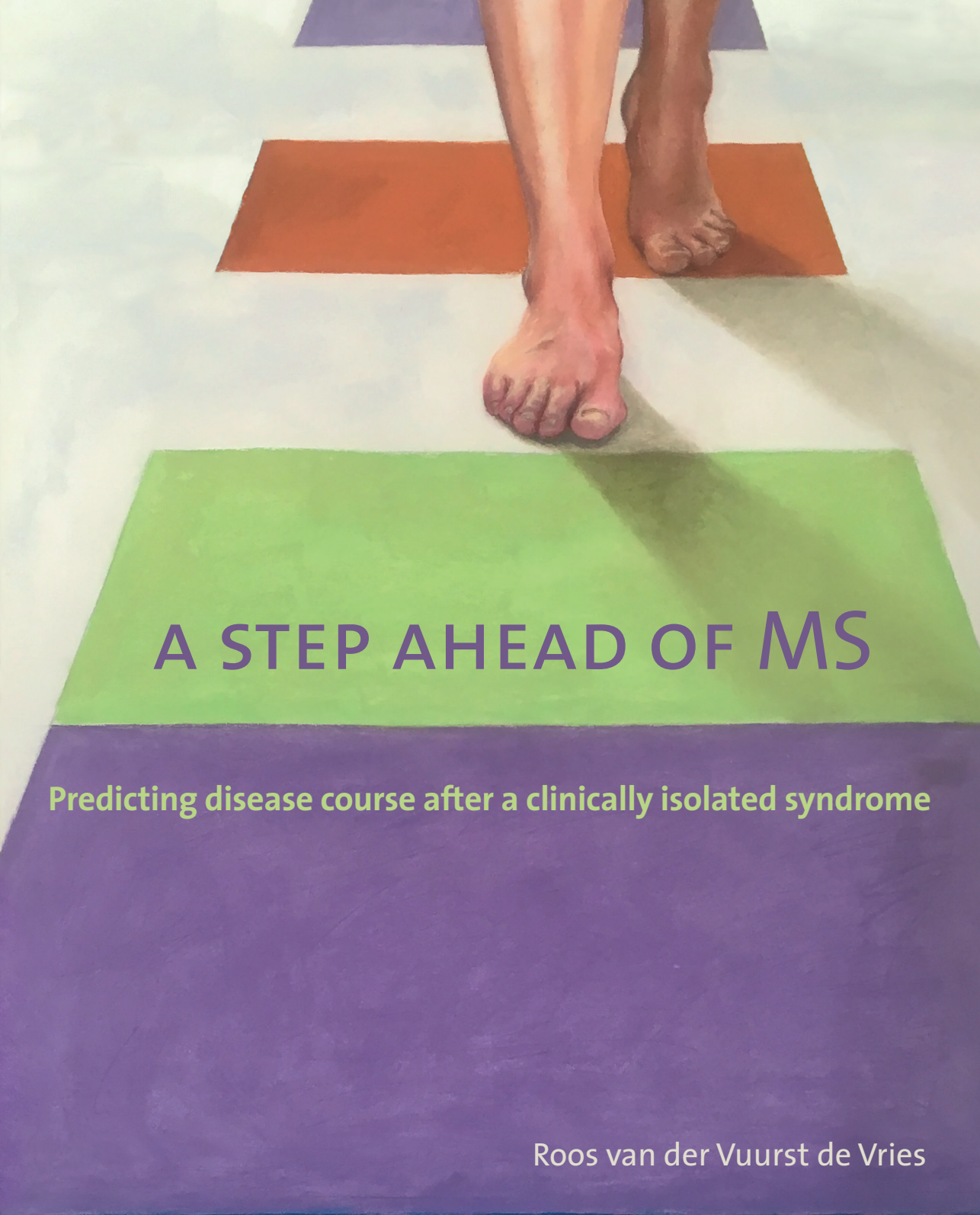

A STEP AHEAD OF MS

Predicting disease course after a clinically isolated syndrome

Roos van der Vuurst de Vries

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Predicting disease course after a clinically isolated syndrome

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A Step Ahead of MS

Predicting disease course after a clinically isolated syndrome

Een stap voor op MS

Het voorspellen van het ziektebeloop na een klinisch geïsoleerd syndroom

Proefschrift

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TABLE OF CONTENTS

Chapter I

General introduction

MULTIPLE SCLEROSIS AND CIS

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), in most cases characterized by relapses of neurological dysfunction and accumulation of disability.[1]
More than 150 years ago, Carswell, Cruveilhier and Charcot already described the pathological and clinical features of MS.[2] Yet, the exact cause of MS is still unrevealed.
The disease mostly affects young women, 70% of patients have an age of onset between 20-40 years.[3] MS also occurs in children,[4] around 3-5% of MS patients have the first attack during childhood.[4,5]
The first generally recognized criteria for MS were established in 1954 by Allison and Millar.[6,7] Ten years later, Schumacher described the 'clinically definite' form of MS (CDMS), defined as: clinical evidence of demyelination in two different neurological localizations at two different time points.[8] After the Schumacher criteria, several revisions were proposed.[7] When using pathologically confirmed cases, the criteria for CDMS proposed by Poser et al in 1983 are shown to be the most sensitive.[9,10] Currently, the McDonald 2017 criteria are used to diagnose MS. However, the Poser criteria for CDMS are still often used in MS research as study endpoint.
The first manifestation of MS is called a clinically isolated syndrome (CIS), a subacute episode of focal neurological symptoms resulting from inflammatory demyelination of the optic nerve, spinal cord, cerebrum, cerebellum or brain stem.[3] The disease course after CIS is highly heterogeneous, most patients will have subsequent relapses after this first manifestation (relapsing remitting MS (RRMS)), however, one third will remain monophasic.[11]

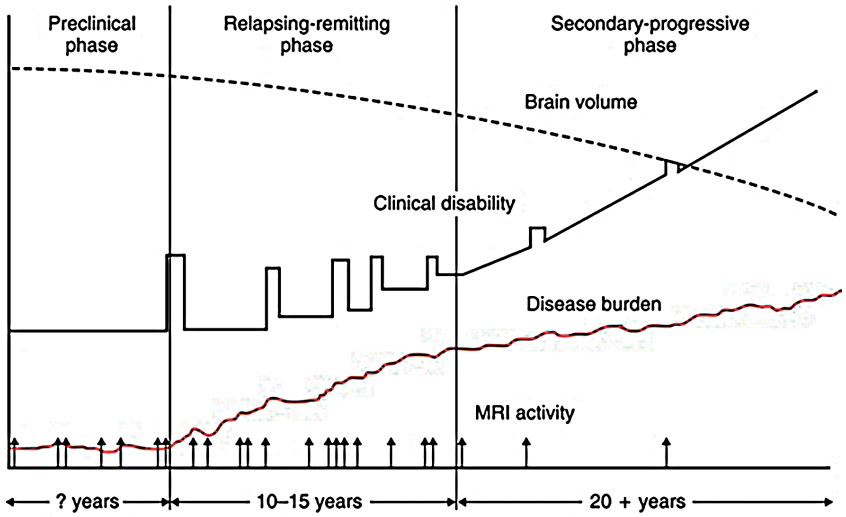


Figure 1. Disease course for the majority of RRMS patients.
The first attack of demyelination (CIS) is followed by a relapsing disease course (RRMS), followed by the progressive phase of the disease (SPMS) (Reference: Fox RJ and Cohen JA, Cleve Clin J of Med 2001; 68: 157-171)

The majority of RRMS patients will reach a progressive phase of MS; secondary progressive MS (SPMS), characterized by irreversible disability progression that is independent of relapses. [12] In this progressive phase, neurodegeneration is more prominent. The transition from RRMS to SPMS usually occurs after approximately 20 years of disease duration.[13]
In ten to fifteen percent of patients, MS starts with a progressive onset and no relapses, these patients have primary progressive MS (PPMS). This thesis focusses on the early presentation of relapse-onset MS. Therefore PPMS is not discussed in this thesis. Figure 1 shows the clinical course, MRI activity and disease burden in RRMS patients.

There is a growing number of disease-modifying therapies (DMT) available that can be administered after the first manifestation of MS (CIS), in patients with a high risk of future disease activity. These therapies are immunomodulatory and taken early in the disease course, delay a second relapse and have a potential to prevent future disability.[3, 14-18] Unfortunately, these therapies do have serious adverse effects.[19-21] To prevent unnecessary treatment of patients with a benign disease course, it is important to identify these patients early. Markers for disease stratification, predicting long-term prognosis and predicting treatment response are crucial to provide more individualized care.[22]
Apart from the important role of risk stratification in the choice for early treatment in patients with a future active disease course, prognostic factors are also essential when counselling patients about the prognosis of this life-changing heterogeneous disease.

CURRENT DIAGNOSTIC TOOLS

In clinical practice, magnetic resonance imaging (MRI) and cerebrospinal fluid (CSF) analyses are routinely used to estimate the risk for MS.

Magnetic resonance imaging (MRI)

MRI of the brain and spinal cord is the most important tool in the diagnostic work-up in patients with a suspicion of CIS and MS. For MS diagnosis two conditions should be present, the first is dissemination in space (DIS), and the second is dissemination in time (DIT). The goal of the use of MRI is not only to demonstrate DIS and DIT but also to exclude alternative diagnoses.[23] MS lesions are typically visible on MRI in the white matter: periventricular, juxtacortical, infratentorial, and in the spinal cord, these lesions are hyper intense on T2-weighted MRI sequences and characteristically ovoid shaped.[24] Figure 2 shows the typical localizations for MS lesions.
The long-term risk for CDMS when T2 lesions are present on MRI at time of the first attack is 60-80%. When these lesions are absent, this risk is 20-25%.[11, 25] Both localization and number of lesions affect the risk of MS. Patients with T2 lesions in the brainstem have a higher MS risk than patients with only supratentorial lesions.[26] Also lesions in the corpus callosum predict CDMS diagnosis in patients with CIS.[27]
Some studies have shown that not only white matter lesions but also grey matter atrophy and brain volume loss, which are signs of axonal loss, were predictive for MS diagnosis.[28, 29] However, another recent study did not find an association between brain volume loss and the risk of CDMS in CIS patients.[30]

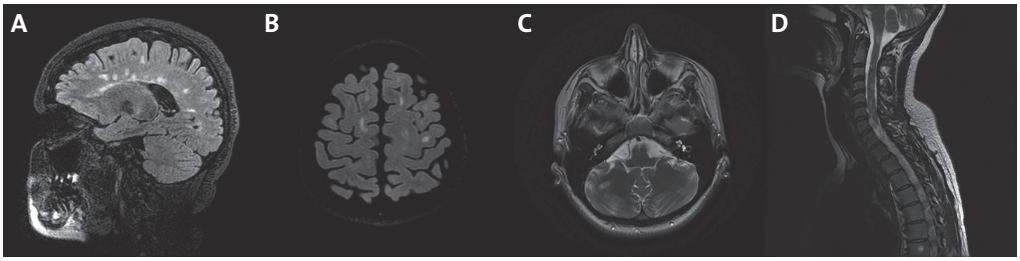
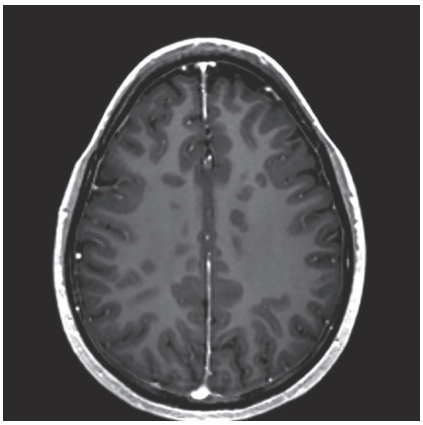


Figure 2. *Typical MS lesions on brain MRI:* **A** *periventricular,* **B** *juxtacortical,* **C** *infratentorial,* **D** *spinal cord*

T1-hypointense lesions, which is another sign of axonal loss, are highly predictive for CDMS diagnosis in children with CIS,[31] but in adults the predictive value is low.[32] Figure 3a shows examples of T1-hypointense lesions.



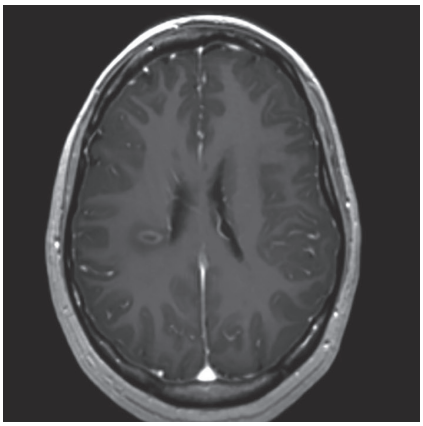


Figure 3. a *T1-hypointense lesions* ***b*** *gadolinium enhancing lesion on brain MRI*

McDonald criteria

The first MRI criteria for MS, established in 2001, were the first version of the McDonald criteria.[33] In the following years multiple updates of these criteria are introduced.[34-36] The criteria are simplified and according to the update in 2010, MS could even be diagnosed at time of CIS when asymptomatic contrast enhancing lesions were seen simultaneously with non-enhancing lesions (DIT).[35] Figure 3b shows a contrast enhancing lesion on brain MRI. The criteria for DIS could be fulfilled when the MRI scan shows at least one clinically silent lesion in two separate characteristic localizations of demyelination.
The latest revisions to the McDonald criteria were proposed in 2017. These new criteria allow an MS diagnosis when the MRI meets the criteria for DIS and unique oligoclonal bands (OCB) are present in CSF, even when there is no evidence of DIT on the MRI scan. Furthermore, not only asymptomatic lesions but also symptomatic lesions can be used to

demonstrate DIS and DIT on MRI.[36] An important note comes with these criteria: they can be applied only when the clinical features are characteristic of MS and other diagnoses are excluded. The MRI scan of patients with other disorders, for example neuromyelitis optica, neurosarcoidosis or CNS lymphoma, can mimic the MRI features used in the MS criteria.[3] Table 1 shows the McDonald 2017 criteria for MS. These newly proposed criteria are validated in this thesis.
In some cases white matter abnormalities fulfilling the MRI criteria for MS are found as a random finding in people without typical MS symptoms. This is called radiologically isolated syndrome (RIS).[37,38] Multiple studies have shown that these people have a chance of 30-40% during a follow-up time of 2-5 years to experience a clinical attack, and therefore be diagnosed with MS.[37,39,40] Hence, this RIS patient group could be considered as at high risk for MS.

	McDonald 2017
DIS	a) Objective clinical evidence of ≥2 lesions, or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack involving a different CNS site b) MRI: ≥1 T2 lesions in at least 2 of 4 MS-typical regions of the CNS: Periventricular, (Juxta)cortical, Infratentorial, Spinal cord
DIT	a) ≥2 attacks separated by a period of at least month b) MRI: Simultaneous presence of gadolinium-enhancing and non-enhancing lesions at any time c) MRI: A new T2 and/or gadolinium-enhancing lesion on follow-up MRI, irrespective of its timing with reference to a baseline scan d) Demonstration of CSF-specific OCBs (as substitute for demonstration of DIT)

Table 1. *Overview of diagnostic criteria (2017) for DIS and DIT, based on the 2017 revisions to the McDonald criteria.[36] MS diagnosis is met when fulfilling DIS criteria (by fulfilling a or b) and DIT criteria (by fulfilling a, b, c or d). Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; OCB, oligoclonal bands*

Cerebrospinal fluid

Unique oligoclonal bands in CSF are a sign of a chronic humoral immune reaction in the CNS.[41] There is a variety of methods to establish OCBs, but a worldwide consensus is to use isoelectric focussing as the correct and standard method to determine OCBs.[42] Oligoclonal bands are not only found in MS. Processes triggering a B-cell response in other neuroimmunological ailments such as neurosarcoidosis, SLE or Behcet's disease may also lead to unique OCBs in CSF.[41] However, the negative predictive value of OCB for MS diagnosis is high, around 90% of patients with MS do have OCBs in CSF.[41,43]

Multiple studies have shown that OCB positivity in CIS patients predicts CDMS diagnosis and disability,[43] even when corrected for MRI measurements.[14, 44, 45] In patients with no T2 lesions on MRI, it is shown that presence of OCB increases the risk for CDMS from 4 to 23%.[46] An other study showed that 50% of MS patients who were initially negative for OCB converted to positive OCB during follow-up.[47] A large study in CIS patients found that the specificity of the 2010 DIS criteria increased from 81% to 88% when adding OCB to the criteria.[48] These results imply that MRI and OCB do complement each other.[49] This is the reason why OCB in CSF are included in the most recent criteria for MS.[36] When OCB are not present in CSF the diagnosis MS should be critically reconsidered.

Visual evoked potentials (VEP)

Visual evoked potentials are used to identify dysfunction of the optic nerve. VEP did not or only moderately improve diagnostic accuracy for MS diagnosis.[50-52] Therefore the diagnostic criteria for MS do not include VEP. However, VEP could be useful to diagnose optic neuritis.

Optical coherence tomography (OCT)

Optical Coherence Tomography is used to measure retinal thickness. This non-invasive technique produces two-dimensional images using low-coherence light.[53] In MS, the retinal nerve fibre layer (RNFL) is of particular interest. Since the retina is not myelinated, myelin is not interfering with OCT measurements. RNFL thickness is negatively correlated with disability and is found to be associated with brain atrophy.[54, 55] Therefore OCT is useful for assessing neuro axonal degeneration. Another extensive study showed that a thinner RNFL in eyes with no history of optic neuritis doubled the risk of disability worsening in the first 3 years and this risk was almost 4 times higher in the 4th and 5th year of follow-up.[56]

PATHOPHYSIOLOGY: NEURO-INFLAMMATION VERSUS NEURODEGENERATION

The exact pathophysiological processes leading to multiple sclerosis pathology have not yet been completely elucidated. Complex mechanisms with neuro-inflammatory and neurodegenerative components have been implicated.[57] Several studies have shown the crucial role for the adaptive immune system in the pathogenesis of MS.[58, 59] In many aspects the disease resembles other so called autoimmune diseases such as rheumatoid arthritis, type I diabetes and psoriasis. Furthermore, inflammatory genes are associated with MS risk and about two third of these genes show overlap with other autoimmune ailments.[60]
Nevertheless, any autoimmune ailment has its target tissue that may be more vulnerable to immune attacks than healthy individuals. Also the enormous differences in disease course between individual MS patients may be related to differences in vulnerability of their brain and/or spinal cord tissue. It is likely that different immune responses play distinct roles in the early versus the progressive phase of the disease.[59, 61] However, it is unclear whether inflammation and neurodegeneration are primary or

secondary processes and how these factors interplay over the disease course. Current therapies for MS are immunomodulating, and relapses are the main target of these MS therapies.[21] Yet, axonal damage is considered one of the major causes of persisting neurological disability.[62] The progressive phase of the disease makes that there is a controversy regarding the importance of relapses, nonetheless relapses early in the disease course do effect later disability and relapses are the main reason for disability in the early disease course.[63]

GENETIC, BIOLOGICAL AND ENVIRONMENTAL FACTORS

Genetic, biological and environmental factors play rolls in the risk for MS. These factors and their relation to MS risk are discussed below.

Latitudinal gradient

The prevalence of MS is unevenly distributed across populations around the world.[64] MS is more common in high-income countries in temperate zones such as Western Europe, North America, and Australia. In low-income countries in tropical zones the prevalence of MS is lower.[65 66] The risk of MS seems to be higher in areas further from the equator. The uneven distribution of MS reflects differences in both genetic predisposition and environmental risk factors.[66] However, there are some controversies about this latitudinal gradient theory. Some studies claim that the latitudinal gradient might be decreasing.[64, 67, 68] These results could be due to genetic factors, but also behavioural factors could be of influence.[66, 69] These controversies could further be the effect of the increased migration of people around the world. Yet, several studies revealed that migration from areas with a low MS risk to areas with a high MS prevalence leads to a higher risk of MS and that migrating from a high risk area to a low risk area results in a reduced risk of MS.[70-74] It has been shown that the second generation of migrants have almost the same MS risk as people in the new country of residence.[70, 71] A recent study showed that disability progression in North Africans living in France is more severe than disease progression in Caucasian patients living in France or North Africans living in North Africa. Resulting in a shorter time to reach higher EDSS scores, a higher relapse rate, and, especially in the second generation, a lower age of onset.[75] These results argue for the fact that the individual risk of MS seems to be mainly set during childhood and is importantly influenced by environmental factors.[76]

Genetics

The latitude theory pleads for a major influence of environmental factors for MS risk. However, genetic factors play an important role as well. Studies in MS patients and their families showed that 20% of MS patients have family members with MS.[77] The fact that the recurrence risk for siblings of MS patients is higher than the risk in the general population and that the concordance in monozygotic twins is higher than in dizygotic twins (25% vs 3%) points to a genetic contribution to the MS risk.[78-80] It seems unlikely that shared environmental factors are of influence on the MS risk in close biological relatives

of MS patients because adoptees of MS patients do not have a higher risk of MS.[81]
The main genetic locus of MS risk is the human leukocyte antigen (HLA) class II region (the classic HLA-DRB1*1501 allele).[82] Up to now over 200 non-HLA MS risk loci, all associated with a small risk of MS, are identified.[83] These loci mainly lie in genes with immunological functions.[60] MS risk seems to be modified by genetic and environmental factors and interactions between the two seems to be of influence here.[84]

Gender

The incidence and prevalence of MS has been increasing in the past decades. This increase is partly due to longer survival, but changes in lifestyle also seem to play an important role, particularly in women.[64, 85] The female/male ratio for MS has been increasing in the last decades.[86, 87] This gender effect mainly occurs early in the disease course, women have a higher relative risk of CIS,[88] but most studies did not find a difference between female and male CIS patients for the risk of developing MS.[14, 88]
A large study containing data from 18885 patients from 25 countries found a higher relapse rate in female patients compared to male patients.[89] However, male patients seem to accumulate disability faster than female patients.[90] In PPMS this difference in disability accumulation was not seen. The increasing female/male risk ratio for CIS can perhaps be explained by changes in lifestyle factors amongst women. There have been important lifestyle changes in past decades, for example in time spent outdoors, eating and drinking behaviour, smoking and obesity, but also life in areas with air pollution.[64] Some changes have been more specific to females, such as birth control and hormonal changes leading to lower age at menarche.

Age

The disease course of MS is influenced by age of onset. Several studies showed that patients who are younger at time of CIS have a higher risk for conversion to CDMS.[14, 44] A younger age of onset is also associated with good response to DMT.[22]
Patient age has been shown a more important determinant of decline in relapse incidence and the secondary progressive phase than disease duration.[89] Furthermore, relapse rate in the relapsing remitting phase of MS does not seem to influence the age of progression to SPMS.[91]

Environmental and clinical factors

Multiple environmental factors are associated with MS risk. These factors modulate disease presentation and therapeutic responsiveness.[92, 93] The most important environmental factors that influence the risk of MS are discussed here.

Vitamin D

An explanation for the above mentioned latitude theory might be the higher load of UV radiation and therefore higher levels of vitamin D in people who live around the

equator. Lack of UVB radiation has been shown to be associated with MS prevalence and lesion load on MRI.[94-96] Many studies found an association between low vitamin D levels and MS risk, both in the general population and in CIS patients.[97-100] Several studies showed that vitamin D measured before the first symptoms of MS is associated with a future MS diagnosis. A population based study in Denmark found that a low concentration neonatal vitamin D measured in dried blood spots samples is associated with an increased risk of MS.[101] A German study showed that in the 24 months before CIS, vitamin D levels were lower than in healthy controls who did not develop CIS.[102] Furthermore, a low vitamin D early in the disease course has been found to be a strong risk factor for long-term disease activity and disability and is associated with a higher relapse rate.[99, 103, 104] Genes involved in the vitamin D metabolism have been detected as risk factors for MS.[60] The main mechanism of action of vitamin D is immunomodulatory,[105] vitamin D supplementation showed multiple immunomodulatory effects.[106, 107] However, it is not exactly clear how vitamin D influences MS.

