiënten was aanzienlijk en correleerde niet met ziekteactiviteit. We vonden een negatief verband tussen de concentratie natalizumab en het lichaamsgewicht.

Toekomstperspectief

Wereldwijd is er een behoefte aan MS biomarkers die kunnen helpen bij het stellen van een tijdige diagnose en die de ziekteactiviteit en de ziekteprogressie kunnen weerspiegelen en voorspellen. Dergelijke biomarkers zijn cruciaal om geneeskunde op maat te kunnen leveren. Dit proefschrift voegt nieuwe informatie toe aan de bestaande wetenschappelijke kennis over potentiele biomarkers. De daadwerkelijke klinische toepassing van MS biomarkers zal nog lang op zich wachten. Er is namelijk tijd en grondig onderzoek nodig.

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Mijn proefschrift is mede tot stand gekomen door een goede samenwerking van de verschillende onderzoeksgroepen binnen het MS Centrum. Hierdoor werkte ik samen met prof. dr. de Vries, prof. dr. Barkhof, prof. dr. Geurts, dr. van Horssen, dr. van Dam, dr. Hulst, dr. Schoonheim en dr. Steenwijk. Wat een geluk dat jullie mij konden bijstaan met al jullie kennis.

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About the author

Cyra Eline Leurs was born in Hilversum, the Netherlands, on the 4th of October 1986. After completing her pre-university education in 2004 at the A. Roland Holst College in Hilversum, she started, in that same year, medical school at the University of Amsterdam.

Throughout the course of clinical internships, her enthusiasm for neurology expanded. This made her apply for an elective internship in neurology at the Academic Hospital in Paramaribo and a final internship in neurology at the OLVG Oost in Amsterdam.

After she obtained her medical degree in 2012, she started working as a junior resident in neurology at the OLVG Oost in Amsterdam.

Between 2014 and 2018 Cyra was appointed as a PhD candidate at the MS center at the VU University Medical Center in Amsterdam, under supervision of professor B.M.J. Uitdehaag, professor C.E. Teunissen and professor J. Killestein.

From July 2018 onwards, she enjoys her work as a resident in training in neurology at the VU University Medical Center in Amsterdam.

During her study in Amsterdam, Cyra was an active member of the Amsterdam Student Rowing Club Nereus. At Nereus, she met her husband Robert Lücken. They live in the beautiful center of Amsterdam together with their dog Bobby.