Pregnancy and hormonal factors

Pregnancy is suggested to be protective against MS disease activity,[108] with a reduced risk of MS during pregnancy.[109] Also the relapse rate seems to be lower especially during the third trimester of pregnancy, but after delivery an increase in relapse rate and an increase in number of MRI lesions is seen.[108, 110] In animal-models anti-inflammatory effects of female sex hormones are observed.[111] Yet, a randomised controlled trial and a cross-over study in women with RRMS did not show a lower relapse rate in the patient group who received a pregnancy-specific form of oestrogen compared to the placebo group.[112, 113] Another finding that argues against a major role for female sex hormones in the protection against MS is that oral contraceptives in the general population do not seem to correlate with a decreased risk of MS.[114-116] However, one of the above mentioned studies showed a lower load of gadolinium-enhancing lesions in patients who were treated with estradiol.[113] How pregnancy results in reduced neuroinflammatory activity in patients with RRMS remains a question to be solved.

Smoking

Several studies provided evidence that smoking results in an increased risk of MS.[117, 118] Smoking influenced the MS risk regardless of age of exposure. Both intensity and duration of smoking was associated with the risk of MS.[118] Not only cigarette smoking but also waterpipe smoking and passive smoking contributed to the risk of MS.[119, 120] Furthermore, it has been shown that smoking worsens the clinical course in MS patients and that smoking shortens the time to the secondary progressive phase of the disease.[121-123] Multiple studies showed that the negative effects slowly decrease after smoking cessation, this is independent of the number of pack years.[118, 121, 122] A Swedish population-based study found that patients who used moist snuff (a form of smokeless tobacco) were at lower risk for MS compared to patients who had never used moist snuff. This risk reduction was even seen in the tobacco smoking population. This indicates that nicotine is not causing the negative effect of smoking, and that nicotine

probably even causes immune-modulating effects that could protect against MS.[124] There are conflicting results about the association between smoking and MS risk in CIS patients. Only a few studies are available in CIS patients and these studies are hampered by methodologic issues.[125-129] The association of smoking and MS risk in CIS patients are described in this thesis.

Alcohol

More conflicting results are found on the effects of alcohol use on MS risk. Some studies suggest that moderate alcohol consumption has a protective effect on MS progression, and even reduces the harmful effect of smoking.[130, 131] But there are also studies showing that cessation of alcohol consumption could improve MS related disability.[132] However, a large cohort study and a meta-analysis of 10 studies did not show an association between alcohol consumption and MS risk.[133, 134] These conflicting results indicate that it is unclear whether alcohol consumption has a protecting or adverse effect on MS.

Hygiene hypothesis

One of the explanations why the increasing incidence of MS mainly occurs in high-income countries is a phenomenon that is called the 'hygiene hypothesis'.[135, 136] Advances in sanitation cause less exposure to infections in childhood, which may result in a higher prevalence of allergic and autoimmune diseases such as MS when encountering infections in adolescence.[136]

EBV virus

Presence of EBV antibodies is observed in almost 100% of MS patients.[137] Also a high EBV antibody titre is associated with risk of MS.[138] Several studies showed that EBV antibodies were increased already in the years before the first symptoms.[102, 138] In young adult patients primary EBV infection manifests in 25-70% as infectious mononucleosis, a clinical manifestation of a primary EBV infection.[139] Patients who have had infectious mononucleosis showed a more than two times higher risk of MS.[140] In both MS and control brain samples EBV infection was present. However, a higher number of EBV proteins has been shown in chronic and active MS plaques compared to controls.[141] It is of note that the prevalence of EBV in the general population is high (94%).[137] However, encountering the EBV virus seems essential for MS development.[140] These results suggest that EBV vaccination would be a potential option to prevent MS.[142] Yet, another possibility is that a shared genetic background contributes to the association between infectious mononucleosis and MS.[143, 144]

Obesity

A large study in 238.371 women found that a BMI of more than 30 kg/m^2 at the age of 18 years increased the risk of MS with a factor two compared to women with a BMI between 18.5 and 21 kg/m^2. In this kind of studies confounders such as smoking, vitamin D, and female sex hormones should be taken into account. In the mentioned study the

findings were corrected for age, latitude of residence, ethnicity, and smoking.[145] Multiple studies confirmed these results in female and in male patients.[146, 147]
Two studies showed that obesity in childhood increased the risk of both paediatric- and adult-onset MS and CIS.[148, 149] On the other hand, more recent studies found that rather adolescent obesity and not childhood obesity increased MS risk.[150, 151] Furthermore, obesity seems to interact with established MS genetic risk loci. A significant interaction is shown between a BMI higher than 27 and carrying 1 or 2 risk alleles of HLA-A*02.[152]
Also a recent study in two datasets found that a higher genetically induced BMI (using a weighted genetic risk score to predict BMI) predicted greater susceptibility to MS, which implies a causal effect of increased BMI on MS risk and an overlap in genetic pathways for obesity and MS.[153] Regarding treatment response, overweight MS patients showed more disease activity while treated with first-line treatment interferon beta than MS patients with a normal weight, implying an effect of BMI on interferon beta treatment response.[154] All these studies found a link between obesity and MS risk, however, it is not known if obesity predicts MS diagnosis in CIS patients. Since the prevalence of obesity is increasing, this may be one of the reasons that MS prevalence is increasing as well.

Salt intake

The main cause of the obesity epidemic is probably the Western diet, which contains increasing amounts of sugar, fat and salt. It has been shown that Th17 cells are more pathogenic under high-salt conditions, showing an upregulation of pro-inflammatory cytokines. The same study showed that a high-salt diet in mice led to a more aggressive course of EAE (the animal model of MS).[155] However, these mice received very high amounts of salt, the equivalent in humans would be 68 grams a day, this is 11 times the advised amount of salt.
Multiple studies showed contradictory results concerning the relation between high salt intake and multiple sclerosis. A study in 2015 showed higher relapse rates and more new lesions on MRI scan in MS patients with medium and high salt intake.[156]
However, more recent studies revealed no influence of salt intake on MS risk in the general population nor in CIS patients.[157, 158] Also two paediatric studies, both performed in 2016, did not show an influence of high salt intake on MS diagnosis or on time to the next relapse.[159, 160] These recent results plead against the former theories about the negative effect of salt on MS disease course.

Stress

There are discordant results concerning the correlation between stress and MS risk.[161]
Most studies show that stressful life events are associated with MS risk and a higher relapse rate and that stress can bring back previous symptoms.[162-164]
However, in a nationwide Danish cohort study no evidence was found for a causal association between well-defined severe stressful life events and MS risk.[165]
Of note is that in this kind of research multiple quality issues arise, such as selection and blinding problems, correction for psychosocial factors and a large heterogeneity in stress measurements.[164]

BIOMARKERS

Finding new biomarkers is an important topic in MS research. There is a need for biomarkers early in the disease course to help diagnosing MS accurately as early as possible and prevent unnecessary treatment, but also for disease stratification, predicting long-term prognosis and predicting and monitoring treatment response.[166, 167] These markers are crucial to provide more individualized care. Up to now multiple biomarkers are identified and have the potential to become clinically useful.[168] A few of the biomarkers that are validated for predicting MS in CIS patients are discussed below.

Chitinase 3 like 1 (CHI3L1) in CSF

Chitinase 3 like 1 was first identified using a proteomic approach.[169] Increased CHI3L1 levels has been associated with a future MS diagnosis in patients with CIS. This finding is validated in independent cohorts of CIS patients.[170, 171] CHI3L1 was not only a predictor for MS diagnosis but also a risk factor for future disability.[170, 172]

Neurofilament light chain (NfL) in serum and CSF

A promising biomarker for axonal damage is neurofilament light chain (NfL).[173] High NfL levels in CSF are associated with MS diagnosis in CIS and RIS patients.[174, 175] Also serum NfL levels were higher in CIS and MS patients compared to healthy controls and associated with disability.[176-178] Serum is a more accessible body fluid than CSF. The correlation between CSF and serum NfL makes serum NfL a promising biomarker to monitor axonal damage longitudinally.

CXCL13

CXCL13 is a chemokine that is involved in B-cell maturation and migration.[179] High CXCL13 levels in CSF are associated with MS diagnosis in CIS patients, relapses and OCBs in CSF. [180, 181]

Kappa and lambda free light chains (KFLC and LFLC)

In case of intrathecal B cell activity, plasma cells secrete free light chains. Kappa free light chains CSF-serum ratios were higher in CIS and MS patients than in controls.[182] Moreover, a lower KFLC/LFLC CSF ratio was associated with a future CDMS diagnosis in CIS patients.[183] whether KFLC is superior to OCB in terms of sensitivity and specificity is not clear.[182, 184]

MULTIPLE SCLEROSIS IN CHILDHOOD

As has been mentioned before, MS also occurs in children. Around 3-5% of MS patients have the first attack during childhood.[4, 5] The differential diagnosis in children with suspected MS is more extensive than in adults.[185, 186] Children with MS tend to have

a higher relapse rate, with more severe attacks than adults with MS.[187, 188] Another sign of higher inflammatory activity is a higher lesion load on MRI in children than in adults.[189, 190] Despite this more inflammatory disease course, disease progression is slower in children. A possible hypothesis for this is that the developing CNS of children has more plasticity to recover.[191] Although progression in childhood-onset multiple sclerosis takes longer, a stage of irreversible disability is reached at a younger age.[191, 192] Furthermore, PPMS in children is rare.[4] Because of these differences it is debated whether adulthood onset MS and childhood onset MS reflects the same disease.[193] The criteria for childhood onset MS are revised in 2012 based on the McDonald 2010 criteria for MS in adulthood. These criteria are developed by the International Paediatric MS Study Group (IPMSSG).[194] In this thesis we compared the clinical features at onset, time to MS diagnosis, relapse rate, and disability between childhood-onset and adulthood-onset CIS and MS.

PROUD STUDIES

From the Rotterdam MS centre ErasMS, two prospective studies in patients who are followed after a first attack of demyelination are coordinated: the PROUD (Predicting the Outcome of a Demyelinating event) study and the PROUD-kids study. Patients are included in Erasmus MC and several collaborating regional hospitals. Both studies have the same prospective study design, giving the opportunity to find markers for predicting long-term prognosis and treatment response to provide a more individualized care.[22] The first cohort consists of adult CIS patients and the second cohort consists of children with a first attack of acquired demyelinating syndromes (ADS). This gives the unique opportunity to compare disease course, biomarkers and clinical factors between paediatric and adult patients with a first attack of possible MS. Patients are included within six months after a first attack of demyelination if they or their families signed an informed consent form. Neurological examination, blood samples, a lumbar puncture (if clinically indicated) and MRI scans are performed at baseline. All patients are included at time of the first attack of demyelination and after that reassessed regularly. The studies described in this thesis are executed within these two patient cohorts.[195-200]

SCOPE OF THIS THESIS

The first attack of MS often occurs in young adults in the prime of their lives. Especially in this young patient group, adequate counselling about their prognosis is important. Furthermore, the increasing amount of available immunomodulatory therapies that could be administered even before MS diagnosis emphasizes the need for better prediction at an early phase of this heterogeneous disease. Adequately predicting disease activity is essential to prevent unnecessary treatment of patients with a benign disease course. This thesis focuses on defining prognostic factors for better prediction of clinical disease activity in adults with CIS and in children with a first attack of ADS.

The first part of this thesis describes prognostic factors in adult patients with CIS.
In **chapter 2**, the new McDonald 2017 criteria are evaluated and compared to the former 2010 McDonald criteria by applying both criteria to our prospective CIS cohort.
Fatigue is a common symptom in patients with MS and CIS. However, not much is known about the course of fatigue after a first attack of demyelination. **Chapter 3** describes the course of fatigue after CIS. In **chapter 4**, the predictive value of the immunological biomarker soluble CD27 (sCD27) for CDMS in CIS patients is evaluated. **Chapter 5** zooms in on distinct effector phenotypes of Th17 cells as key regulators of MS onset. We evaluated the correlation of these cells with disease activity in CIS and MS and examined the association with natalizumab treatment response.
Smoking is a well-established factor that influences disease course of patients with MS. However, there are conflicting results about the association of smoking and MS risk in CIS patients. In **chapter 6**, we aimed to determine the risk of CDMS in smoking and non-smoking patients at time of CIS.

The second part of this thesis focuses on the search of prognostic markers for disease course in children with ADS. We compared our prospective cohort of children with ADS to our prospective cohort of adult CIS patients.
In **chapter 7**, we compared multiple clinical parameters, time to MS diagnosis and relapse rate between adults and children after CIS. The above described biomarker sCD27 is in **chapter 8** evaluated in children with ADS. Another promising biomarker is neurofilament light chain (NfL), a marker for axonal damage. It is shown that NfL in CSF predicts MS diagnosis in adult patients with CIS, in children this was not yet investigated. In **chapter 9**, we compared NfL levels between paediatric and adult CIS patients and explored the predictive value of NfL levels for CDMS diagnosis. Finally, the key findings of this thesis and implications for future research are summarized and discussed in **chapter 10**.

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Part I

Disease course after clinically isolated syndrome in adults

Chapter 2

Application of the 2017 revised McDonald criteria for multiple sclerosis to patients with a typical clincally isolated syndrome

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ABSTRACT

Importance: Recently, the International Panel on Diagnosis of Multiple Sclerosis revised the McDonald 2010 criteria for the diagnosis of multiple sclerosis (MS). The new criteria are easier to apply and could lead to more and earlier diagnoses. It is important to validate these criteria globally for their accuracy in clinical practice.

Objective: To evaluate the diagnostic accuracy of the 2017 criteria versus the 2010 criteria to predict clinically definite MS (CDMS) in patients with a typical clinically isolated syndrome (CIS).

Design, setting and patients: 251 Patients fulfilled the inclusion criteria. 13 Patients received another diagnosis early in the diagnostic process and therefore were excluded from the analyses. 9 CIS patients declined to participate in the study. This left 229 patients who were included between March 2006 and August 2016 in this prospective CIS cohort. Patients underwent a baseline MRI scan within three months after onset of symptoms and, if clinically required, a lumbar puncture was performed.

Main outcome and measures: Sensitivity, specificity, accuracy, positive and negative predictive value were calculated after 1, 3 and 5 years for the 2017 versus the 2010 criteria.

Results: Among the 229 CIS patients, 167 were female (73%). The mean (SD) age was 33.5 (8.2) years. Hundred and thirteen patients (49%) were diagnosed with CDMS during a mean (SD) follow-up time of 65.3 (30.9) months. Sensitivity for the 2017 criteria was higher than for the 2010 criteria (68%; 95% CI, 57-77%, vs 36%; 95%CI, 27-47%; p<0.001), but specificity was lower (61%; 95% CI, 50-71% vs 85%; 95%CI, 76-92%; p<0.001). Using the 2017 criteria more MS diagnoses could be made at baseline (97 (54%; 95%CI, 47-61%) vs 46 (26%; 95%CI, 20-32%) p<0.001). In the group with at least 5 years of follow-up, 33% of patients who were diagnosed with MS using the 2017 criteria did not experience a second attack during follow-up versus 23% when using the 2010 criteria.

Conclusion and relevance: The 2017 revised McDonald criteria are more sensitive but less specific for a second attack than the previous 2010 criteria. The trade-off is that it leads to a higher number of MS diagnoses in patients with a less active disease course.

KEY POINTS

Question What is the diagnostic accuracy of the 2017 criteria versus the 2010 criteria to predict clinically definite MS (CDMS) in patients with a typical clinically isolated syndrome (CIS)?

Findings This study included 229 patients with a clinically isolated syndrome. The sensitivity of the revised McDonald 2017 criteria was higher than for the 2010 criteria, and specificity was lower for the 2017 criteria.

Meaning The 2017 revision of the McDonald MS criteria leads to a higher number of MS diagnoses in patients with a less active disease course.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) with substantial heterogeneity in severity and prognosis.[1]
In 85% of patients, MS starts with a clinically isolated syndrome (CIS), a first clinical episode of CNS demyelination.[2] CIS can remain a single event, but can also be followed by the relapsing disease MS. MS is diagnosed based on clinical or MRI evidence of dissemination in space (DIS) and time (DIT). The diagnostic criteria for MS evolved over the years to diagnose MS earlier and more easily.[3-6]
Up to now, MS can be diagnosed when a typical CIS is followed by a new clinical event or when there are new lesions on T2 weighted images on a follow-up MRI scan. Since the McDonald 2010 criteria, MS can also be diagnosed based on a single baseline MRI scan showing asymptomatic contrast enhancing lesions.[6]
Disease-modifying therapies (DMTs) can be administered early in the disease course, even at time of CIS. DMTs can delay a second attack after CIS,[7-9] and have potential to prevent future disability.[10, 11] However, DMTs have side effects. To select patients for early treatment, it is important to predict accurately who will develop a relapsing disease course and who will not.[12] Accurate diagnostic criteria are therefore essential.
Recently, new diagnostic criteria have been proposed for MS in patients with a typical CIS.[13] These criteria are easier to apply than the McDonald 2010 criteria (Table 1). The most important addition is that the new criteria allow MS diagnosis when the MRI scan meets criteria for DIS and unique oligoclonal bands (OCB) are present in CSF, even in absence of DIT on the MRI scan. The other major difference is that not only asymptomatic but also symptomatic lesions can be used to demonstrate DIS and DIT on MRI. Furthermore, cortical lesions can be used to demonstrate dissemination in space.[13]
These revisions will presumably lead to a higher number of MS diagnoses at time of CIS. However, the revised criteria may be accompanied by a higher rate of false positive diagnosis. Therefore, it is of great importance to apply these criteria to available cohorts of CIS patients and validate their accuracy in clinical practice.
We aimed to evaluate the diagnostic accuracy of these novel criteria.

	McDonald 2010	McDonald 2017
DIS	a) Objective clinical evidence of ≥2 lesions, or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack involving a different CNS site b) ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS: Periventricular Juxtacortical Infratentorial Spinal cord **(symptomatic lesions in patients with brainstem or spinal cord syndrome are excluded)**	a) Objective clinical evidence of ≥2 lesions, or objective clinical evidence of 1 lesion with reasonable historical evidence of a prior attack involving a different CNS site b) ≥1 T2 lesion in at least 2 of 4 MS-typical regions of the CNS: Periventricular **(Juxta)cortical** Infratentorial Spinal cord
DIT	a) ≥2 attacks separated by a period of at least 1 month b) Simultaneous presence of **asymptomatic** gadolinium-enhancing and non-enhancing lesions at any time c) A new T2 and/or gadolinium-enhancing lesion on follow-up MRI, irrespective of its timing with reference to a baseline scan	a) ≥2 attacks separated by a period of at least 1 month b) Simultaneous presence of gadolinium-enhancing and non-enhancing lesions at any time c) A new T2 and/or gadolinium-enhancing lesion on follow-up MRI, irrespective of its timing with reference to a baseline scan **d) Demonstration of CSF-specific OCBs (as substitute for demonstration of DIT)**

Table 1. McDonald 2010 and 2017 criteria

Overview of diagnostic criteria (2010 and 2017) for DIS and DIT. Based on the 2010 revisions to the McDonald criteria and 2017 revisions to the McDonald criteria.

Abbreviations: DIS, dissemination in space; DIT, dissemination in time; CNS, central nervous system; MRI, magnetic resonance imaging; CSF, cerebrospinal fluid; OCB, oligoclonal bands.

METHODS

Study participants

Patients with a suspected first episode of MS were included in our prospective cohort of CIS patients (Predicting the Outcome of a Demyelinating event, PROUD study), an multicentre study in Erasmus MC in Rotterdam, a tertiary referral centre for MS patients (MS Centre ErasMS) in collaboration with several regional hospitals. Patients were between 18 and 50 years of age and were included between March 2006 and August 2016, within 3 months after onset of clinical symptoms, and with at least 1 year of follow-up. Patients were assessed at baseline and reassessed annually. No patients with a previous history of neurological symptoms suggestive for CNS demyelination were included. Patients underwent baseline MRI and routine laboratory tests to rule out other possible diagnoses. When an alternative diagnosis was made, the patient was excluded from analyses.

Standard protocol approvals and patient consents

The study protocol was approved by the Ethics Committee of Erasmus MC Rotterdam and of the other participating centres. All patients provided written informed consent.

Definitions

A relapse was defined as new symptoms of neurological dysfunction or subacute worsening of existing symptoms after 30 days of improvement or stable disease and no evidence of an alternative diagnosis.[14] Symptoms had to exist for longer than 24 hours and not be preceded by fever. Exacerbations were confirmed by neurological examination. CDMS was defined as clinical dissemination in space and time as described by Poser et al.[3]

Procedures

MRI scans were performed on a 1.5T magnet with a standard head coil (Philips, Best, The Netherlands, or General Electric, Milwaukee, WI, USA). Images were obtained in the axial plane and the following pulse sequences were used: T1-weighted conventional spin-echo(SE), SE proton-density-weighted (PDW), T2 weighted SE, and/or fluid-attenuated inversion recovery (FLAIR) sequence, with 2-5mm images.

Patient selection

All baseline MRI scans were scored for DIS using the McDonald 2010 criteria and the 2017 revisions to the McDonald criteria (n=229). To evaluate the 2010 and 2017 criteria for DIT and DIS+DIT, we selected patients who had a baseline MRI scan that included T1 images after gadolinium administration or scans that did not show any T2 hyper intense lesions (n=180).

We performed sub-analyses for patients of whom we had data on OCB (n=124), patients with baseline spinal cord MRI available (n=79), and patients who were not treated with DMT before CDMS diagnosis (n=135).

Statistical analyses

For statistical analyses we used SPSS software, version 24.0 (SPSS Inc) and GraphPad Prism5. Patients who fulfilled the diagnostic criteria at time of the first attack and were diagnosed with CDMS during follow-up were considered as true positives (TP). False positives (FP) were defined as fulfilling the diagnostic criteria for MS at baseline but not diagnosed with CDMS during follow-up. Patients who did not fulfil the diagnostic criteria and who were not diagnosed with CDMS during follow-up were considered as true negatives (TN) and patients who did not fulfil the diagnostic criteria at baseline but were diagnosed with CDMS were considered as false negatives (FN).

The following ratios were calculated:
Sensitivity: [TP / (TP+FN)] x100
Specificity: [TN / (TN+FP)] x100
Positive predictive value (PPV): [TP / (TP+FP)] x100
Negative predictive value (NPV): [TN / (TN+FN)] x100
Accuracy: [(TP + TN)/(TP + TN + FP + FN)] x100

Ratios were calculated with a 95% confidence interval (CI), for DIS, DIT (with and without oligoclonal bands) and MS diagnoses on baseline MRI scans using the 2010 and 2017 McDonald criteria after 1, 3 and 5 years of follow-up. We compared sensitivity and specificity between the 2010 and 2017 criteria using McNemar's test.
For group comparison of continuous parametric variables, we used 2-tailed t test and for non-parametric data Mann-Whitney U test. For categorical data, we applied Chi-square. Time to diagnosis using the 2010 and 2017 criteria and time to CDMS were analyzed using Kaplan-Meier curves and compared using log-rank test. Hazard ratios for time to CDMS were calculated using COX proportional hazard regression analysis. Patients without a second attack during follow-up were considered as censored observations. P-values less than 0.05 were considered significant.

RESULTS

Patient characteristics

In total 251 patients fulfilled the inclusion criteria, 13 patients were diagnosed with other diagnoses than MS, all <3 months of follow-up (neuromyelitis optica, spinal cord tumor, chronic relapsing inflammatory optic neuropathy, meningioma, vascular, sarcoidosis, vitamin B12 deficiency, psychogenic). Nine patients declined to participate in the study. After these exclusions, 229 CIS patients were eligible for analysis. All patients had at least 1 year follow-up time.
One hundred thirteen patients (49%) were diagnosed with CDMS during a mean (SD)

follow-up of 65.3 (30.9) months. Median (IQR) time to CDMS diagnosis was 23.4 (10.2-45.3) months. Fifty-five patients (24%) were treated with DMT before CDMS diagnosis. The median (IQR) time between onset of symptoms and baseline MRI scan was 3.7 weeks (1.6-6.3). One hundred eighty (79%) had MRI scans with post-gadolinium images available or scans that did not show any abnormalities. Baseline spinal cord MRI was performed in 107 patients (47%), of whom 78 (73%) had spinal cord symptoms. In 148 patients(65%) oligoclonal bands were assessed. Table 2 shows the baseline characteristics of the included patients.

Characteristic	CIS-patients (n=229)	CDMS (n=113)	Monophasic (n=116)	p-value[a]
Female sex, no. (%)	167 (72.9)	85 (75.2)	82 (70.7)	0.44
Age[b], mean (SD), years	33.5 (8.2)	32.2 (7.7)	34.8 (8.4)	0.01
Follow-up time, mean (SD), months	65.3 (30.9)	73.1 (29.3)	57.6 (30.5)	<0.01
Clinical syndrome type, no. (%)				
-optic nerve	88 (38.4)	41 (36.3)	47 (40.5)	0.51
-brainstem	44 (19.2)	29 (25.7)	15 (12.9)	0.01
-spinal cord	79 (34.5)	37 (32.7)	42 (36.2)	0.94
-cerebral hemispheres	39 (17.0)	21 (18.6)	18 (15.5)	0.54
-cerebellar	14 (6.1)	9 (8.0)	5 (4.3)	0.25
DMT at time of CIS no. (%)	55 (24.0)	35 (31.0)	20 (17.2)	0.02
OCB, (> 1 band), (n=148) (%)	111 (75.0)	65 (83.3)	46 (65.7)	0.01
Time to baseline MRI, median (IQR)	4.0 (1.7-6.9)	4.3 (2.0-7.5)	3.6 (1.6-6.3)	0.14
MS 2010 criteria, no. (%)	46 (20.1)	33 (29.2)	13 (11.2)	<0.01
MS 2017 criteria, no. (%)	110 (48.0)	73 (64.6)	37 (31.9)	<0.01

Table 2. Patient characteristics

[a] P value calculated between CDMS and Monophasic

[b] Age at symptom onset

Abbreviations: CIS, clinically isolated syndrome; CDMS, clinically definite multiple sclerosis; SD, standard deviation; DMT, disease-modifying therapies; OCB, oligoclonal bands; MRI, magnetic resonance imaging; IQR, interquartile range.

DIS and DIT criteria at baseline

The 2010 criteria for DIS were fulfilled for 124 of 229 patients (54%). Of them 74 (60%) experienced a second relapse (CDMS) during follow-up. One hundred forty-nine patients (65%) fulfilled the 2017 criteria for DIS and 89 (60%) were diagnosed with CDMS.
To evaluate the 2010 and 2017 criteria for DIT we selected patients who had a baseline MRI scan including post-gadolinium T1 images (n=180).
The 2010 criteria for DIT were fulfilled for 46 of 180 patients (26%). Thirty-three of these

patients (72%) were diagnosed with CDMS. Hundred and twenty-six of 180 patients (70%) fulfilled DIT according to the 2017 criteria. Of them 76 (60%) experienced a second attack. Table 3 shows sensitivity, specificity, and accuracy with 95%CI for DIS and DIT according to the 2010 and 2017 criteria.

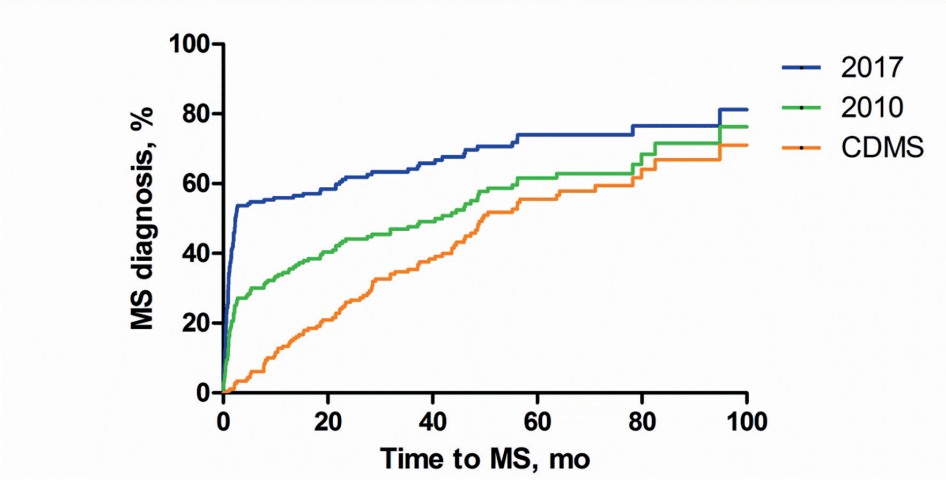
	DIS (n=229)	**DIT** (n=180)	**DIS+DIT** (n=180)
2010			
Sensitivity (95%CI)	66 (56-74)	36 (27-47)	36 (27-47)
Specificity (95%CI)	57 (47-66)	85 (76-92)	85 (76-92)
Accuracy (95%CI)	61 (55-67)	61 (54-68)	61 (54-68)
Hazard ratio (95%CI)	2.0 (1.3-2.9)	1.9 (1.2-2.9)	1.9 (1.2-2.9)
2017			
Sensitivity (95%CI)	79 (70-86)	84 (74-90)	68 (57-77)
Specificity (95%CI)	48 (39-58)	44 (34-55)	61 (50-71)
Accuracy (95%CI)	63 (57-70)	64 (57-71)	64 (57-71)
Hazard ratio (95%CI)	2.7 (1.7-4.2)	2.6 (1.5-4.6)	2.0 (1.3-3.1)

***Table 3**. Test characteristics for the 2010 and 2017 McDonald criteria*
Abbreviations: DIS, dissemination in space; DIT, dissemination in time; 95%CI, 95% confidence interval; PPV, positive predictive value; NPV, negative predictive value

2010 vs 2017 revised criteria

Using the McDonald 2010 criteria, 46 of 180 patients with CIS (26%; 95%CI, 20-32%) were diagnosed with MS at baseline. When using the 2017 criteria, 51 more patients are diagnosed with MS at baseline: 97 patients (54%; 95%CI, 47-61%) (p<0.001). Thirty-three of 46 patients with MS (72%) using the 2010 criteria and 62 of 97 patients with MS (64%) using the 2017 criteria had a second attack during follow-up. Resulting in a sensitivity of 36%; 95%CI, 27-47%; (2010) vs 68%; 95%CI, 57-77% (2017) (p<0.001) and a specificity of 85%; 95%CI, 76-92%; (2010) vs 61%; 95%CI, 50-71% (2017) (p<0.001). Table 3 shows sensitivity, specificity, and accuracy for the 2010 and 2017 criteria.
For patients with at least five years follow-up (n=88), 14 of 22 patients (64%) diagnosed with MS using the 2010 criteria had a second attack before year five, for the 2017 criteria 26 of 48 (54%).
This implies that 22 of 48 patients (46%), with at least 5 year follow-up and diagnosed with MS at baseline using the 2017 criteria were not yet diagnosed with CDMS after five years. For the 2010 criteria this were 8 of 22 patients (36%). For the total follow-up time in the group with at least 5 year follow-up, these numbers were 16 of 48 (33%) (2017) and 5 of 22 (23%) (2010). Sensitivity, specificity, PPV, NPV and accuracy at one, two, five years and for the total follow-up time for DIS, DIT for the 2010 and 2017 criteria are shown in Supplementary Table 1.

Figure 1 shows the survival curves for CDMS according to the poser criteria,[3] the McDonald 2010 and the revised 2017 criteria. MS diagnosis is made earlier using the 2017 compared to the 2010 criteria (p<0.001). Table 3 shows the hazard ratios for DIS, DIT and DIS+DIT for the 2010 and 2017 criteria.



No. at risk						
2017 criteria	180	62	39	24	10	3
2010 criteria	180	96	65	38	12	3
CDMS	180	128	81	46	16	5

Figure 1. Time from CIS to CDMS, McDonald 2010, and 2017 MS

Survival curves for time from clinically isolated syndrome to multiple sclerosis for CDMS, the 2010 and revised 2017 criteria. Abbreviations: CDMS, clinically definite MS

Contribution of OCB and symptomatic enhancing lesions separately

Using the new 2017 criteria, twice as many patients could be diagnosed with MS at baseline compared to the 2010 criteria (97 vs 46 patients). In 32 (63%) of these extra MS diagnoses the diagnosis could be made based on DIT fulfilment with OCB in CSF. Of these 32 patients, 47% was not diagnosed with CDMS during follow-up. The other major difference between the criteria is that not only asymptomatic but also symptomatic lesions can be used to demonstrate DIS and DIT on MRI. This led to the other 19 (37%) more MS diagnosis at baseline. Of these patients, 31% had no second attack.

Sub-analyses

For all sub-analyses we selected patients who had a baseline MRI scan that included T1 images after gadolinium administration (n=180).

Patients with known OCB status were selected for the first sub-analysis (n=124, 69%). Sensitivity for the 2010 versus the 2017 criteria was respectively: 32% (95% CI, 21-45%) and 70% (95% CI, 57-80%) (p<0.001). The specificity was: 75% (95% CI, 55-89%) vs 53% (95% CI, 40-67%) (p<0.001). In 79 of 180 patients (44%), a baseline spinal cord MRI was performed. Here sensitivity for the 2010 criteria was 29% (95% CI, 16-45%) and for the 2017 criteria: 72% (95% CI, 58-86%) (p<0.001). The specificity was 84% (95% CI, 67-93%) vs 49% (95% CI, 32-65%) (p<0.001)
Because DMT could have postponed a second attack, we performed another sub-analysis excluding the patients treated with DMT before CDMS diagnosis, leaving 135 of 180 patients (75%) eligible for analyses. Here sensitivity for the 2010 criteria was 27% (95% CI, 17-40%) and for the 2017 criteria: 58% (95% CI, 45-70%) (p<0.001). Specificity was 90% (95% CI, 81-96%) vs 73% (95% CI, 61-82%) (p<0.001)

DISCUSSION

We evaluated the accuracy of the 2017 revisions of the McDonald criteria versus the McDonald 2010 criteria to predict CDMS at the moment of a first demyelinating attack. The criteria for DIS and DIT used in the 2010 and 2017 criteria were applied to a prospective cohort of 229 CIS patients during a mean follow-up of 5.4 years.
We observed higher sensitivity for the 2017 criteria than for the 2010 criteria (68% vs 36%). However, as expected, specificity for the 2017 criteria was lower (61% vs 85%). The accuracy did not differ significantly (accuracy: 61% (2010) and 64% (2017)). Accuracy of the McDonald 2010 criteria was similar to earlier studies validating the 2010 criteria in CIS cohorts.[15-18]
High sensitivity of diagnostic criteria is important to allow earlier initiation of DMT, which has been shown beneficial for disease outcome.[7, 9, 10] On the other hand, incorrect diagnoses and unnecessary treatment should be avoided. In our data, specificity of the 2017 criteria was significantly lower than for the 2010 criteria. Earlier data showed that the previous McDonald criteria lead to a higher number of MS diagnoses in patients who will not have a second attack.[19] When new less strict criteria are introduced, the Will Roger's phenomenon could be observed.[20] This refers to the statistical observation that when a boundary on a scale is moved to the left, the outcome for both patient groups improves. More CIS patients will be moved to the MS group, therefore the MS group will probably have a more favourable outcome with a lower attack frequency. This makes comparisons between different time periods in MS research challenging, since the overall prognosis for MS patients is getting better.
With these new MS criteria, MS diagnosis includes a single CIS attack without subsequent clinical disease activity and therefore comes even closer to the group with radiologically isolated syndrome (RIS). Patients with RIS cannot be diagnosed with MS using the current criteria. Since DMTs can delay a second attack in patients with CIS and have a potential to prevent future disability, the discussion comes closer to whether RIS patients who are at high risk for future attacks and already have cognitive problems[21] should also be diagnosed with MS and be treated with DMT.
We found that after five years of follow-up the number of patients fulfilling the criteria, but with no second attack was higher for the 2017 than for the 2010 criteria

(46% vs 36%). However, in the period after these five years of follow-up, eight more patients were diagnosed with CDMS. Of them six fulfilled the 2017 criteria at baseline, and three fulfilled the 2010 criteria. This shows that a longer follow-up will probably lead to a higher positive predictive value of the criteria.
Another potential explanation for the high number of patients that fulfilled the criteria but did not experience a second event is treatment with DMT before CDMS diagnosis. This could have postponed or prevented a second attack and therefore lowered the specificity of the criteria. We did not exclude patients who used DMT before CDMS diagnosis, this could have introduced selection bias. Instead of exclusion, we performed a separate analysis with the group that did not receive DMT before second attack. In this sub-analysis the specificity increased for both criteria. But after at least five years of follow-up, even in this untreated group, 4 of 15 (27%) patients who fulfilled the 2010 criteria and 11 of 33 (33%) patients who fulfilled the 2017 criteria did not experience a second attack.
Sixty-three % Of the extra MS diagnoses using the new diagnostic 2017 criteria were made based on OCB in CSF, 47% of these patients did not experience a second attack. The other 37% of new MS diagnoses could be made based on demonstrating DIS or DIT with asymptomatic enhancing lesions on MRI, 31% of these did not experience a second attack. Especially inclusion of OCB in the 2017 criteria seems to contribute to the lower specificity.
There are some limitations to this study. Although there was a sufficient follow-up to draw conclusions (mean FU-time: 5.4 years), the range is rather wide (SD: 2.6 years). The possibility remains that after further follow-up, additional patients will have a second attack. However, total number of patients in this study was large enough to allow sub-analyses for different minimum follow-up times and this demonstrated maintenance of accuracy together with a limited decrease of sensitivity (Supplementary Table 1).
Secondly, not all patients underwent a baseline spinal cord MRI scan and lumbar puncture. Though it is known to increase sensitivity,[22, 23] it is not common clinical practice to include spinal cord MRI in the routine diagnostic work-up when there are no spinal cord symptoms. Also a lumbar puncture was not always clinically required and we did not have ethical permission for obtaining CSF for research purposes alone. Therefore, the decision to perform a lumbar puncture or a spinal cord MRI was not random. To avoid selection bias, we did not exclude the patients who did not have CSF or spinal cord MRI data available. In this way, our findings are more applicable to the general clinical practice. However, we did perform sub-analyses and specificity and sensitivity did not differ from the total group.
Thirdly, though applied in most studies on MS criteria for CIS patients,[24-26] the use of sensitivity, specificity, NPV and PPV is somewhat problematic. They imply reference to a gold standard. Obviously, in this patient group we do not have neuropathological information. Earlier studies used pathologically confirmed CDMS cases to demonstrate that the highest rate of correct diagnoses could be made when using the Poser CDMS criteria.[27, 28] Therefore we accepted the use of CDMS as a proxy to pathological evidence to confirm MS diagnosis.
Lastly, in the 2017 criteria, cortical lesions are added as an extra parameter to show DIS. Advanced MRI techniques are required to visualize cortical lesions. These techniques are

hardly available in routine clinical practice,[13] therefore at present, we cannot study the contribution of this parameter. A recent study, in which the MAGNIMS 2016 MRI criteria were validated in a large retrospective CIS cohort, showed that inclusion of cortical lesions did not affect DIS criteria performance.[23, 25]
In conclusion, the 2017 revisions to the McDonald criteria are more sensitive than the previous 2010 criteria. Therefore, the new diagnostic criteria will probably increase the proportion of MS diagnoses. However, the specificity is significantly lower when applied to our cohort of CIS patients, leading to a higher number of MS diagnoses with a less active disease course, at least in the first years after onset.

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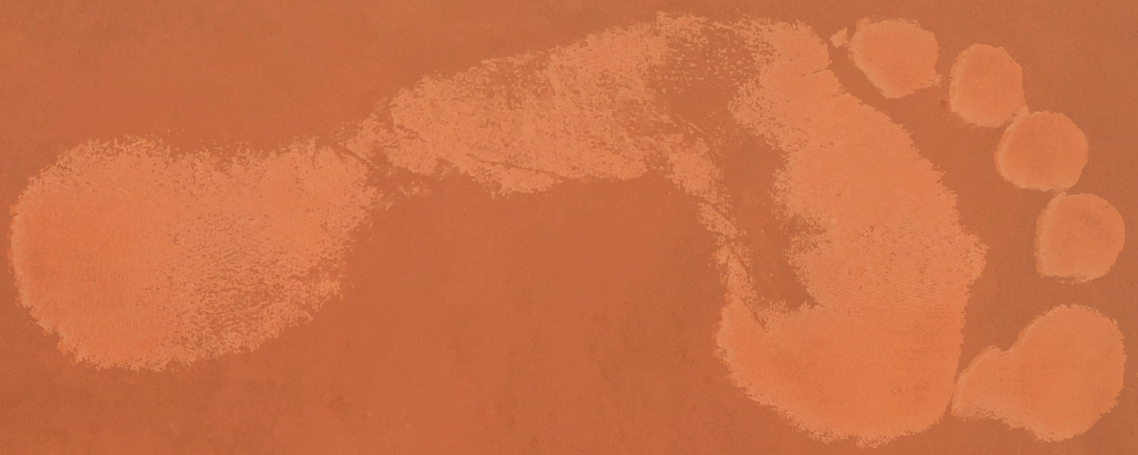
	DIS 2010 (n=229)	DIS 2017 (n=229)		DIT 2010 (n=180)	DIT 2017 (OCB excluded) (n=180)	DIT 2017 (OCB included) (n=180)	DIS+DIT 2010 (n=180)	DIS+DIT 2017 (OCB excluded) (n=180)	DIS+DIT 2017 (OCB included) (n=180)
Sensitivity (95%CI)			**Sensitivity (95%CI)**						
1yr (n=229)	71 (52-85)	84 (66-94)	1yr (n=180)	33 (16-55)	58 (37-77)	96 (77-100)	33 (16-55)	58 (37-77)	79 (57-92)
2yr (n=208)	72 (58-83)	83 (70-92)	2yr (n=159)	31 (18-47)	50 (34-66)	83 (68-93)	31 (18-47)	50 (34-66)	67 (50-80)
5yr (n=125)	61 (48-74)	75 (62-86)	5yr (n=88)	33 (20-49)	49 (34-64)	81 (66-91)	33 (20-49)	47 (32-62)	61 (45-75)
Total fu (n=229)	66 (56-74)	79 (70-86)	Total fu (n=180)	36 (27-47)	51 (40-61)	84 (74-90)	36 (27-47)	50 (39-60)	68 (57-77)
Specificity (95%CI)			**Specificity (95%CI)**						
1yr (n=229)	49 (41-56)	38 (31-45)3	1yr (n=180)	76 (68-82)	65 (57-73)	34 (27-42)	76 (68-82)	67 (59-75)	50 (42-58)
2yr (n=208)	49 (41-58)	9 (31-47)	2yr (n=159)	75 (66-83)	65 (56-73)	33 (25-42)	75 (66-83)	68 (58-76)	47 (38-56)
5yr (n=125)	56 (43-68)	49 (36-61)	5yr (n=88)	82 (67-92)	71 (56-83)	40 (26-56)	82 (67-92)	73 (32-62)	51 (36-66)
Total fu (n=229)	57 (47-66)	48 (39-58)	Total fu (n=180)	85 (76-92)	75 (65-84)	44 (34-55)	85 (76-92)	78 (67-85)	61 (50-71)
PPV (95%CI)			**PPV (95%CI)**						
1yr (n=229)	18 (12-26)	17 (12-25)	1yr (n=197)	17 (83-32)	21 (12-32)	18 (12-26)	17 (8.3-32)	22 (13-34)	20 (13-29)
2yr (n=208)	33 (25-43)	32 (25-41)	2yr (n=159)	31 (18-47)	34 (23-47)	31 (23-40)	31 (18-47)	36 (24-49)	31 (22-42)
5yr (n=125)	54 (41-66)	55 (44-66)	5yr (n=88)	63.6 (41-82)	62 (44-77)	57 (43-69)	64 (41-82)	63 (44-78)	55 (39-68)
Total fu (n=229)	60 (51-68)	60 (51-68)	Total fu (n=180)	72 (56-83)	68 (55-78)	60 (51-69)	72 (56-84)	69 (56-80)	64 (54-73)
NPV (95%CI)			**NPV (95%CI)**						
1yr (n=229)	91 (84-96)	94 (85-98)	1yr (n=180)	88 (81-93)	91 (84-95)	99 (89-100)	88 (81-93)	91 (84-96)	94 (86-98)
2yr (n=208)	84 (74-90)	87 (76-94)	2yr (n=159)	75 (66-83)	78 (69-86)	84 (70-93)	75 (66-83)	79 (70-86)	80 (68-88)
5yr (n=125)	63 (50-75)	70 (55-82)	5yr (n=88)	56 (43-68)	59 (45-72)	69 (48-85)	56 (43-68)	59 (45-72)	58 (41-73)
Total fu (n=229)	63 (53-72)	70 (59-80)	Total fu (n=180)	57 (48-65)	60 (50-69)	72 (58-83)	57 (48-65)	60 (50-69)	65 (54-75)
Accuracy (95%CI)			**Accuracy (95%CI)**						
1yr (n=229)	52 (45-58)	44 (38-51)	1yr (n=180)	70 (63-77)	64 (57-71)	42.2 (35-49)	70 (63-77)	66 (59-73)	54 (48-61)
2yr (n=208)	55 (49-62)	51 (44-57)	2yr (n=159)	64 (56-71)	61 (53-69)	45.9 (38-54)	64 (56-71)	63 (56-71)	52 (44-60)
5yr (n=125)	58 (50-67)	61 (52-69)	5yr (n=88)	58 (48-68)	60 (50-70)	60 (50-70)	58 (48-68)	60 (50-70)	56 (55-66)
Total fu (n=229)	61 (55-67)	63 (57-70)	Total fu (n=180)	61 (54-68)	63 (56-70)	64 (57-71)	61 (54-68)	63 (57-70)	64 (57-71)
Hazard ratio (95%CI)	2.0 (1.3-2.9)	2.7 (1.7-4.2)	**Hazard ratio (95%CI)**	1.9 (1.2-2.9)	2.0 (1.3-3.0)	2.6 (1.5-4.6)	1.9 (1.2-2.9)	2.1 (1.4-3.2)	2.0 (1.3-3.1)

Supplementary Table 1. Test characteristicsfor 2010 and 2017 criteria
Abbreviations: DIS, dissemination in space; DIT, dissemination in time; OCB, oligoclonal bands; 95%CI, 95% confidence interval; fu, follow-up; PPV, positive predictive value; NPV, negative predictive value



Chapter 3

Fatigue after a first attack of suspected multiple sclerosis

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ABSTRACT

Background: Fatigue is reported by more than 75% of of multiple sclerosis (MS) patients. In an earlier study we showed that fatigue is not only a common symptom in patients at time of clinically isolated syndrome (CIS; fatigued 46%), but also predicts subsequent diagnosis of clinically definite MS (CDMS). The course of fatigue after CIS is unknown.

Objective: We aimed to explore the long-term course of fatigue after CIS.

Methods: In this study, 235 CIS patients, aged 18-50 years, were prospectively followed. Patients filled in the Krupp's Fatigue Severity Scale (FSS) and the Hospital Anxiety and Depression Scale (HADS) at baseline and annually. After reaching CDMS diagnosis, EDSS (Expanded Disability Status Scale) was obtained annually. Mixed-effects models were used to analyse longitudinal FSS measurements.

Results: Fatigue at baseline was an independent predictor for CDMS diagnosis (hazard ratio (HR): 2.6, 95% confidence interval (CI): 1.6-4.4). The evolution of FSS was the same in CIS patients who remained monophasic and patients who were diagnosed with CDMS during follow-up. However, FSS increased by 0.86 units after reaching CDMS diagnosis (p=0.01). After this increase, the FSS course remained unaltered (p=0.44).

Conclusion: Fatigue, which is often present at time of CIS, probably persists over time and increases after a second attack.

INTRODUCTION

Fatigue is a common symptom in MS, reported by more than 75% of patients,[1-3] and is described as the most debilitating symptom by 15-40% of MS patients.[4] In MS fatigue is defined as 'a subjective lack of physical and/or mental energy, perceived by the individual or caregiver to interfere with usual and desired activities'[5]
The pathophysiological mechanisms of MS-related fatigue are not yet fully understood. Fatigue can result from MS-related symptoms such as sleep deprivation, MS-related neuro-psychiatric disorders such as depression and anxiety, and side effects of medication (secondary fatigue),[6,7] but fatigue can also be the direct result of MS-related pathophysiological processes such as inflammation, demyelination, and axonal loss (primary fatigue).[6,7] Several studies also indicated an independent relation between inflammation and fatigue.[8,9] In a previous study we showed that fatigue was present in 46% of patients at time of the first attack, independent of the neuroanatomical localization of this first attack, with a severity similar to fatigue in patients with MS.[10] This indicates that the MS-related fatigue presents itself already early in the disease at the moment of the first symptoms, not related to the attack itself. Few relatively short-term studies in patients diagnosed with MS suggested that fatigue persists over time. Those studies had a maximum follow-up of 3 years.[1,11-14] The long-term course of fatigue after clinically isolated syndrome (CIS) is not known. The aim of this study was to explore the course of fatigue, during follow-up after CIS. The second aim was to study the association between fatigue at time of CIS and disability after a second clinical attack.

METHODS

Patients

All patients with CIS, a suspected first episode of MS, who visited Erasmus Medical Centre (EMC) University Hospital in Rotterdam, a tertiary referral centre for multiple sclerosis, or one of the collaborating regional hospitals, were enrolled in this study. All the patients were included and followed between July 2006 and December 2015 in the multicentre prospective observational study on Predicting the Outcome of a Demyelinating event (PROUD study). Study protocols have been described previously.[10] Patients were between 18 and 50 years of age, and were included in the study within six months after the onset of CIS. At baseline patients underwent an MRI scan and routine laboratory tests to rule out alternative diagnoses.[15] Patients with alternative diagnoses and patients who suffered from life-threatening comorbidities (i.e. malignancies, acquired immunodeficiency syndrome (AIDS)) were excluded. Patients with comorbidities likely to cause fatigue, other than depression, were excluded from the analyses. After inclusion patients were reassessed annually.

Definitions

An exacerbation was defined as new symptoms or subacute worsening of existing symptoms after 30 days of improvement or stable disease and no evidence of alternative

diagnosis.[16] To be regarded as exacerbation, symptoms had to exist for longer than 24 hours and not to be preceded by fever.[17] All exacerbations were confirmed by neurological examination. Clinically definite multiple sclerosis (CDMS) was defined as clinical dissemination in space and time as described by Poser et al.[17] Disability was measured using the Expanded Disability Status Scale (EDSS).[18] After a diagnosis of CDMS, EDSS was obtained at annual reassessments by a trained physician. In case of an exacerbation, the physicians made sure that an EDSS was obtained at least three months after the exacerbation.

Questionnaires

Krupp's Fatigue Severity Scale (FSS) was used to assess fatigue.[19] This self-administered questionnaire is validated for use in patients with MS.[19-21] FSS consists of nine questions with seven possible answers for each question, ranging from strong disagreement to strong agreement. The FSS score ranges from one to nine; fatigue is defined as an FSS of 5.0 or higher.[22]
Hospital Anxiety and Depression Scale (HADS) was used to measure anxiety and depression.[23] This is also a self-administered questionnaire, that has been validated in MS patients.[24] It contains seven items measuring symptoms of anxiety and seven items measuring symptoms of depression. For both anxiety and depression a score of 11 points or higher (out of 21 points) was considered as being anxious or depressed. HADS was obtained at the same time as FSS to correct for depression and anxiety, as fatigue is associated with these factors.[25,26] FSS and HADS were obtained at baseline and annually.

Standard protocol approvals and patient consent

This study was approved by the Medical Ethics Committee of Erasmus MC Rotterdam. Written informed consent was obtained from all patients.

Data analysis

Statistical analyses were done using SPSS version 21 (SPSS Inc., Chicago, Illinois, USA) for Windows, R statistical software version 3.2.4 and GraphPad Prism5 (GraphPad, San Diego, USA) for Windows. We compared continuous data using a two-tailed t-test (for age, follow-up time, Time CIS to baseline FSS, FSS and HADS-A at baseline) or a Mann-Whitney U-test (for HADS-D and IgG index) if the data were not normally distributed. Chi-square or Fisher's exact test were applied to analyse categorical data (gender, ethnicity, immunomodulating therapy (IMT), dichotomized FSS, clinical syndrome type, oligoclonal bands (OCB) and MRI features). Time to CDMS was calculated from the onset of the first symptoms to the second clinical attack (confirmed by a neurological examination). Patients who did not experience a second attack were considered as censored observations. Survival data were analysed using Kaplan Meier survival analyses with Log-rank test, univariable, and multivariable Cox proportional hazard regression models. To analyse the longitudinal FSS measurements and to evaluate the association between baseline FSS and EDSS during follow-up, we used mixed effects models. To model the

time effect flexibly we utilized natural cubic splines with two internal knots placed at the 33.3% and 66.6% percentiles of the observed follow-up times. In the linear mixed model used to analyse the longitudinal FSS measurements we corrected our findings for ethnicity, gender, anatomical localization of the first symptoms, age at time of CIS, and HADS. To analyse the change in evolution of FSS after CDMS diagnosis we included an interaction term between CDMS at time of questionnaire and the nonlinear time. In the model where we analysed EDSS (as continuous variable) we corrected for localization of the first symptoms. To analyse the correlation between FSS at baseline and evolution of EDSS during follow-up after CDMS we included the interaction term between FSS at baseline and time. For both analyses the model with random intercept and random slopes were deemed the most appropriate based on likelihood ratio tests between nested random-effects structures. Different F-tests were used to evaluate whether the time effect was nonlinear, whether FSS altered after being diagnosed with CDMS, and whether EDSS was dependent on the baseline FSS.

RESULTS

Patient characteristics

At time of the analyses, 281 patients were enrolled in the study. In total, 46 patients were excluded from further analysis because of alternative diagnoses (n=12), comorbidities other than depression, that are likely to cause fatigue (Crohn's disease (n=1), ulcerative colitis (n=1), hypothyroidism (n=7), pan-hypopituitarism (n=1), newly diagnosed diabetes mellitus (n=1)), and missing data on the questionnaires (n=23). After exclusions, 235 patients were left for the analyses. A total of 825 questionnaires were obtained (median number of questionnaires per person is: 3.0 (interquartile range (IQR): 2.0-5.0)). During the mean follow-up of 51.9 months, 89 patients (37.9%) were diagnosed with CDMS. Fifteen of 825 questionnaires were obtained within three months after CDMS diagnosis. Fifty-nine patients received IMT before CDMS diagnosis. Patient characteristics are shown in Table 1. CIS patients are stratified into CDMS and monophasic.

Characteristic	All patients n=235 (100%)	CDMS n=89 (37.9%)	Monophasic n=146 (62.1%)	p-value [a]
Gender, female n (%)	178 (75.7)	74 (83.1)	104 (71.2)	0.04
Age (years) mean (SD) [b]	34.2 (8.3)	33.6 (8.1)	34.5 (8.4)	ns (0.38)
Caucasian ethnicity, n (%)	187 (79.6)	76 (85.4)	111 (76.0)	ns (0.08)
Follow-up time (months), mean (SD)	51.9 (29.5)	65.1 (25.0)	43.9 (29.1)	p<0.01
Time CIS to baseline FSS (weeks), mean (SD)	12.8 (8.6)	12.3 (6.9)	13.2 (9.6)	ns (0.50)
Time CIS to CDMS (months), median (IQR)	na	19.0 (8.4-42.3)	na	na
Immunomodulating therapy, n (%)	101 (43.0)	74 (83.1)	27 (18.5)	<0.01
Immunomodulating therapy at time of CIS, n (%)	59 (25.1)	32 (36.0)	27 (18.5)	<0.01
Questionnaires at baseline				
FSS, mean (SD)	4.2 (1.8)	4.8 (1.8)	3.8 (1.7)	<0.01
FSS ≥ 5.0, n (%)	83 (35.3)	47 (52.8)	36 (24.7)	<0.01
HADS-A, mean (SD)	7.1 (4.1)	7.6 (4.4)	6.7 (3.9)	ns (0.14)
HADS-D, median (IQR)	4.0 (1.3-7.0)	4.0 (2.0-8.0)	3.0 (1.0-6.0)	<0.01
Presenting phenotype at CIS				
-Optic neuritis	94 (40.0)	29 (32.6)	65 (44.5)	ns (0.07)
-Brainstem	25 (10.6)	15 (16.9)	10 (6.8)	ns (0.06)
-Spinal cord	62 (26.4)	23 (25.8)	39 (26.7)	ns (0.88)
-Cerebellum	3 (1.3)	0 (0.0)	3 (2.1)	ns (0.29)
-Cerebral hemispheres	21 (8.9)	8 (9.0)	13 (8.9)	ns (0.98)
-Multifocal	30 (12.8)	14 (15.7)	16 (11.0)	ns (0.29)
CSF findings at baseline				
Positive OCB, n (%) (n=142)	115 (81.0%)	57 (93.4%)	58 (71.6%)	<0.01
IgG index, median (IQR) (n=137)	0.83 (0.57-1.30)	1.00 (0.62-1.39)	0.78 (0.56-1.11)	0.03
Features baseline MRI scan				
≥ 9 T2 lesions on T2-weighted images, n (%)	90 (38.3)	48 (53.9)	42 (28.8)	<0.01
Gadolinium-enhancing lesions, n (%) (n=158)	65 (41.1)	31 (48.4)	34 (36.2)	ns (0.12)

Table 1. Patient characteristics

[a] p-value calculated between CDMS and monophasic. [b] Age at time of CIS

Abbreviations: CIS, clinically isolated syndrome; CDMS, patients who are diagnosed with CDMS during follow-up after CIS defined by Poser criteria; Monophasic, not diagnosed with CDMS; ns, not significant; na, not applicable; HADS-A, HADS-anxiety; HADS-D, HADS-depression; OCB, oligoclonal bands; Ig Immunoglobulin

Fatigue predicts CDMS diagnosis

Fatigue at baseline was associated with a shorter time to CDMS diagnosis (HR: 2.5, 95% CI 1.5-3.9; p<0.001) using a univariable Cox regression model. After adjustments (sex, age, ethnicity, localization of symptoms, anxiety, depression, number of T2 lesions, gadolinium enhancement at baseline MRI and IMT before CDMS diagnosis), multivariable COX analysis showed that fatigue at time of CIS was associated with CDMS diagnosis both as a dichotomous variable (HR 2.7, 95% CI 1.6-4.4; p<0.001) and as continuous variable (HR 1.4, 95% CI 1.2-1.6; p<0.001). Kaplan-Meier curves for time to CDMS in fatigued and non-fatigued patients are shown in Figure 1.
There were no differences between FSS scores in patients who received IMT versus patients who did not receive IMT in the CDMS group (mean FSS, respectively 4.6 vs 4.4; p=0.19) and in the monophasic CIS group (mean FSS, respectively 3.8 vs 3.4; p=0.26). Patients who were diagnosed with CDMS during follow-up showed higher scores on the depression scale (HADS-D) compared to patients who remained monophasic (median: 4.0 vs 3.0 p<0.01). However, the hazard ratio per point elevation in HADS-D was small (HR: 1.075, 95% CI 1.02-1.14; p=0.01).
From 112 patients, we knew the level of education. There was no difference in baseline FSS between patients with the lowest (n=65) and highest (n=47) level of education. Level of education did not correlate with CDMS diagnosis during follow-up in this cohort.

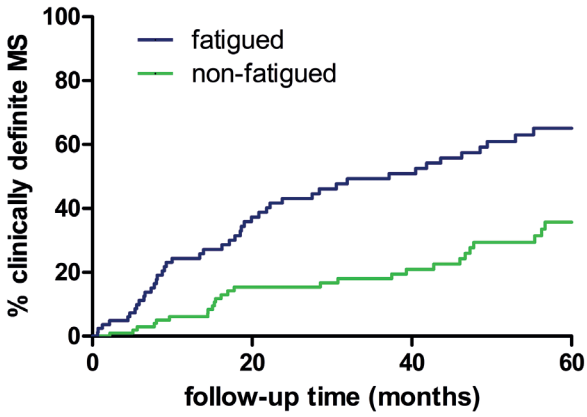

Figure 1. Time to CDMS
Kaplan-Meier curves for time to CDMS. Patients were stratified at fatigued (FSS≥5) vs non-fatigued FSS<5), (log-rank test, p<0.01)

Longitudinal follow-up of fatigue

FSS at time of CIS did not differ between male and female patients (male vs female, mean FSS 3.90 vs 4.31 (p=0.18)). However, during follow-up female patients had a 0.68 units higher FSS than males (mixed effects model analysis: estimated main effect for female:0.68 (p<0.01)). There was no significant difference in FSS between Caucasian and non-Caucasian patients at baseline (Caucasian vs non-Caucasian, mean baseline FSS:

4.20 vs 4.30 (p=0.76)) and during follow-up (estimated main effect for non-Caucasian: 0.23 (p=0.27)). Age at time of CIS did not correlate with FSS at baseline (Pearson's rho: 0.02 (p=0.78) nor during follow-up (estimated main effect per year older: 0.01 (p=0.30)). Anatomical localization of the first presenting symptom did not influence the FSS at baseline (optic neuritis vs other localization, mean baseline FSS: 3.93 vs 4.40 (p=0.10) nor during follow-up (estimated main effect for optic neuritis: 0.17 (p=0.38). The mean time between first neurological symptoms and baseline FSS was 12.8 weeks (SD: 8.6), no correlation between this time and baseline FSS score was found.
An increase of >1 point in FSS was seen in 25% of patients who remained CIS during follow-up. In the patients who were diagnosed with CDMS 30% showed an increase of >1 point in FSS, this difference was not significant (p=0.34).
The longitudinal evolution of FSS in time was nonlinear (p<0.01). Therefore we utilized a mixed effects model with natural cubic splines. The evolution of FSS was not altered after CDMS diagnosis (p=0.44 for interaction between the nonlinear effect for time and CDMS diagnosis). However, there was a significant increase of FSS by 0.86 units after CDMS diagnosis (estimated main effect for FSS at time of CDMS: 0.86 (p=0.01)). After this increase in FSS the FSS course remained unaltered, comparable to the course of FSS in monophasic CIS patients. These results were adjusted for ethnicity, gender, anatomical localization of first symptoms, age at time of CIS, and HADS. The evolution of FSS for monophasic CIS patients and CIS patients diagnosed with CDMS during follow-up are shown in Figure 2A and 2B, Figure 2B depicts the FSS evolution for CIS patients with two years between CIS and CDMS. The figures for CIS patients with different times between CIS and CDMS look similar, with the sole difference that the increase in FSS takes place at time of CDMS diagnosis.

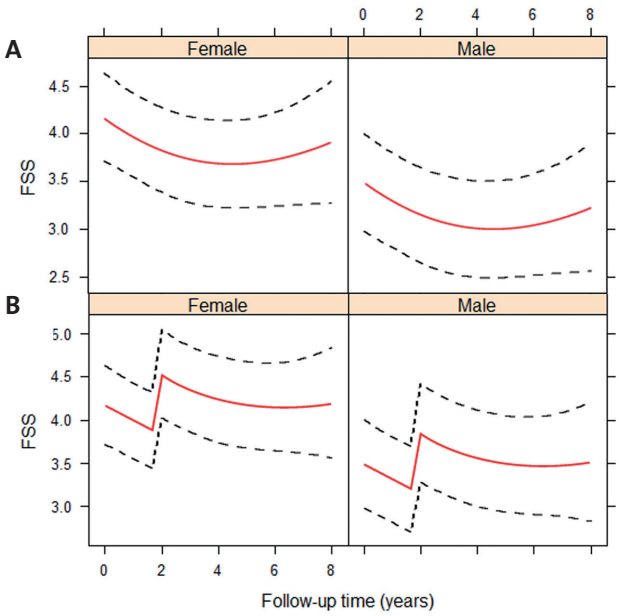

Figure 2 Follow-up FSS in males and females
Time 0 refers to study entry.
A. *FSS evolution in patients who are not diagnosed with CDMS during follow-up.*
B. *FSS evolution in patients who are diagnosed with CDMS during follow-up. The illustration depicts the model for CDMS diagnosis at year 2. For patients with other times to CDMS diagnosis, the figure looks similar with the increase in fatigue at time of CDMS diagnosis*

EDSS

When patients were diagnosed with CDMS (n=89), EDSS was obtained annually. There is a trend towards a 0.9 units higher EDSS during follow-up in patients with FSS ≥5.0 at time of CIS, compared to patients with FSS <5.0 (mixed effects model analysis: estimated fixed effect for FSS≥5.0: 0.90 (p=0.10)). Those results were adjusted for localization of the first symptoms. Patients who changed >1 point in FSS score during follow-up did not show higher EDSS scores during follow-up than patients who did not change >1 point in FSS score.

DISCUSSION

In this study we explored the course of fatigue after CIS. The first part of the study replicates our earlier observation that a higher FSS at time of CIS, unrelated to disability or time between first neurological symptoms and baseline FSS, predicts a subsequent diagnosis of CDMS.[10] Patients with a future diagnosis of CDMS already had a higher FSS at time of CIS than patients who remained monophasic during follow-up. Here we showed that after the second attack (and thus after CDMS diagnosis), the FSS increased even more.

We found that fatigue is predictive for a shorter time to CDMS diagnosis. There is some evidence that a shorter time to CDMS diagnosis might correlate with a more severe disease course with more disability.[27] Cavallari et al. found that fatigue in patients with MS was predictive for disease worsening.[28] Therefore we tested whether fatigue present even earlier in the disease also predicts future disability. In a mixed-effects model, with correction for localization of CIS, we did not find a correlation between fatigue at time of CIS (FSS ≥5.0) and EDSS during follow-up. However, we did find a trend towards a 0.9-point higher EDSS score during follow-up in patients who were fatigued at baseline; a 0.9-point increase in EDSS might be clinically relevant.

A strength of our study was the large sample size, almost twice as large as our previous cohort (235 vs 127).[10] Earlier studies looked at the course of fatigue in patients who were already diagnosed with MS with a much shorter follow-up of 1 to a maximum of 3 years with various results in the persistency of fatigue.[1, 11-13, 29, 30] To our knowledge, this is the first study that examined fatigue annually after onset of CIS with a long follow-up (mean: 4.3 years). Since we had a large sample size we could adjust our findings for multiple factors including HADS. Anxiety and depression are known confounders and were associated with FSS, therefore the analyses were adjusted for these factors.[25, 26, 31]

Although part of the patients received IMT, it did not influence the results, because FSS did not differ between those who used IMT and those who did not. This is in line with previous literature.[12, 32] Fifty-nine patients received IMT prior to CDMS diagnosis, which may have postponed CDMS diagnosis. Therefore our results may even be an underestimation. However, in the multivariable COX model we corrected for IMT prior to CDMS diagnosis.

One explanation for the observed increase in FSS score after CDMS diagnosis could be that this increase is related to the second attack itself, however, only a small proportion of questionnaires was obtained in a short period after the second attack (15 of 825 questionnaires within three months); this indicates that it is more likely that the observed increase in FSS is the MS-related fatigue and not the attack related fatigue.

The FSS is a self-administered questionnaire, and easy to fill in. Although it is a subjective scale, it is as efficient as other scales measuring fatigue.[33] Its validity has been proven in MS; hence it has been used widely in MS studies. Because of its short length and simplicity we had a preference for specifically this scale in order to limit the withdrawal due to repetitive annual measurement of fatigue.

In this study less than 40% of CIS patients experienced a second attack during a mean follow-up time of more than 4 years. Using the newest diagnostic criteria,[34] a part of the remaining 60% of patients, having a less active disease, would be diagnosed with MS. This underlines that not only predictors for MS diagnosis are important but also predictors for disease activity and disability.
There were a few limitations to our study. First, because patients were included over a long period of time, there was a wide range in follow-up, resulting in different numbers of completed questionnaires per patient. To overcome this problem a mixed-effects model was used which allowed us to correct for different follow-up times and use all available questionnaires in the analyses. Second, we did not measure EDSS scores at the moment of CIS; therefore, we could not adjust for baseline EDSS. All EDSS scores were obtained at least 3 months after an attack because we did not want a resent relapse influencing the EDSS score. However, although we could not adjust for baseline EDSS, we did adjust for localization of CIS.
We did not have reliable information on level of education for all patients. However, in a subgroup of 112 patients we had this information. In this subgroup the results were not altered after adding level of education to the COX regression model.
Last, since we did not perform a follow-up MRI scan regularly, we used the Poser criteria for defining CDMS diagnosis.[17] In this way, we did show an effect on a second attack, this might be clinically more relevant, than showing an effect on MRI.
In summary, using a large prospective cohort of CIS patients, we show that fatigue at time of CIS predicts a subsequent CDMS diagnosis. Our findings indicate that fatigue exists early in the disease course of MS, probably will remain present during the further follow-up, and increases after CDMS diagnosis. Despite the long follow-up, we did not find an association between presence of fatigue at the moment of the first symptoms and future disability. However, we did find a trend towards a higher EDSS in patients who were fatigued at baseline. It would be interesting to follow our cohort to see whether, with a longer follow-up, the correlation of fatigue at first attack and disability during follow-up will reach significance. Fatigue can interfere with a person's ability to function at home and work, our advice for clinical practise is to give proper attention to symptoms and management of fatigue already early in the disease course.

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Chapter 4

Soluble CD27 levels in cerebrospinal fluid as a prognostic biomarker in clinically isolated syndrome

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ABSTRACT

Importance: There is a growing number of therapies that could be administered after the first symptom of CNS demyelination. These drugs can delay multiple sclerosis (MS) diagnosis and slow down future disability. However, treatment of patients with benign course may not be needed, therefore there is a need for biomarkers to predict long-term prognosis in patients with clinically isolated syndrome (CIS).

Objective: To investigate whether the T-cell activation marker soluble CD27 (sCD27), measured in cerebrospinal fluid (CSF) of patients at time of a first attack, predicts a subsequent diagnosis of MS and is associated with a higher relapse rate.

Design, setting and patients: This prospective study included 77 patients with CIS between March 2002 and May 2015 in a tertiary referral centre for multiple sclerosis, in collaboration with several regional hospitals. Patients with CIS underwent a lumbar puncture and MRI scan within 6 months after first onset of symptoms.

Main Outcome and Measures: Soluble CD27 levels were determined in cerebrospinal fluid using a commercially available Enzyme-Linked Immuno Sorbent Assay (ELISA). Cox regression analyses was used to calculate univariate and multivariable hazard ratios for MS diagnosis. Association between sCD27 levels and relapse rate was assessed using a negative binomial regression model.

Results: Among 77 patients with CIS, 50 were female (79.5%), and mean (SD) age was 32.7 (7.4) years. Mean (SD) age in the control individuals was 33.4 (9.5) years, and 20 were female (66.7%). Patients with CIS had higher CSF sCD27 levels than control individuals (geometric mean (95% CI): 31.3 U/mL (24.0-40.9) vs 4.67 U/mL (2.9-7.5); p<0.001). During a mean follow-up of 54.8 (±35.1) months 39 of 77 (50.6%) patients were diagnosed with MS. In a model adjusted for MRI and CSF measurements, sCD27 levels were associated with a diagnosis of MS (hazard ratio: 2.4 per 100 U/mL increase in sCD27 levels 95% CI, 1.27-4.53; p=0.007). Additionally, MS patients with high sCD27 levels (>31.4 U/mL (median)) at time of CIS had a 5.5 times higher annualized relapse rate than patients with low sCD27 levels (annualized relapse rate: 0.06 vs 0.33; p=0.02).

Conclusion and relevance: Soluble CD27 in cerebrospinal fluid of CIS patients predicts MS diagnosis and a high relapse rate. Therefore, sCD27 is an activation molecule directly related to the immunopathology of the disease, and is a potential clinical marker to help in treatment decisions after a first attack of suspected MS.

KEY POINTS

Question: What is the value of soluble CD27 in predicting long-term prognosis in patients with clinically isolated syndrome?

Findings: In this prospective study that included 77 patients with clinically isolated syndrome, soluble CD27 was independently associated with MS diagnosis. Additionally, soluble CD27 was associated with a 5.5-fold higher annualized relapse rate.

Meaning: Soluble CD27 in cerebrospinal fluid of patients with clinically isolated syndrome could be used to predict which patients will have an active disease course at the time of a first attack.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory disease of the CNS (central nervous system) with large heterogeneity in severity and prognosis.[1] There is a growing number of therapies that could be administered after the first symptom of CNS demyelination (clinically isolated syndrome (CIS)), even before MS diagnosis. Disease-modifying therapies (DMTs) delay MS diagnosis, and have a potential to prevent future disability.[2-7] However, these therapies have side effects, therefore, treating CIS patients who will not reach MS diagnosis should be prevented. This underscores the need for biomarkers to predict long-term prognosis at an early phase of the disease.[8,9]
Many studies have shown the crucial role for T cells and B cells in the pathogenesis of MS.[10-13] T cells activated by the TCR/CD3 complex release a soluble form of CD27 (sCD27).[14] CD27 and sCD27 co-stimulate maturing of T cells, and induce activation and proliferation of T and B cells.[15,16] Increased sCD27 levels have been reported in various autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis,[17-20] and MS.[21,20] Soluble CD27 is considered a biomarker of active intrathecal T-cell-mediated inflammation. For distinguishing MS from non-inflammatory diseases sCD27 showed a higher discriminatory power than common humoral markers for active intrathecal inflammation as IgG index or presence of oligoclonal bands (OCB).[22] Soluble CD27 levels in serum did not discriminate normal individuals from patients with MS.[21] It is not known if CSF sCD27 levels at time of CIS have a predictive value for disease course. The aim of this study was to examine whether CSF sCD27 levels in CIS patients, predict MS diagnosis and relapse rate.[23]

METHODS

Study participants

For this study, data were prospectively collected between March 2002 and May 2015 from patients with a first episode of demyelinating disease who visited the neurological clinic of Erasmus MC University Hospital in Rotterdam, a tertiary referral centre for patients

with MS, in collaboration with several regional hospitals. Patients with CIS were eligible for this study if they were between 18-50 years of age. Patients with alternative diagnoses and patients who suffered from life-threatening comorbidities (i.e. malignancies, acquired immunodeficiency syndrome (AIDS)) were excluded from analysis. At baseline patients underwent an MRI-scan with gadolinium and routine laboratory tests to rule out alternative diagnoses.[24] If a lumbar puncture was performed, extra CSF was collected for the study. CSF samples were aliquoted and stored at -80 degrees. For this study we included patients with a CSF sample collected within 6 months after onset of CIS and at least 6 months follow-up after CIS. After inclusion, patients were reassessed at least annually at the neurology outpatient department. For control group, we used CSF from symptomatic controls (SCs), patients with neurological symptoms, but have no objective clinical findings to define a specific neurological disease, as defined by Teunissen et al.[25] Control group patients underwent a lumbar puncture at the neurology department of Erasmus MC between January 2002 and April 2014 for reasons other than neuroinflammatory diseases. Those were acute headache with no subarachnoid hemorrhage, no idiopathic intracranial hypertension and no inflammation (n=15), exclusion of neurosyphilis (n=7), and other (e.g. muscle pain, sensory disturbances without CNS pathology) (n=8).

Standard protocol approvals, registrations, and patient consents

The study protocol was approved by the Ethics Committee of the Erasmus MC University Hospital. All prospectively included patients provided written informed consent.

Definitions

An exacerbation of MS was defined as per acute worsening of existing symptoms or new symptoms after 30 days of improvement or stable disease and no evidence of alternative diagnosis. To be regarded as exacerbation, symptoms should exist for more than 24h and not be preceded by fever. All exacerbations were confirmed by neurological examination.[26] The diagnosis of MS in all patients was made according to the McDonald criteria (revised in 2010) based on a second clinical relapse or the baseline MRI scan.[27] All patients underwent a baseline MRI scan with gadolinium. A follow-up MRI scan was not performed regularly, therefore we did not take the follow-up MRI scan into account when defining MS diagnosis. Patients with monophasic CIS did not fulfill McDonald criteria (revised in 2010) criteria during follow-up. Disability was measured using the Expanded Disability Status Scale (EDSS).[28] When patients experienced a second attack, EDSS was performed annually. EDSS scores performed within 3 months after exacerbation were not used in the analyses. Follow-up was calculated by subtracting CIS date from the last visit date.

Sample preparation and sCD27 ELISA

Collected CSF samples were immediately centrifuged for 10 minutes at 3000 rpm to separate the supernatant from cells and cellular elements. After centrifugation, CSF was

aliquoted and stored at -80 °C until use for this study.[29] Routine CSF diagnostics including IgG index, OCBs, cell count and total protein was performed. Soluble CD27 levels were measured twice for each sample using the commercially available enzyme-linked-immunosorbent assay (ELISA) kit (PeliKine compact human sCD27 kit; Sanquin)[14] The ELISA was performed according to the manufacturer's instructions. Concentrations were expressed in U/mL by reference to a standard curve supplied with the ELISA kit. The analysts who performed the experiments were blinded for the clinical diagnosis of patients. The detection limit of the ELISA was 5 U/ml.

Statistical analysis

Statistical analyses were done using SPSS 21.0 (SPSS Inc., Chicago, Illinois, USA) for Windows and Graphpad Prism5 (GraphPad, San Diego, USA) for Windows. To assess normality of data distribution, we performed the Kolmogorov-Smirnov test. The distribution of sCD27 was nonparametric and after log transformation the data were normally distributed. Therefore, geometric means were calculated. For group comparison of continuous variables, we applied two-tailed t-test (age, follow-up, sCD27) or nonparametric Mann-Whitney U test (IgG index, time between CIS and lumbar puncture (LP)). Nominal data comparison between groups was done using Chi-square or Fisher's exact test (gender, clinically isolated syndrome type, treatment before MS diagnosis, OCB). Time to MS was calculated from onset of first symptoms to MS diagnosis (McDonald criteria, revised in 2010).[27] Patients who were not diagnosed with MS during follow-up were considered as censored observations. Cox proportional hazard regression analysis was used to calculate univariate and multivariable hazard ratios. The proportional hazard assumption was tested by including time dependent covariate in the model. Odds ratios for MS in the subgroup with >0 subclinical T2 lesions on the MRI scan were calculated using binary logistic regression. Annualized relapse rate (ARR) was compared between groups using a negative binomial regression model with the natural logarithm of number of follow-up years after a second clinical attack as offset. This offset corrects for the difference in follow-up between included patients. Because of overdispersion of data, we did not use a Poisson regression model. P-values <0.05 were considered significant.

RESULTS

Patient characteristics

Inclusion criteria for this study were met by 77 patients with CIS. The mean follow-up time for the total CIS group was 52.4 months (SD ±34.5). The median time from CIS to MS diagnosis (McDonald criteria, revised in 2010) (n=39) was 7.8 months (IQR: 2.0-41.9). Nine patients (11.4%) received DMT (Glatiramer acetate (n=3) or interferon-beta (n=6)) in the period between CIS and MS diagnosis. No patients were treated with DMT at time of lumbar puncture. Thirty symptomatic controls were age and gender matched. Patient and control characteristics are shown in Table 1. Patients with CIS were further stratified into MS and monophasic.

Characteristic	SCs (n=30)	CIS-patients (n=77)	MS (n=39)	Monophasic (n=38)	p-value [a]
Gender, female, n (%)	20 (66.7)	50 (64.5)	31 (79.5)	19 (50.0)	0.007
Age (years), mean (SD) [b]	33.4 (9.5)	32.7 (7.4)	31.8 (6.8)	33.7 (8.0)	0.27
Follow-up time (months), mean (SD)	na	54.8 (35.1)	71.4 (37.5)	37.7 (22.2)	<0.001
Clinical syndrome type, n (%)					
-ON	na	38 (49.4)	19 (48.7)	19 (50.0)	0.91
-Brainstem	na	12 (15.6)	6 (15.4)	6 (15.8)	0.96
-Spinal cord	na	18 (23.4)	8 (20.5)	10 (26.3)	0.55
-Cerebral hemispheres	na	8 (10.4)	5 (12.8)	3 (7.9)	0.71
-Multifocal	na	1 (1.3)	1 (2.6)	0 (0.0)	0.99
DMT at time of CIS, n (%)	na	9 (11.4)	4 (10.3)	5 (13.2)	0.69
OCB (> 1 band), (%)	na	54/73 (78.1)	31/37 (91.9)	23/36 (63.9)	0.05
IgG index, median (IQR) (n=75)	na	0.70 (0.52-1.20)	1.00 (0.54-1.40)	0.61 (0.49-0.92)	0.06
Elevated IgG index (cut-off: 0.68), n (%)	na	40/75 (53.3)	23/37 (62.2)	17/38 (44.7)	0.13
White blood cell count, median (IQR)	na	4.0 (0.5-12.0)	4.0 (0.0-12.0)	3.5 (1.0-12.0)	0.98
Time CIS to LP (weeks) median (IQR)	na	6.0 (2.7-13.1)	6.0 (2.9-13.0)	5.9 (2.4-14.0)	0.78
Features first MRI					
≥9 lesions on T2-weighted images, n (%)	na	24 (31.1)	19 (48.7)	5 (13.2)	0.001
Normal brain MRI, n (%)	na	6 (7.8)	2 (5.1)	4 (10.5)	0.43
MS only based on first MRI, n (%)	na	8 (10.4)	8 (20.5)	na	na
MS based on second attack, n (%)	na	31(40.3)	31 (79.5)	na	na
sCD27, geometric mean (95% CI)	4.7 (2.9-7.5)	31.3 (24.0-40.9)	42.0 (29.1-60.6)	23.2 (15.8-33.9)	0.03

Table 1. Patient characteristics
[a] p-value calculated between MS and monophasic [b] for CIS patients: age at CIS, for SCs: age at LP
Abbreviations: SCs, symptomatic controls; CIS, clinically isolated syndrome; MS, fulfilling McDonald criteria (revised in 2010); Monophasic, not fulfilling McDonald criteria (revised in 2010); na, not applicable; SD, standard deviation; IQR, interquartile range; OCB, Oligoclonal bands; Ig, Immunoglobulin; sCD27, soluble CD27

Soluble CD27 levels are higher in CIS patients than in symptomatic control individuals

We first compared sCD27 levels in CSF between CIS patients and controls. After log transformation sCD27 levels were normally distributed. Soluble CD27 levels were significantly higher in CIS patients than in controls. The geometric mean (95% CI) was respectively 31.3 U/mL (24.0-40.9) vs 4.7 U/mL (2.9-7.5); p<0.01. (Figure 1A)

Soluble CD27 levels are associated with MS diagnosis

Next, we compared CIS patients who were diagnosed with MS (n=39) (mean follow-up time: 52.4 months (SD ±34.5)) to patients who remained CIS (n=38) during follow-up. Soluble CD27 levels were significantly increased in CIS patients with a future MS diagnosis (geometric mean (95%CI) respectively 42.0 U/mL (29.1-50.6) vs 23.2 U/mL (15.8-33.9); p=0.03. (Figure 1B)

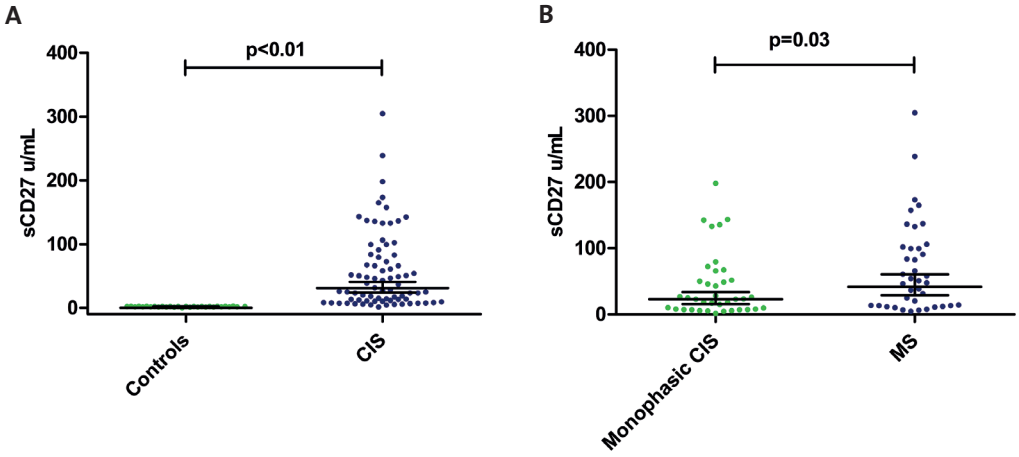

Figure 1. Comparison of CSF sCD27 levels

A. *Comparison of CSF sCD27 levels between CIS versus controls.* **B**. *Comparison of CSF sCD27 levels between monophasic CIS versus MS. Horizontal lines with error bars indicate geometric mean with 95% CI*

High sCD27 levels are associated with shorter time to MS

We stratified data by sCD27 levels using the median sCD27 level (31.4 U/ml) of CIS patients as cut-off. In Figure 2, we show that CIS patients with high levels sCD27 had a shorter time to MS diagnosis than patients with low sCD27 levels (log-rank test: p=0.02). After adjustments (≥9 T2 lesions, contrast enhancement on baseline MRI-scan, presence of OCB and IgG index), multivariable Cox regression analyses showed that sCD27 is an independent predictive factor for time to MS diagnosis. The HR was 2.4 per 100 U/mL increase of sCD27 (p=0.007).

We performed a sub-analysis where we excluded monophasic CIS patients with less than two years of follow-up (n=12). The HR in univariate and multivariable Cox analyses remained significant (HR: 2.3 per 100 U/mL; p=0.009).
Nine patients (11.8 %) received DMT before MS diagnosis. Seven of nine patients (77.8%) had sCD27 levels higher than 2 times the maximum level of the control group. Four of them (44.4%) were diagnosed with MS. The log-rank test for time to MS diagnosis was more significant when we excluded these nine patients with DMT before MS diagnosis (p=0.004). The corrected HR for MS diagnosis was 2.4 per 100 U/mL increase of sCD27 (p=0.007).

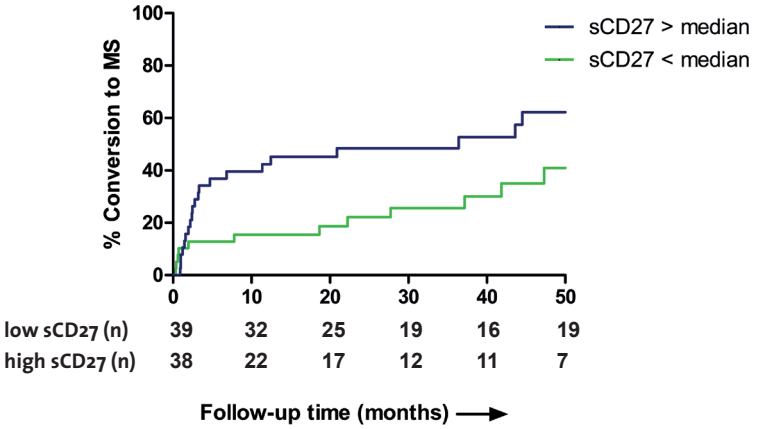

Figure 2. Time from CIS to MS in patients with high and low sCD27 levels
Kaplan-Meier curve for time from CIS to MS for patients with and without high sCD27 levels (log-rank test p=0.02). Cut-off value: median: 31.4 U/mL

Annualized relapse rate is higher in patients with high sCD27 levels at time of CIS

The total number of relapses in patients who experienced a second clinical attack (n=31) was 26 in a total of 120.9 person-years at risk. The group with high sCD27 levels at time of CIS (n=18, using the median sCD27 level in CIS patients as cut-off (31.4 U/mL)) had a 5.5 times higher ARR during follow-up after the second attack, estimated by negative binomial regression analysis with log link, corrected for follow-up time (ARR: 0.06 vs 0.33; p=0.02). Other possible factors effecting ARR, such as contrast enhancement, dissemination in space (McDonald criteria, revised in 2010), more than 9 T2 lesions on the first MRI scan, unique OCBs in CSF and proportion of time on DMT, did not have significant influence on ARR. However, IgG index was significantly correlated with ARR. One point elevation in IgG index was associated with 3.7 times higher ARR (p<0.01). Soluble CD27 titer did not correlate with EDSS later in the disease course (data not shown). We collected EDSS scores of 24 of 31 patients with a second attack. During follow-up only 3 patients reached an EDSS score of 4 or more. Of these, all had high sCD27 levels (greater than median of 46.3 U/mL).

High sCD27 levels at time of CIS are associated with OCB and IgG index

There was a positive correlation between IgG index and sCD27 in CSF at time of CIS (Spearman's rho: 0.68; p<0.01). CIS patients with more than one unique OCB in CSF (n=54) had significantly higher sCD27 levels than CIS patients with no OCBs (n=19) (p<0.01). (Figure 3)

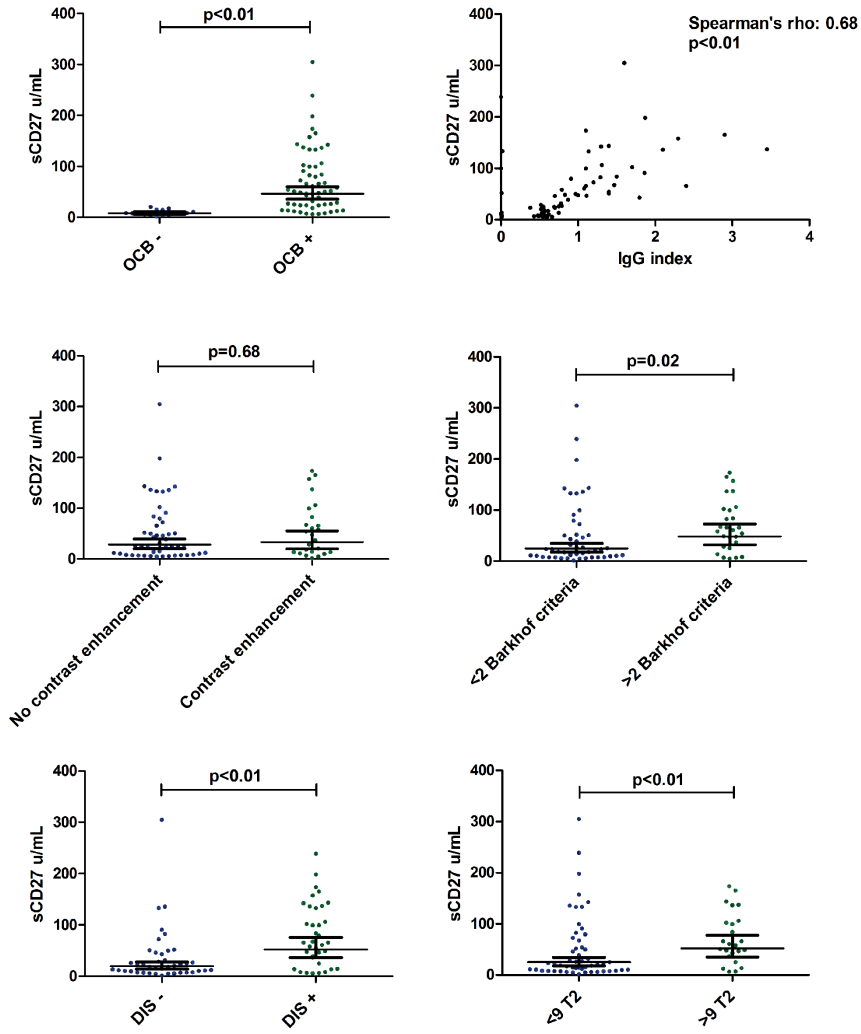

Figure 3. Soluble CD27 levels in CIS patients, CSF and MRI measurements
Horizontal lines with error bars indicate geometric mean and 95% CI

High sCD27 levels at time of CIS are associated with MRI abnormalities

Patients with more than 9 T2 lesions on the baseline MRI scan (n=24) had higher sCD27 levels (geometric mean, 52.3 U/mL vs 24.8 U/mL; p=0.009). Patients with no subclinical T2 lesion on the brain MRI at time of CIS (n=14) had lower sCD27 levels than patients with at least one subclinical T2 lesion (geometric mean: 14.8 vs 37.0; p=0.008). In a sub-analysis we selected patients with at least one subclinical T2 lesion (n=63). Within this group the odds ratio of high sCD27 (using the median sCD27 level in CIS patients as cut-off (31.4 U/mL)) for MS diagnosis was 3.0; p=0.037. In the same subgroup the odds ratio for presence of oligoclonal bands did not reach significance (OR: 2.9; p=0.09) Contrast enhancement on the MRI scan at time of CIS was not associated with sCD27 levels. (Figure 3.)

DISCUSSION

The rationale behind this study is based on the observation that CSF levels of sCD27 are significantly elevated in MS versus symptomatic controls.[21,22,30] We reasoned that a MS-associated biomarker could perhaps predict disease course at first attack, in analogy with MS-related MRI abnormalities that can predict future definite MS.[27,31,32] Indeed we demonstrate here that high sCD27 predicts a subsequent diagnosis of MS, and is associated with higher attack frequency after diagnosis. 21 of 77 CIS patients (27%) had sCD27 levels below the maximum level of controls. These patients clearly had a less active disease course.

Soluble CD27 is a stable molecule, the immune assay is quite reproducible and performed consistently in the literature.[21,22,30] It carries some extra attraction as this T-cell activation marker appears directly related to the core of immunopathology in MS.[10,11,33] It is of note that Bielekova et al. have found a clear relation between sCD27 levels and the presence of intrathecal T cells.[22] Amongst an impressive set of CSF inflammatory parameters, sCD27 performed best in identifying local inflammation and appeared correlated with progression. It seems not farfetched to suggest that higher T-cell activation is related to higher lesion load and a more active disease course.

The number of T2 lesions on brain MRI at time of CIS is currently the most predictive tool for MS diagnosis. A larger number of T2 lesions result in a shorter time to MS diagnosis. Also unique OCBs in CSF are predictive for a second attack in CIS patients.[3]

In a multivariable analysis we found that the association of sCD27 with future MS diagnosis remained after adjustment for known predictive factors such as OCB, IgG index, and MRI abnormalities (more than nine T2 lesions, and contrast enhancement on baseline MRI-scan).

In line with previous studies, we observed a correlation between sCD27 and IgG index.[21,22,26] (Figure 3) Whether there is a direct role for sCD27 released by T cells on IgG production by B cells is speculative, but such a functional effect has been shown in vitro by Dang et al.[34] They showed that the binding of sCD27 to the CD27 ligand CD70 on memory B cells, stimulates those B cells to differentiate into IgG secreting plasma cells.[20,34]

At time of lumbar puncture none of the patients were treated with DMT in this study cohort. Nine patients (11.8 %) received DMT before MS diagnosis. Excluding these

nine patients from the survival analyses does not have major effects on our results. Although the log-rank test for time to MS diagnosis is more significant when we exclude the patients with DMT before MS diagnosis.
In a subgroup of patients with at least one subclinical T2 lesion, high sCD27 was associated with MS diagnosis. The sample size was too small for analyses in more subgroups. In combination with MRI findings, sCD27 quantification could be helpful in decision making for starting immunomodulatory therapy because sCD27 is a marker for neuro-inflammation and predicts MS diagnosis and subsequent relapse rate.
There are some limitations of this study. First, the follow-up time has a wide range. To address this problem, we corrected for follow-up in the statistical analysis. We also did a sub-analysis in which we excluded monophasic CIS patients with less than 2 years follow-up. The HR in univariate and multivariable Cox analyses remained significant. Second, all patients underwent a baseline MRI scan with gadolinium, but a follow-up MRI scan was not performed uniformly according to a strict protocol. Therefore, we did not take the follow-up MRI scan in account when defining patients with MS but used the classical Poser criteria based on clinical manifestations. Third, though the sample size was sufficient for multivariable analysis, validation in a bigger cohort is required.

CONCLUSIONS

This study demonstrates that sCD27 levels in CSF of CIS patients helps to predict MS diagnosis and a high relapse rate. Therefore, CSF sCD27 could be an activation marker directly related to the immunopathology of the disease with potential value for selecting patients with higher subsequent disease activity.

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Chapter 5

T helper 17.1 cells associate with multiple sclerosis disease activity: perspectives for early intervention

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ABSTRACT

Interleukin-17-expressing $CD4^+$ T helper 17 (Th17) cells are considered as critical regulators of multiple sclerosis disease activity. However, depending on the species and pro-inflammatory milieu, Th17 cells are functionally heterogeneous, consisting of subpopulations that differentially produce interleukin-17, interferon-gamma and granulocyte macrophage colony-stimulating factor. In the current study, we zoomed in on distinct effector phenotypes of human Th17 cells and their correlation with disease activity in multiple sclerosis patients. Th memory populations single- and double-positive for C-C chemokine receptor 6 (CCR6) and CXC chemokine receptor 3 (CXCR3) were functionally assessed in blood and/or cerebrospinal fluid from a total of 59 clinically isolated syndrome, 35 untreated and 24 natalizumab-treated relapsing-remitting multiple sclerosis, as well as 9 end-stage multiple sclerosis patients. Within the clinically isolated syndrome group, 23 patients obtained a second attack within 1 year and 26 patients did not experience subsequent attacks during a follow-up of more than 5 years. Low frequencies of T helper 1 (Th1)-like Th17 ($CCR6^+CXCR3^+$), and not Th17 ($CCR6^+CXCR3^-$) effector memory populations in blood strongly associated with a rapid diagnosis of clinically definite multiple sclerosis. In cerebrospinal fluid of clinically isolated syndrome and relapsing-remitting multiple sclerosis patients, Th1-like Th17 effector memory cells were abundant and showed increased production of interferon-gamma and granulocyte macrophage colony-stimulating factor compared to paired $CCR6^+$ and $CCR6^-$ $CD8^+$ T-cell populations and their blood equivalents after short-term culturing. Their local enrichment was confirmed ex vivo using cerebrospinal fluid and brain single-cell suspensions. Across all pro-inflammatory Th cells analyzed in relapsing-remitting multiple sclerosis blood, Th1-like Th17 subpopulation T helper 17.1 (Th17.1; $CCR6^+CXCR3^+CCR4^-$) expressed the highest very late antigen-4 levels and selectively accumulated in natalizumab-treated patients who remained free of clinical relapses. This was not found in patients who experienced relapses during natalizumab treatment. The enhanced potential of Th17.1 cells to infiltrate the central nervous system was supported by their predominance in cerebrospinal fluid of early multiple sclerosis patients and their preferential transmigration across human brain endothelial layers. These findings reveal a dominant contribution of Th1-like Th17 subpopulations, in particular Th17.1 cells, to clinical disease activity and provide a strong rationale for more specific and earlier use of T cell-targeted therapy in multiple sclerosis.

INTRODUCTION

Multiple sclerosis (MS) is mediated by effector T cells trafficking from the periphery into the central nervous system to trigger local inflammation, demyelination and neurodegeneration.[1] Although current T cell-directed treatment attenuates disease activity, it often causes serious complications and does not prevent disease progression in MS.[2] To improve treatment efficacy and risk management, more in-depth insight into human effector T cells during MS onset is warranted. In the earliest clinical presentation of MS, clinically isolated syndrome (CIS), increased peripheral $CD4^+$ T cell activation is linked to the occurrence of a second attack.[3] However, substantial knowledge has been gained about specific human T helper (Th) functions, and the exact nature of the pro-inflammatory Th subsets involved in MS is incompletely understood.

Both Th1 and Th17 cells are known to be encephalitogenic, but use distinct transmigration routes to enter the central nervous system. In experimental autoimmune encephalomyelitis, Th1 cells preferentially migrate into the spinal cord, while Th17 cells mainly infiltrate the brain.[4] This is facilitated by their differential expression of pro-inflammatory cytokines, chemokine receptors and integrins.[5-7] Interleukin-17 (IL-17) and C-C chemokine receptor 6 (CCR6) are key determinants for Th17 transmigration across the blood-brain barrier.[7,8] IL-17 is generally considered as the signature cytokine produced by CCR6-positive Th17 cells. However, this greatly underestimates Th17 effector function, since subpopulations also (co-)produce interferon-gamma (IFN-γ) and granulocyte macrophage colony-stimulating factor (GM-CSF). Next to IL-17, also IFN-γ and GM-CSF are strongly produced by myelin-specific CCR6-positive Th cells in MS.[9] Th17 polyfunctionality is differently regulated between species, as reflected by the antagonistic regulation of IL-17 and GM-CSF expression in human compared to murine Th cells.[10-12] Particularly GM-CSF produced by Th cells is implicated as a critical mediator of MS onset.[13,14]

The surface expression of another chemokine receptor, CXCR3, defines Th17 cells with Th1-like features.[15] CCR6 and CXCR3 expression on $CD4^+$ T cells is controlled by transcription factors RORγt and T-bet, respectively, which were originally associated with IL-17/IFN-γ double-production.[15] However, recent findings demonstrate more heterogeneous IL-17, IFN-γ and GM-CSF expression profiles in Th17 cells, depending on the inflammatory milieu.[16] Besides CCR6 and CXCR3, also the presence of the α4β1 integrin very late antigen-4 (VLA-4), which is abundant on Th17 cells in MS cerebrospinal fluid,[17] determines T-cell transmigration capacities. Anti-VLA-4 monoclonal antibody natalizumab is currently one of the most effective therapies in MS, but relapses are still encountered after one year in about one-third of treated patients.[18] Understanding which distinct pro-inflammatory Th subsets are targeted by natalizumab will help to better predict treatment response in MS.[19]

Here, blood and cerebrospinal fluid samples from CIS and both untreated and natalizumab-treated relapsing-remitting MS (RRMS) patients were assessed for distribution, memory phenotype, activation and pro-inflammatory capacity of Th17 subsets. We reveal that IFN-γ-/GM-CSF-producing ($CCR6^+CXCR3^+$), but not IL-17-producing ($CCR6^+CXCR3^-$) Th17 effector cells are key regulators of MS onset. A Th1-like Th17 subpopulation termed Th17.1 ($CCR6^+CXCR3^+CCR4^-$) is selectively targeted by natalizumab in MS patients who remained free of clinical relapses. This work supports the design and early use of therapeutic strategies against Th17.1 cells to prevent relapses in MS.

MATERIALS AND METHODS

Patients

Characteristics of patients and controls in the screening cohorts are summarized in Table 1. Main experimental results were confirmed using additional cohorts (Supplementary Table 1). All CIS and RRMS patients as well as controls were included at Erasmus MC (Rotterdam, The Netherlands), which is a national tertiary referral centre for MS patients (MS Centre ErasMS). All primary material was collected between 2007 and 2017.
For blood analyses, we selected 23 CIS patients who did not experience a second clinical attack for at least 5 years of follow-up (CIS-CIS) and 26 CIS patients who were diagnosed with clinically definite MS (CDMS) within 1 year after CIS (CIS-CDMS) from our prospective cohort. None of these patients were treated with disease-modifying therapies before or at time of sampling. CIS was defined as a first clinical attack of demyelination in the central nervous system.[20] CDMS diagnosis was made when a patient experienced two attacks with clinical evidence of two separate lesions according to the Poser criteria.[21] A relapse was defined as sub-acute worsening of existing symptoms, or new symptoms after at least 30 days of improvement or stable disease.[22] Fatigue scores were acquired at time of the first attack using the self-administered Krupp's Fatigue Severity Scale (FSS), as shown previously.[23] Anti-EBNA1 IgG levels were determined in plasma using a well-validated chemiluminescent assay and analyzer (Liaison XL; both Diasorin, Saluggia, Italy) according to manufacturers' instructions at our local referral centre for virus diagnostics (Erasmus MC).
RRMS patients were diagnosed according to the McDonald 2010 criteria.[24] Blood Th cell analyses were performed for 31 treatment-naive RRMS patients, as well as for 24 RRMS patients before start and after both 6 and 12 months of natalizumab therapy. The median time between the last clinical attack and first administration of natalizumab was 2.8 months (IQR: 1.7-6.3). Seventeen of these patients (70.8%) were treated with disease-modifying therapy before initiation of natalizumab (14 with interferon (IFN)-β, 1 with both dimethylfumarate and fingolimod, 1 with glatiramer acetate and 1 with mitoxantron).
Cerebrospinal fluid with and without paired blood samples were obtained from 14 early-stage MS patients (ErasMS) and 9 late-stage MS patients (Netherlands Brain Bank, Amsterdam). In the early-stage MS group, 10 patients were CIS at the time of lumbar puncture and 4 patients were diagnosed with RRMS within 6 months before sampling. The median time between sampling and the last clinical attack was 2.8 months (IQR: 1.3-5.8). Additional autopsied brain tissues were obtained from 5 late-stage MS patients and 2 non-demented controls (Netherlands Brain Bank). All study protocols were approved by the medical ethics committee of the Erasmus MC (Rotterdam) and VUmc (Amsterdam, The Netherlands). Written informed consent was obtained from all included patients and controls.

Blood, ex vivo					
Cohort	HC	CIS-CIS	CIS-CDMS	RRMS, no Tx[g]	RRMS, Nat Tx[g]
Patients, n	19	16	16	18	17[a]
Gender, female n (%)	16 (84)	11 (69)	13 (81)	15 (83)	12 (71)
Age (years), median (IQR)[b]	45 (35-49)	36 (27-40)	33 (28-37)	46 (37-50)	38 (30-46)[c]
Follow-up time (years), median (IQR)	na	6.8 (6.2-7.3)	4.1 (3.1-5.7)	na	na
Disease duration (months), median (IQR)[d]	na	2.0 (1.3-3.1)	2.0 (1.2-3.0)	120 (48-193)	92 (48-160)[c]
≥9 T2 lesions at baseline, n (%)	na	3 (19)	10 (63)	na	na
CSF/brain, ex vivo				**CSF, TCC**	
Cohort[e]	Early MS	Late MS	Late NDC	Early MS	Late MS
Patients, n (paired blood)	4 (4)	5 (5)	2 (2)	10 (4)	7 (7)[f]
Gender, female n (%)	2 (50)	5 (100)	1 (50)	10 (100)	5 (71)
Age in years, median (IQR)[b]	32 (18-41)	62 (44-72)	78 (NA)	33 (23-38)	70 (60-82)
Follow-up time (years), median (IQR)	0.3 (0.3-0.5)	na	na	1.5 (0.6-5.5)	na
Disease duration (months), median (IQR)[d]	3.8 (2.7-5.2)	na	na	3.8 (1.0-22.4)	na
PMD (hours), median (IQR)	na	8.5 (8.4-9.2)	6.1 (NA)	na	8.6 (8.3-9.3)
pH CSF, median (IQR)	na	6.3 (6.3-6.7)	6.5 (NA)	na	6.5 (6.3-6.7)

Table 1. Characteristics of patients and controls in screening cohorts

[a]Fourteen patients were included for in-depth analysis of Th17 subpopulations in blood. For three patients, Th subsets were only used for analysis of pro-inflammatory cytokine expression.

[b]At time of sampling

[c]At time of pretreatment sampling

[d]Time from CIS diagnosis to sampling

[e]Samples obtained from either CIS and RRMS patients ('early') or deceased MS patients and NDC ('late')

[f]Three patients were also used for ex vivo CSF/brain T-cell analysis

[g]RRMS according to the McDonald 2010 criteria

Abbreviations: CDMS, clinically definite MS; CIS, clinically isolated syndrome; CSF, cerebrospinal fluid; IQR, interquartile range; na, not applicable or available; Nat, natalizumab; NDC, non-demented control; PMD, post-mortem delay; RRMS, relapsing-remitting MS; TCC, T-cell culture; Tx, treatment

Mononuclear cell isolation from blood, cerebrospinal fluid and brain tissue

Blood from patients and matched controls was collected using Vacutainer CPT tubes (BD Biosciences, Erembodegem, Belgium) containing sodium heparin. Peripheral blood mononuclear cells were isolated according to manufacturer's instructions. After centrifugation, cells were taken up in RPMI1640 (Lonza, Verviers, Belgium) containing 40% fetal calf serum (FCS) and 20% dimethyl sulfoxide (Sigma-Aldrich, Saint-Louis, MO) and stored

in liquid nitrogen until further use. Surplus cerebrospinal fluid of early-stage MS patients was obtained through lumbar puncture for diagnostic purposes. Blood and cerebrospinal fluid samples from late-stage MS patients were acquired post-mortem through heart puncture and ventricle drainage, respectively. Collection tubes with cerebrospinal fluid were centrifuged for 10 min at 500g. Paired blood and blood from buffy coats were diluted in PBS, after which mononuclear cells were isolated by density gradient centrifugation using Ficoll-Paque Plus (GE Healthcare, Freiburg, Germany). Cerebrospinal fluid and blood mononuclear cell fractions were resuspended in RPMI 1640 containing with 10% heat inactivated human AB serum (Sanquin, Rotterdam, The Netherlands) and 1% Pen/Strep (Lonza) and left to rest at 37oC until further use. Brain tissue samples were processed and single-cell suspensions were obtained as recently published.[25]

Short-term cerebrospinal fluid and blood T-cell cultures

Short-term culturing of cerebrospinal fluid-derived T cells was required to obtain sufficient cell numbers for FACS sorting and intracellular cytokine staining of the Th subsets of interest. Cerebrospinal fluid and blood T cells were cultured as previously described.[26] In short, mononuclear cell fractions were treated for 13 to 15 days with γ-irradiated feeder cells ($10x10^6$ PBMC and $1x10^6$ EBV- B-cell lines HAL-02 and RS-411), phytohemagglutinin-L (1 ng/ml; Sigma-Aldrich), IL-2 (25 U/ml; Erasmus MC) and IL-15 (12.5 ng/µl; Miltenyi Biotec, Leiden, The Netherlands) in RPMI1640 containing L-glutamine (Lonza), 1% Pen/Strep and 10% heat-inactivated human AB serum. IL-2 and IL-15 were added every 3 to 4 days. Post-mortem cerebrospinal fluid samples were re-stimulated using the same protocol.

Antibodies and flow cytometry

Multicolor flow cytometric analysis was performed using the following fluorochrome-labeled monoclonal anti-human antibodies: CD3 BV785 (SK7), CD8 FITC (SK1), CD45RA APC-H7 (HI100), HLA-DR FITC and BB515 (G46-6), VLA-4 BV711 and APC (9F10), CD45RO PerCP-Cy5.5 (UCHL1), CD25 BV605 and APC-R700 (2A3), CD226 BB515 (DX11), MCAM PerCP-Cy5.5 (P1H12), PSGL-1 APC (KPL-1), GM-CSF BV421 and PE-CF594 (BVD2-21C11; all BD Biosciences), CD4 BV510 (OKT4), CD38 BV711 and PerCP-Cy5.5 (HIT2), CXCR3 BV421, PE-Cy7 and APC (G025H7), CCR6 PE (G034E3), CCR7 PE-CF594 (150503), CCR4 PE-Cy7 and PE-Dazzle (L291H4), CD161 BV605 (HP-3G10), IFN-γ BV421 and BV711 (4S.B3), and IL-17A APC (BL168; all Biolegend, London, UK). Cells were stained for 30 min at 4oC, measured with an LSRII-Fortessa flow cytometer and analyzed using FACSDiva 6.1.2 software (both BD Biosciences). Th1, Th17 and Th1-like Th17 cells were defined based on markers CCR6 and CXCR3 with and without the use of CCR4. For analyses without CCR4, total $CD4^+$ T cells were subdivided into $CCR6^-CXCR3^+$ (Th1), $CCR6^+CXCR3^-$ (Th17) and $CCR6^+CXCR3^+$ (Th1-like Th17) subsets. In each Th subset, the proportion of effector memory ($CCR7^-CD45RA^-$) and central memory ($CCR7^-CD45RA^+$) cells was analyzed. CCR4 was used as a marker to discriminate $CCR6^-CXCR3^+CCR4^-$ (Th1), $CCR6^+CXCR3^-CCR4^+$ (Th17), $CCR6^+CXCR3^+CCR4^-$ (Th17.1) and $CCR6^+CXCR3^+CCR4^+$ (Th17 double-positive, DP) subpopulations.[27]

Intracellular cytokine staining

Th1 ($CCR6^-CXCR3^+$), Th17 ($CCR6^+CXCR3^-$), Th1-like Th17 ($CCR6^+CXCR3^+$), as well as $CCR6^-$ and $CCR6^+$ $CD8^+$ T cells were sorted from blood and cerebrospinal fluid T-cell memory pools ($CD3^+CD25^{-/int}$ $CD45RO^+CD45RA^-$) using a BD FACSAria™ III cell sorter. Prior to isolation of Th memory subsets from buffy coats, $CD4^+$ cells were purified from the mononuclear cell fraction using CD4 microbeads and the autoMACS Pro Separator (both Miltenyi Biotec). Cells were stimulated with phorbol 12-myristate 13-acetate (PMA; 1:2000) and ionomycin (1:500; both Sigma-Aldrich) for 5 h. GolgiStop (1:1500; BD Biosciences) was added during the last 2.5 h of stimulation. Stimulated cells were fixed and permeabilized using the BD Cytofix/CytopermTM kit (BD Biosciences) according to the provided protocol, and stained for IFN-γ, GM-CSF and IL-17A within the same tube.

RNA isolation and quantitative PCR

Sorted T-cell subsets were washed with PBS and resuspended in RNA lysis solution with 1% 2-ME. Total RNA was extracted using the GenElute™ Total RNA Purification kit (Sigma-Aldrich) and treated with DNase I (Invitrogen, Carlsbad, CA). Complementary DNA (cDNA) was synthesized from total RNA using a reaction mix containing Tris-aminomethane (200 mM), KCl (500 mM), $MgCl_2$ (0.2M; Sigma-Aldrich), DTT (100mM; Invitrogen), random hexamers (50μM; Invitrogen), oligo(dT) 15 primer (100 μg/ml; Promega, Madison, WI), dNTP mix (10 mM; Promega), RNAsin (40 U/μl; Promega) and superscript II (200 U/μl; Invitrogen). After incubation at 42°C for 50 min and inactivation at 990C for 3 min, cDNA was diluted and stored at -200C until use. For quantitative PCR, 0.2μM forward and reverse primer (Sigma-Aldrich), 10 μM probe (Universal Probe Library; Roche Applied Science, Penzberg, Germany) and diluted cDNA were added to Taqman Universal PCR Master Mix. Target gene expression was measured using optimal primer/probe assays and Taqman 7900HT (Applied Biosystems, Foster City, CA). We used the following thermal cycle protocol: 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. CT values were analyzed using SDS 2.4.1 software (Applied Biosystems). Expression levels of target genes were normalized using 18S rRNA as a reference. Primer sequences are indicated in Supplementary Table 2.

T-cell transmigration assays

$CD4^+CD25^{-/int}$ memory T cells depleted from naive populations ($CCR7^+CD45RA^+$) were sorted by FACS and added at $2x10^5$ cells/well to 3 μm pore size transwell plates (Corning, Amsterdam, The Netherlands). Migration of Th17 subsets towards medium or CXCL10 (900ng/ml; R&D Systems, Abingdon, UK) was analyzed after 3 h incubation at 37°C using FACS. To assess transendothelial migration of Th17 subsets, migration experiments were performed across confluent monolayers of human brain endothelial cells (hCMEC/D3 cell line)[28] on collagen-coated 5 μm pore size transwells, as previously described.[29] In this system, $5x10^5$ Th memory cells were added per well and migration was assessed after 4 h.

Statistical analyses

Statistical analyses were carried out using Graphpad Prism Software, version 5.04. We used the two-tailed Mann-Whitney U test to compare two independent groups and the Wilcoxon matched-pairs signed rank test to compare samples of the same persons. Correlations were tested using Spearman's rank. A logistic regression model was used to correct for MRI measurements in the multivariable analyses. Experimental data are depicted as mean and standard error of the mean. P values less than 0.05 were considered significant.

RESULTS

Low frequencies of Th1-like Th17 and not Th17 effector cells in CIS blood associate with rapid MS onset

To search for pro-inflammatory Th subsets that are critically involved in early diagnosis of clinically definite MS (CDMS), we used PBMC at time of CIS from age- and gender-matched patients who remained monophasic for at least 5 years (CIS-CIS, n=16) and from patients who experienced a second attack within 1 year (CIS-CDMS, n=16; Table 1). Flow cytometric analysis of $CD4^+$ T cells showed decreased Th1-like Th17 ($CCR6^+CXCR3^+$) frequencies in the CIS-CDMS group compared to the CIS-CIS group (median: 5.9% vs 11.2%, p=0.011; Fig. 1A). After correction for lesion load on MRI at baseline, using a logistic regression model, the association remains significant (OR: 0.78 per percent increase in Th1-like Th17; p=0.026). In CIS-CDMS, additional reductions in effector memory (EM) to central memory (CM) ratios were found for Th1-like Th17 (mean: 0.30 vs 0.50, p=0.005; Fig. 1B). Similar but less strong reductions were observed for Th1 ($CCR6^-CXCR3^+$; 10.0% vs 12.5%, p=0.070 and mean EM/CM ratio: 0.21 vs 0.29, p=0.021). Frequencies and EM/CM ratios for Th17 ($CCR6^+CXCR3^-$) did not differ between CIS-CIS and CIS-CDMS (Fig. 1A and B). Th subset distribution in CIS patients was not affected after stratification for methylprednisolone treatment in the last 3 months prior to sampling (data not shown). Finally, Th1-like Th17 EM/CM ratios in CIS blood inversely correlated to anti-EBNA1 IgG titers (p=0.013; Fig. 1C) and fatigue (p=0.001; Fig. 1D), which were reported as independent predictive markers for early CDMS diagnosis.[23,30]

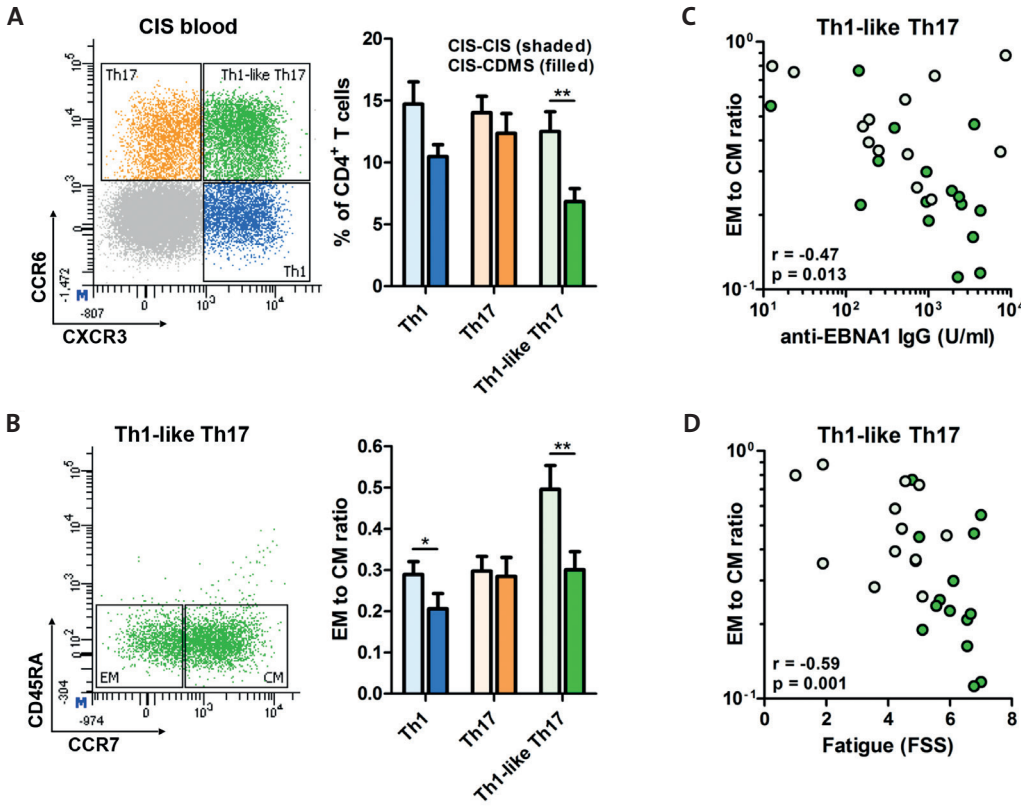


Figure 1. Reduction of Th1-like Th17 effector cells in the blood of CIS patients with short time to CDMS

*CIS patients were selected based on blood sampling within 4 months after diagnosis and time between CIS and CDMS. 'CIS-CDMS' patients were diagnosed with CDMS within 1 year (n=16; filled bars), while 'CIS-CIS' patients were not diagnosed with CDMS for at least 5 years (n=16; shaded bars). $CD4^+$ T cells in the blood were compared for (**A**) Th1 ($CCR6^-CXCR3^+$), Th17 ($CCR6^+CXCR3^-$) and Th1-like Th17 ($CCR6^+CXCR3^+$) cell distribution, as well as (**B**) effector memory (EM; $CCR7^-CD45RA^-$) to central memory (CM; $CCR7^+CD45RA^-$) cell ratios within each of these subsets, as determined by flow cytometry. Th1-like Th17 effector to central memory cell ratios were correlated to reported predictors of early CIS to CDMS transition, anti-EBNA1 IgG blood titer (**C**) and fatigue severity scale (FSS; **D**). * $p < 0.05$; ** $p < 0.01$*

Effector populations of highly activated Th1-like Th17 cells are reduced in blood after MS diagnosis

To verify that these selective differences in Th subsets are associated with MS diagnosis, we explored total frequencies of blood Th1 EM and Th1-like Th17 EM cells in treatment-naive RRMS patients (n=18, Table 1), and age-/gender-matched healthy controls (HC, n=19). Strongly reduced frequencies were found for both these subsets in RRMS (median: 1.1% and 0.7%) compared to CIS-CIS (1.9%, $p < 0.001$ and 2.8%, $p < 0.0001$) and

HC (2.8%, p < 0.001 and 3.3%, p < 0.0001; Fig. 2A). These frequencies did not significantly differ between the RRMS and CIS-CDMS (1.0% and 1.3%) group. In RRMS, a significant proportion of blood Th1-like Th17 cells was positive for both CD38 and HLA-DR (Fig. 2B), indicating a highly activated phenotype after MS diagnosis. This was not seen for Th1 cells (Fig. 2B). These data suggest that Th1-like Th17 effector cells are selectively activated in the periphery and recruited to the central nervous system during MS onset.

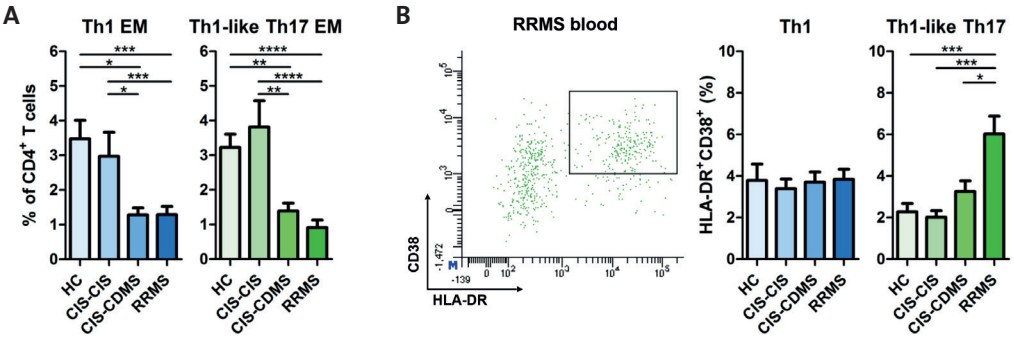


Figure 2. Th1-like Th17 effector cells are highly activated and less present in the blood after MS diagnosis
*(**A**) Th1 and Th1-like Th17 effector memory (EM) frequencies in CD4+ T cells from CIS-CIS (n=14) and CIS-CDMS (n=16) as well as RRMS (n=18) and both age- and gender-matched healthy control (HC; n=19) blood.*
*(**B**) Highly activated fractions of blood Th1 and Th1-like Th17 cells in CIS-CIS (n=14), CIS-CDMS (n=16) and RRMS (n=18) patients as well as HC (n=19), as defined by co-expression of late T-cell activation markers HLA-DR and CD38. * p < 0.05; ** P < 0.01; *** p < 0.001; **** p < 0.0001*

Predominant expression of T-bet and RORγt, as well as IFN-γ and GM-CSF, but not IL-17A by activated blood Th1-like Th17 cells

Human CCR6+ CD4+ T cells are not only strong producers of IL-17, but also express IFN-γ and GM-CSF.[9,27] To explore how these pro-inflammatory cytokines are co-regulated in our phenotypically defined Th1-like Th17 (CCR6+CXCR3+) cells, T-bet and RORγt, as well as IFN-γ, GM-CSF and IL-17A expression was compared to paired Th1 (CCR6-CXCR3+) and Th17 (CCR6+CXCR3-) populations from healthy blood donors. Th1-like Th17 expressed both T-bet and RORγt mRNA at higher levels than Th1 (p=0.016 and p=0.004) and Th17 (p=0.008 and p=0.039, respectively; Fig. 3A). IFN-γ mRNA levels were similar between Th1 and Th1-like Th17, while GM-CSF mRNA levels in Th1-like Th17 were higher than in Th1 (p=0.008) and Th17 (p=0.020). Th1-like Th17 cells only moderately expressed IL-17A mRNA (Fig. 3B). Differences in IFN-γ, GM-CSF and IL-17A expression were verified on protein level (Fig. 3C). The percentage of GM-CSF-positive cells was 2- to 3-fold higher in Th1-like Th17 than in Th1 and Th17. IL-17A-positive cells were approximately 4-fold less present in Th1-like Th17 compared to Th17. At single-cell level, IFN-γ was mainly co-expressed with GM-CSF and not with IL-17A in Th1-like Th17 cells (Fig. 3D). These cytokine profiles were the same for Th subsets from CIS and RRMS blood (data not shown). TNF-α expression was comparable between Th1, Th17

and Th1-like Th17 subsets (Fig. 3B). CD226 was higher, while CD25 and FoxP3 were lower expressed by Th1-like Th17 as compared to Th17 (Fig. 3A and Supplementary Table 3), which supported their pro-inflammatory potential.[31, 32] Th1-like Th17 cells also showed sustained CD161 expression (Supplementary Table 3), reflecting an ex-Th17 phenotype.[33]

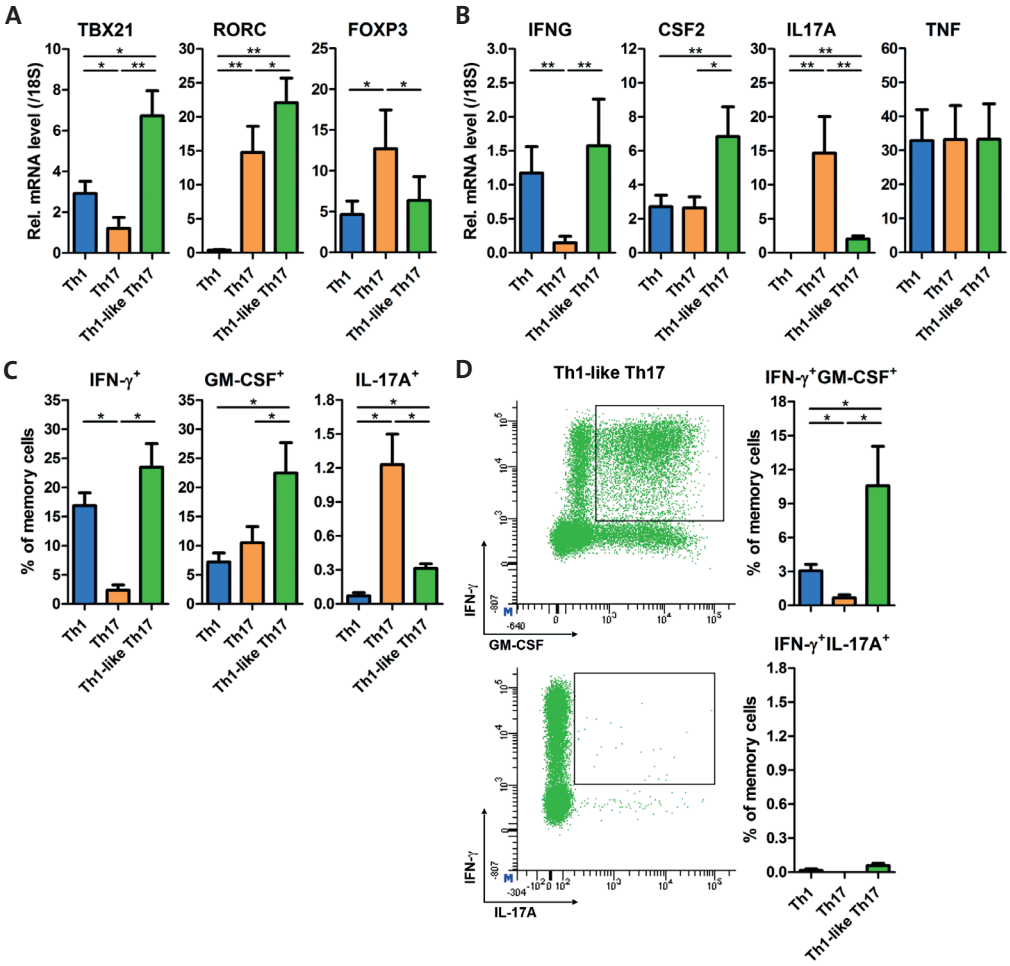


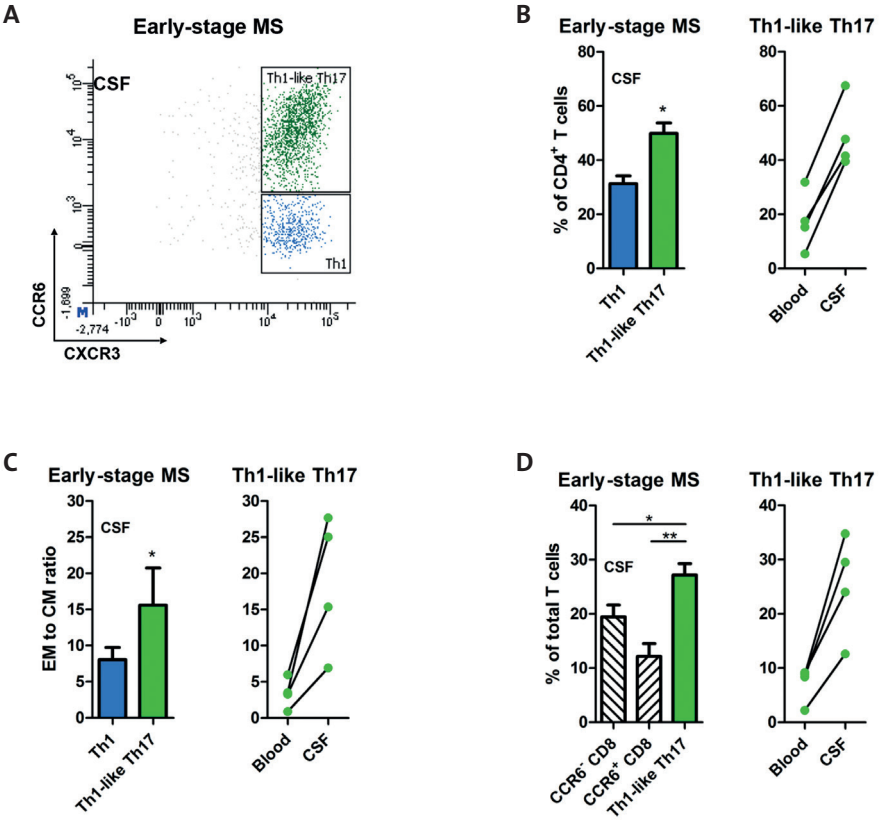
Figure 3. Blood Th1-like Th17 cells predominantly express T-bet and RORγt, and are high IFN-γ and GM-CSF, but low IL-17A producers

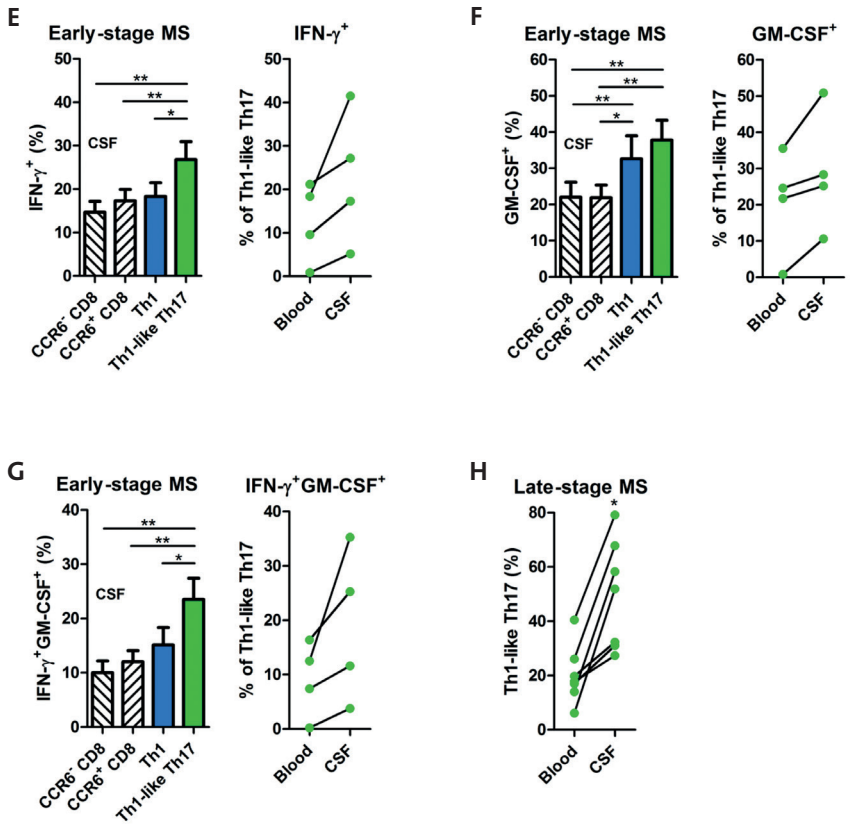
*Buffy coats from 9 healthy blood donors were used to assess gene expression of TBX21, RORC and FOXP3 (**A**), as well as IFNG, CSF2, IL17A and TNFA (**B**) in sorted Th1 (CCR6$^-$CXCR3$^+$), Th17 (CCR6$^+$CXCR3$^-$) and Th1-like Th17 (CCR6$^+$CXCR3$^+$) memory populations. Cells were stimulated with PMA and ionomycin and mRNA levels were measured using quantitative PCR. (**C**) Flow cytometric analysis of intracellular IFN-γ, GM-CSF and IL-17A expression in PMA- and ionomycin-stimulated Th1, Th17 and Th1-like Th17 memory cells from the same blood donors (n=7). (**D**) Representative dot plots and quantification of co-expression of IFN-γ with GM-CSF and IL-17A in Th1-like Th17 memory cells (n=7). * $p < 0.05$; ** $p < 0.01$*

Th1-like Th17 cells of MS patients are more abundant and pro-inflammatory in cerebrospinal fluid than in paired blood T-cell cultures

To explore the local pro-inflammatory capacity of Th1-like Th17 cells in early MS, cerebrospinal fluid (n=10) and paired blood (n=4) T-cell subsets from early-stage MS patients (Table 1) were analyzed after short-term culturing. Th1 and Th1-like Th17 were the main populations in the cerebrospinal fluid CD4$^+$ T-cell pool (Fig. 4A). Proportions of Th1-like Th17 were higher than those of Th1 (p=0.020) and their equivalents in blood (2- to 3-fold increase; Fig. 4B). Similar results were obtained with EM/CM ratios, which were high for both subsets but most prominent in Th1-like Th17 cells in cerebrospinal fluid (Fig. 4C). Within the total cerebrospinal fluid T-cell pool, Th1-like Th17 cells were enriched and co-produced more IFN-γ and GM-CSF compared to CCR6$^-$ and CCR6$^+$ CD8$^+$ T cells, and paired blood counterparts (Fig. 4D to G). The percentage of IFN-γ-positive cells was increased in cerebrospinal fluid Th1-like Th17 versus Th1 (Fig. 4E). The enrichment of Th1-like Th17 in cerebrospinal fluid compared to blood (Fig. 4A and B) was also found in T-cell cultures from late-stage MS patients (n=7, p=0.016; Table 1 and Fig. 4H), suggesting that Th1-like Th17 recruitment to the central nervous system also occurs at later stages of the disease.

Figure 4. Prevalence of pro-inflammatory Th1-like Th17 cells in CIS and MS CSF compared to blood T-cell cultures.

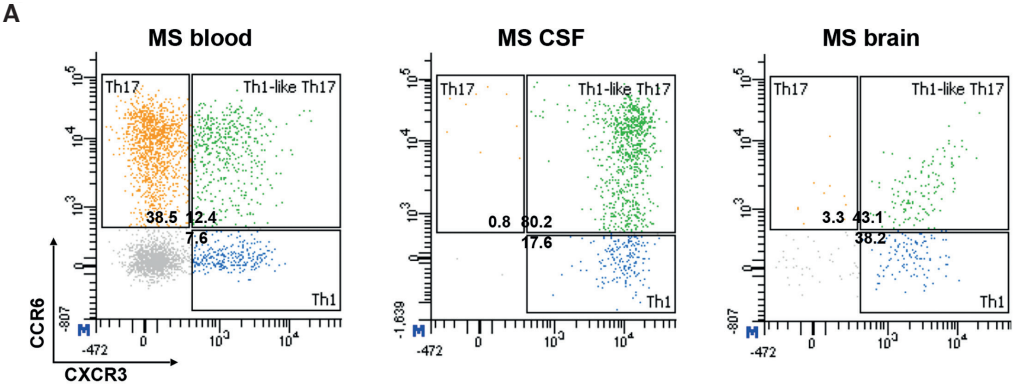




*(**A-D**) Presence of Th1-like Th17 ($CCR6^+CXCR3^+$) subsets in short-term cerebrospinal fluid T-cell cultures from 10 early-stage MS patients (CIS, n=7; RRMS, n=3). Cerebrospinal fluid Th1-like Th17 were compared to Th1 cells and their equivalents in blood for percentages in the total $CD4^+$ T-cell pool (**A, B**) and for effector memory (EM) to central memory (CM) ratios (**C**) from the same patients. Similar analyses were performed for cerebrospinal fluid Th1-like Th17 and both $CCR6^-$ and $CCR6^+$ $CD8^+$ T-cell subsets within the total T-cell pool (**D**). These T-cell subsets were separated, stimulated with PMA and ionomycin and assessed for intracellular expression of (**E**) IFN-γ, (**F**) GM-CSF and (**G**) IFN-γ with GM-CSF. For each analysis, Th1-like Th17 subsets were compared between paired cerebrospinal fluid and blood T-cell cultures. (**H**) Th1-like Th17 frequencies in T-cell cultures from paired blood and cerebrospinal fluid of late-stage MS patients (n=7). * $p < 0.05$; ** $p < 0.01$*

Ex vivo Th1-like Th17 cells are enriched in the central nervous system and accumulate in the blood after natalizumab treatment of MS patients

To confirm their recruitment to the central nervous system, we compared ex vivo Th1 and Th1-like Th17 frequencies in single-cell suspensions of 10 brain tissues and paired cerebrospinal fluid and blood samples of 5 late-stage MS patients (Table 1, Fig. 5A and B). Th1 and Th1-like Th17 cells were overrepresented, while Th17 cells were hardly seen in MS brain tissues and cerebrospinal fluid, in contrast to blood. The enrichment of Th1-like Th17 was also found in cerebrospinal fluid, but was less in brain tissues from 2 non-demented controls (Fig. 5B), suggesting that enhanced infiltration of Th1-like Th17 cells into the brain parenchyma is associated with MS.[34-36]
In addition to chemokine receptors and pro-inflammatory cytokines,[6,7] adhesion molecules play a key role in migration of peripheral Th cells into the central nervous system, including VLA-4, MCAM and PSGL-1.[37] Interestingly, VLA-4 but not MCAM and PSGL-1 was the most abundant on Th1-like Th17 cells (Supplementary Table 3). In RRMS patients (n=14), blood Th1-like Th17 proportions were elevated after 6 months of treatment with natalizumab (anti-VLA-4 monoclonal antibody; median pre- vs post-Tx: 7.7% vs 10.4%, p=0.006; Fig. 5C and Table 1). Th1-like Th17 cells did not show differences in EM/CM ratio (data not shown), but their activation state (see also Fig. 2B) was significantly reduced after natalizumab therapy (Fig. 5D). Th1-like Th17 showed increased capacity to produce IFN-γ and GM-CSF in post- versus pre-treatment blood samples (Fig. 5E and F). These results show that the effects of natalizumab in MS are associated with an accumulation of Th1-like Th17 cells in the blood.



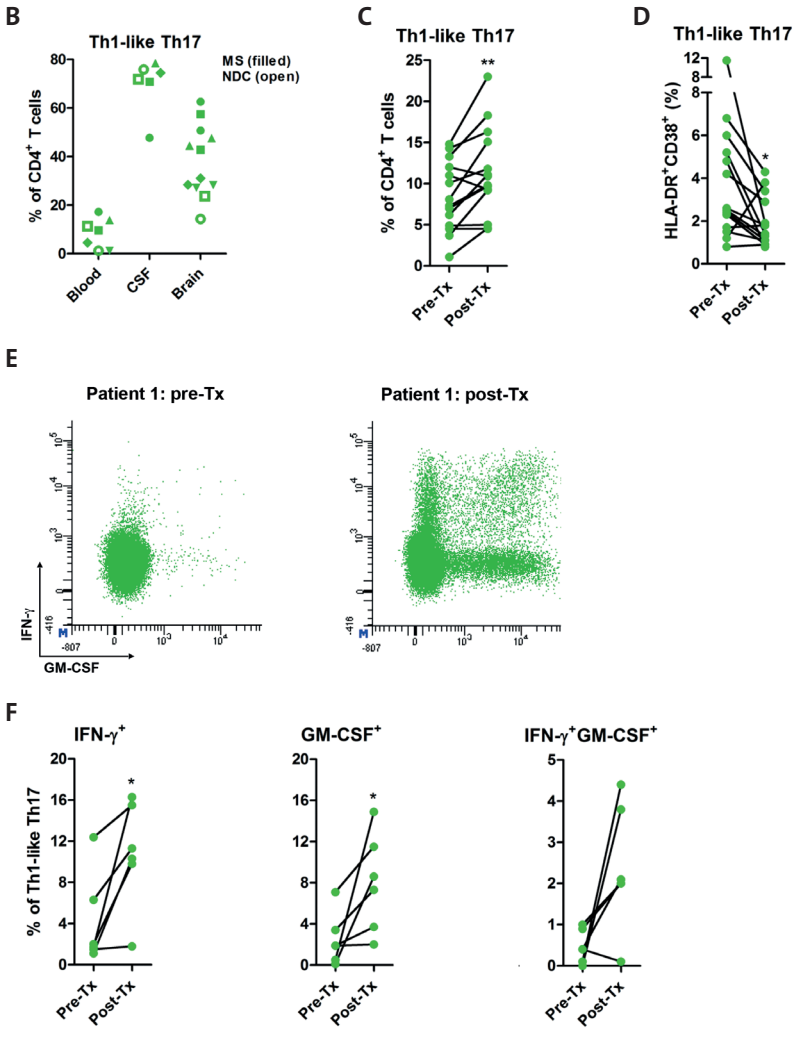
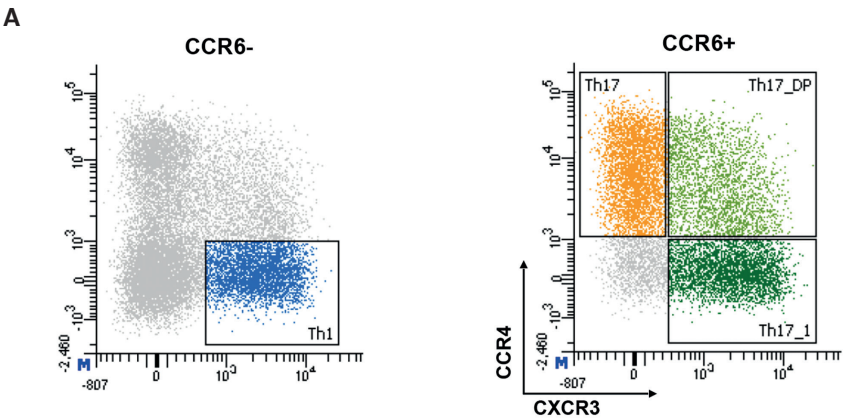


Figure 5. Th1-like Th17 recruitment to the central nervous system and targeting by natalizumab in MS patients. *(**A**) Presence of Th1 (CCR6$^-$CXCR3$^+$), Th17 (CCR6$^+$CXCR3$^-$) and Th1-like Th17 (CCR6$^+$CXCR3$^+$) cells in single-cell suspensions from brain tissue, cerebrospinal fluid and blood of an MS patient, as determined by FACS. (**B**) Th1-like Th17 frequencies in 10 brain tissues and paired cerebrospinal fluid and blood samples from 5 different MS patients (filled shapes). Similar analyses were performed for 2 non-demented controls (NDC; open shapes). Each shape represents a different donor. For Th1-like Th17 cells in MS blood, frequencies (n=14; **C**), activation (n=14; **D**) as well as pro-inflammatory capacities (n=6; **E** and **F**) were determined before and 6 months after natalizumab treatment. T-cell activation was assessed by surface expression of both HLA-DR and CD38. To determine their pro-inflammatory capacity, Th1-like Th17 memory cells were isolated from pre- and post-treatment blood, stimulated with PMA and ionomycin, and stained for intracellular IFN-γ and GM-CSF. * $p < 0.05$; ** $p < 0.01$*

Predominant targeting of VLA-4high Th17.1 cells by natalizumab in MS patients who do not experience clinical relapses

To assess the selectivity of natalizumab effects on pro-inflammatory Th populations in MS patients, CCR4 was included as a surface marker in our flow cytometric panels for subdivision of Th1-like Th17 into recently described pathogenic Th17.1 (IFN-γ^{high}GM-CSF^{high}IL-17^{low}) and Th17 double-positive (DP; IFN-γ^{low}GM-CSF^{low}IL-17^{int}) subpopulations [27,38]. Th17.1 (CCR6$^+$CXCR3$^+$CCR4$^-$) frequencies were significantly increased in RRMS blood samples after both 6 and 12 months of treatment (median: 5.3% and 6.1%) versus pre-treatment (3.7%; n=14, both p=0.0002; Table 1; Fig. 6A and B). No significant differences were found in Th1 (CCR6$^-$CXCR3$^+$CCR4$^-$), Th17 (CCR6$^+$CXCR3$^-$CCR4$^+$) and Th17 DP (CCR6$^+$CXCR3$^+$ CCR4$^+$) cells (Fig. 6A and B). Importantly, this accumulation of Th17.1 was most pronounced in natalizumab-treated patients who were free of clinical relapses (n=9; pre-Tx, 3.8% vs 6m post-Tx, 6.5% and 12m post-Tx, 6.8%; p=0.008 and p=0.004, respectively). As compared to patients who had relapses during treatment (n=5; pre-Tx, 3.2% vs 6m post-Tx, 4.0% and 12m post-Tx, 4.1%; Fig. 6C). The accumulation of Th17.1 cells in the blood of clinical responders was validated using a second cohort (Supplementary Table 1; Supplementary Fig. 1A).
In pre-treated RRMS blood, VLA-4 surface expression on Th17.1 (mean MFI: 2603) was the highest of all pro-inflammatory Th subsets analyzed, including Th1 (MFI: 1328, p=0.001), Th17 (MFI: 1255, p=0.0001) and Th17 DP (MFI: 2038, p=0.002; Fig. 6D and Supplementary Fig. 1B). After natalizumab treatment, VLA-4 was downregulated on all subsets analyzed, but this was most prominent for Th17.1 (mean reduction: 6m post-Tx, 56%, 12m post-Tx, 58%), as compared to Th1 (6m post-Tx, 52%, p=0.038; 12m post-Tx, 54%, p=0.005), Th17 (6m post-Tx, 37%, p=0.003; 12m post-Tx, 38%, p=0.0009) and Th17 DP (6m post-Tx, 49%, p=0.002; 12m post-Tx, 50%, p=0.002; Fig. 6E). This indicates that Th17.1 cells are preferentially targeted by natalizumab treatment, preventing their transmigration into the central nervous system of MS patients.



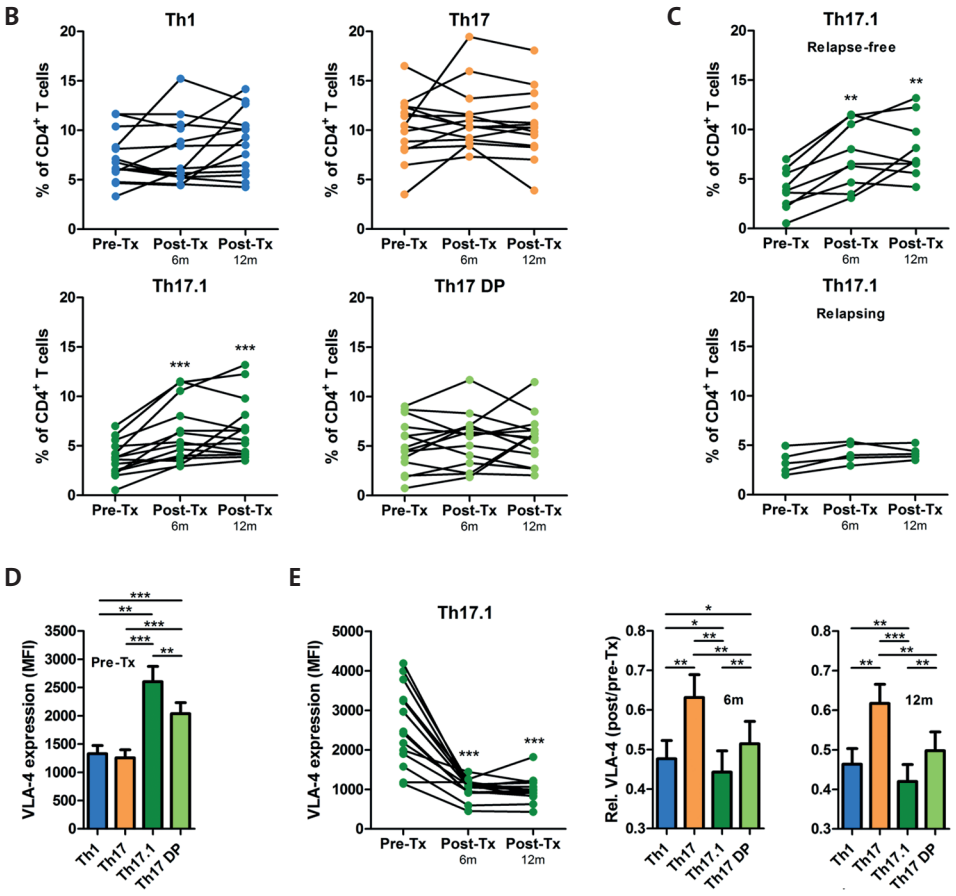


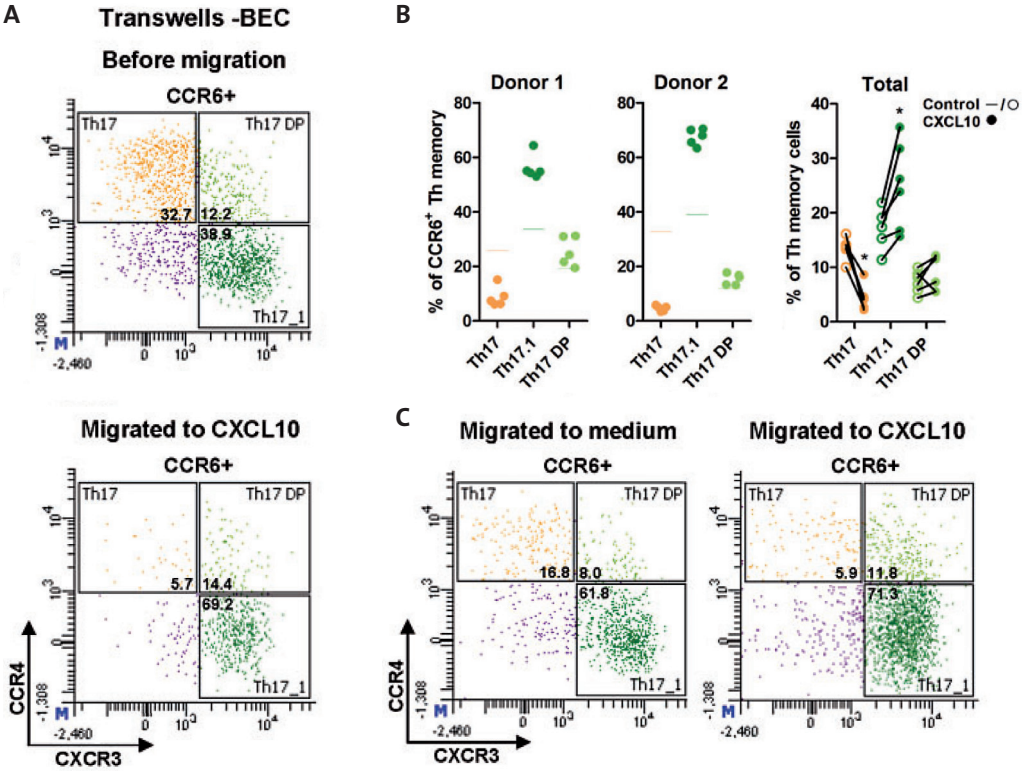
Figure 6. Selective accumulation of Th17.1 cells in natalizumab-treated MS patients who do not experience clinical relapses.

*Using CCR4 as an additional marker, Th1-like Th17 cells were subdivided into Th17.1 ($CCR6^+CXCR3^+CCR4^-$) and Th17 DP ($CCR6^+CXCR3^+CCR4^+$) subsets and analyzed in natalizumab-treated RRMS patients by flow cytometry (**A**). Th1 ($CCR6^-CXCR3^+CCR4^-$), Th17 ($CCR6^+CXCR3^-CCR4^+$), Th17.1 and Th17 DP cells were monitored in pre- and both 6 and 12 months post-treatment blood samples (n=14; **B**). Th17.1 proportions were separately evaluated in relapse-free (n=9) and relapsing (n=5) treatment groups (**C**). VLA-4 surface expression levels were determined on these Th subpopulations before (**D**) and both 6 and 12 months after (**E**) natalizumab treatment. * $p < 0.05$; ** $P < 0.01$; *** $p < 0.001$*

Pathogenic Th17.1 cells have a superior capacity to transmigrate into the central nervous system in early MS

To further study the central nervous system transmigration potential of Th17.1 in MS, we performed different in vitro transwell migration assays using total Th memory cell fractions. Th17.1 was the main Th17 subpopulation migrating across transwell filters towards inflammatory mediator CXCL10 (Fig. 7A and B).[39] No migration was observed towards

medium only (data not shown). Particularly Th17.1 cells did show spontaneous transmigration across human brain endothelial layers (hCMEC/D3), which was enhanced under CXCL10-attracting conditions (Fig. 7C and D).
In addition, ex vivo flow cytometric analysis revealed an enrichment of Th17.1 versus Th17 and Th17 DP cells in cerebrospinal fluid versus paired blood samples from 4 early-stage MS patients (3 CIS and 1 RRMS; Table 1; Fig. 7E and F). Consistently, lowered Th17.1 frequencies were found in the blood from 26 CIS-CDMS versus 21 CIS-CIS patients (p=0.019), as well as 13 RRMS patients versus 12 matched HC (p=0.031; Fig. 7G and Supplementary Table 1). Both Th17.1 and Th17 DP cells were abundant in cerebrospinal fluid compared to blood from late-stage MS patients (Supplementary Fig. 2).
Finally, to confirm that Th17.1 is a distinct pro-inflammatory Th17 subset, we evaluated the expression of key regulators of Th17 differentiation and pathogenicity. Along with VLA-4 (see also Fig. 6D), CD161, CD226, MDR1 (ABCB1), IL-23R, STAT4, TOSO and GZMB (granzyme B; all upregulated), as well as CD25 and BATF (downregulated) were discriminative markers for Th17.1 (Supplementary Table 3 and Supplementary Fig. 3). The abundant expression of T-bet, RORγt, IFN-γ and GM-CSF in Th17.1 cells (Supplementary Fig. 4) confirmed the pronounced Th1 features of this Th17 subset.[27]
Collectively, these data demonstrate the propensity of Th17.1 cells to recruit to the central nervous system and mediate disease activity in early MS.



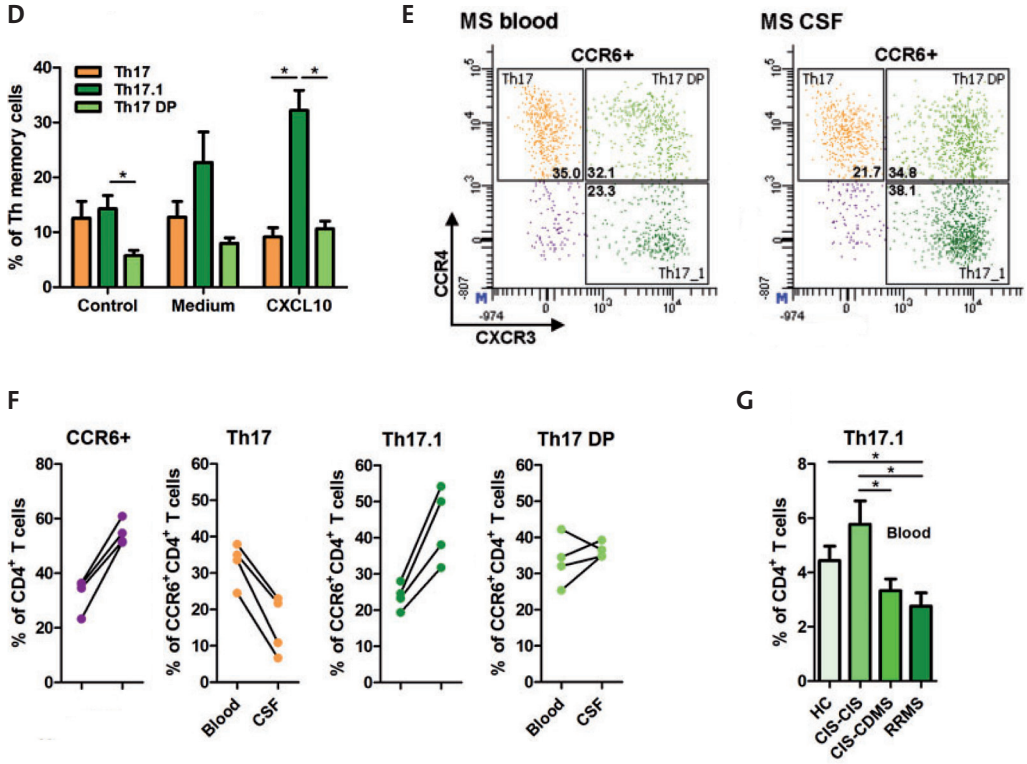


Figure 7. Enhanced central nervous system transmigration potential of Th17.1 cells and their recruitment to cerebrospinal fluid in early MS.

*Total Th memory cells were sorted from healthy blood and used to assess the in vitro transmigration capacities of Th17, Th17.1 and Th17 DP cells across transwell membranes (**A** and **B**; n=6) and monolayers of human brain endothelial cells (BEC; **C** and **D**, n=4) towards CXCL10. Each experiment was performed in quintuplicate. Th17 subset distribution was assessed before ('control') and after migration towards medium or CXCL10 using FACS. (**E** and **F**) Ex vivo Th17, Th17.1 and Th17 DP frequencies of CCR6+ Th cells in paired cerebrospinal fluid and blood from 4 early MS patients. (**G**) The presence of Th17.1 cells in blood samples from 21 CIS-CIS, 26 CIS-CDMS and 13 treatment-naive RRMS patients, as well as 12 healthy controls (HC), as determined by FACS. * $p < 0.05$*

DISCUSSION

In this study, we demonstrate that IFN-γ- and GM-CSF-expressing Th1-like Th17 ($CCR6^+CXCR3^+$) cells are selectively associated with early disease activity in MS patients. During disease onset, highly activated and effector memory Th1-like Th17 cells are markedly reduced in the peripheral blood and represents the main pro-inflammatory T-cell population within cerebrospinal fluid. This local recruitment seemed to be preferentially targeted by natalizumab treatment to prevent subsequent MS relapses, since a Th1-like Th17 subpopulation termed Th17.1, and no other Th subsets, predominantly accumulated in the blood of relapse-free patients. The current work provides in-depth insights into the pro-inflammatory capacity of distinct $CCR6^+$ Th subpopulations during the course of MS,[9] and offers new possibilities to fine-tune currently approved T-cell directed treatment for MS patients.

The use of both CCR6 and CXCR3 as discriminating markers for Th17 cells does not only reflect their pro-inflammatory state, but also their capability to migrate into local inflammatory sites. Previous studies on Th17 cells in experimental autoimmune encephalomyelitis and MS primarily focused on single expression of CCR6,[9] or IL-17, which is increased in blood and is further upregulated in cerebrospinal fluid during a relapse.[17] Here, we demonstrated that additional expression of CXCR3 subdivides human $CCR6^+$ Th17 into high ($CXCR3^-$) and low ($CXCR3^+$) producers of IL-17A. In these $IL\text{-}17^{low}$ producers, which were overrepresented in early-stage MS cerebrospinal fluid compared to blood T-cell cultures, GM-CSF is the major pro-inflammatory cytokine expressed together with IFN-γ. This is likely caused by their elevated levels of T-bet, and not RORγt, as previously reported for human Th cells.[11] The association of Th1-like Th17 (T-bet-dependent) and not Th17 (RORγt-dependent) with a short time to CDMS diagnosis is supported by the expression of T-bet, and not RORγt in $CD4^+$ T cells during rapid MS onset.[40] Th1-like Th17 cells were also highly activated after MS diagnosis, which links to the important role of $CD4^+$ T-cell activation in CIS progression.[3] This suggests that during MS disease onset, the loss of T regulatory function[41] results in the activation of peripheral Th1-like Th17 subsets, which infiltrate the central nervous system to mediate local inflammation. Indeed, memory Th cells of relapsing MS patients were more capable of differentiating into Th1-like Th17 cells, albeit co-producing IFN-γ and IL-17.[35] These Th cells were cultured in the presence of IL-23, prompting RORγt and subsequently IL-17 expression.[11] In our CCR6- and CXCR3-based approach, we defined pro-inflammatory cytokine profiles of Th17 and Th1-like Th17 populations directly from the blood. This could explain why we identified IFN-γ/GM-CSF- and not IL-17-producing Th17 cells as the most pro-inflammatory subset in early MS, and also agrees with the minimal influence of IL-17 and strong impact of GM-CSF on experimental autoimmune encephalomyelitis induction.[13, 42, 43] For proper analysis of cytokine production by Th1-like Th17 cells in cerebrospinal fluid, we had to add IL-2 to short-term T-cell cultures, inducing GM-CSF expression.[11] Th1-like Th17 subsets co-produced more IFN-γ and GM-CSF than other T-cell subsets in cerebrospinal fluid and their counterparts in blood. Our finding that pro-inflammatory Th1-like Th17 and especially Th17.1 cells were highly enriched in cerebrospinal fluid of early-stage MS patients is in line with their reduced frequencies in the blood (this study), and the increased cerebrospinal fluid CD4 to CD14 ratios in CIS patients with a short time to CDMS.[44]

Consistent with *in situ* observations in MS brain tissue,[35] a small fraction of blood and cerebrospinal fluid Th1-like Th17 and Th17.1 cells did co-produce IFN-γ and IL-17, but this was considerably less than their co-production of IFN-γ and GM-CSF. Besides Th17.1, also Th17 DP (IL-17^{int}) cells were enriched in cerebrospinal fluid of late-stage MS patients, suggesting that local IL-17 production is mainly involved in disease progression. Nevertheless, the predominance of Th1-like Th17 cells in MS cerebrospinal fluid and brain tissues as observed in this study corresponds to more recent findings that central nervous system inflammation in MS is largely mediated by infiltrating IFN-γ- and not IL-17-producing Th cells.[34, 36, 45]

Th1-like Th17 cells contain several features promoting their selective intrusion into the central nervous system, although local Th17 plasticity cannot be completely ruled out.[46] Th1-like Th17 cells produce high levels of IFN-γ, triggering CXCL10 expression by endothelial cells to favor CXCR3-mediated migration into the central nervous system,[36, 47] and thereby MS disease activity,[48] which is supported by our in vitro and ex vivo transmigration results. Additional expression of GM-CSF by this subset may further dysregulate the blood-brain barrier, as described for monocytes.[49] Prior to their extravasation, Th17 cells make use of distinct molecules involved in the rolling on and adhesion to endothelial cells, which are activated by pro-inflammatory cytokines and chemokines.[37, 50, 51] One of these molecules is the α4β1-integrin VLA-4, which is targeted by natalizumab to cause a strong reduction of lymphocytes in MS cerebrospinal fluid.[52] In addition to previous work,[53] we now show that only a particular Th1-like Th17 subpopulation termed Th17.1 accumulates in the blood from MS patients who clinically respond to natalizumab treatment. These selective effects may thus be useful for predicting freedom from MS activity,[19] and understanding the potential lethal MS rebounds that occur in patients who have to stop this treatment due to increased risk of progressive multifocal leukoencephalopathy (PML).[54, 55] MS rebounds are characterized by a rapid influx of pro-inflammatory cells into the central nervous system to cause excessive inflammation, potentially resulting in PML-immune reconstitution inflammatory syndrome (IRIS).[56] Although not proven yet, the marked accumulation of Th17.1 in natalizumab-treated MS blood puts forward their transmigration into the central nervous system as a critical process during these complications. Out of all pro-inflammatory Th subsets defined by CCR6, CXCR3 and CCR4, Th17.1 revealed the strongest VLA-4 surface expression levels in MS blood, which explains their restricted targeting by natalizumab. Consistent with our results, VLA-4 levels were found to be higher on Th17 than on Th1 cells in MS patients, probably mediating their trafficking into the central nervous system.[17] However, when we compared individual Th17 subpopulations, i.e. CCR6$^+$ Th17 (CXCR3$^-$CCR4$^+$; IL-17^{high}), Th17 DP (CXCR3$^+$CCR4$^+$; IL-17^{dim}) and Th17.1 (CXCR3$^+$CCR4$^-$; IL-17^{low}),[27] VLA-4 surface expression seemed to be inversely associated with their ability to produce IL-17, as also described for mice.[57] The predominant expression of VLA-4 on Th17.1 cells closely parallels the dependence of IFN-γ- and not IL-17-producing Th cells on this integrin for their entry into the central nervous system during experimental autoimmune encephalomyelitis.[57, 58] However, adhesion molecules other than VLA-4 must be taken into account for alternative transmigration routes of pro-inflammatory Th17 cells as well,[50] especially considering the rebound effects after natalizumab discontinuation in MS.

This cross-sectional study exemplifies that a more refined evaluation of chemokine

surface receptors, pro-inflammatory cytokines and adhesion molecules is warranted to better understand the contribution of human Th1 and Th17 to MS and other autoimmune and neuroinflammatory diseases. Based on CCR6/CXCR3, IFN-γ/GM-CSF and VLA-4 expression, we identify Th1-like Th17 as a clinically relevant $CD4^+$ T-cell population during disease onset and treatment in MS patients. Future work on the localization and antigen specificity of these subsets in human brain lesions will be critical to determine their local impact on myelin and axonal loss in MS. The prominent association of Th1-like Th17 cells, in particular Th17.1, with MS activity suggests the possibility for more specific T cell-targeted therapies, and pleads for further assessment of the use of natalizumab earlier in the disease course of MS.[59]

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SUPPLEMENTARY TABLES AND FIGURES

Blood, ex vivo					
Cohort	HC	CIS-CIS	CIS-CDMS	RRMS, no Tx	RRMS, Nat Tx
Patients, no.	12	21[a]	26[a]	13	9[b]
Gender, female no. (%)	8 (67)	15 (71)	21 (81)	10 (77)	5 (56)
Age in years, median (IQR)[c]	33 (28-48)	35 (29-39)	33 (27-36)	45 (37-54)	28 (21-43)[d]
Follow-up time in years, median (IQR)	na	7.0 (6.1-7.3)	3.7 (2.5-5.8)	na	na
Disease duration in months, median (IQR)[e]	na	2.4 (1.3-3.8)	2.6 (1.4-3.4)	113 (38-130)	33 (24-57)[d]
≥9 lesions on T2-weighted images at baseline, no. (%)	na	6 (29)	13 (50.0)	na	na

Supplementary Table 1. Characteristics of patients and controls in additional cohorts

[a]14 CIS-CIS and 16 CIS-CDMS were also included in the screening cohorts (see Table 1); [b]2 natalizumab-treated RRMS patients were also used for analysis of pro-inflammatory cytokine expression only (Table 1); [c]at time of sampling; [d]at time of pre-treatment sampling. RRMS according to the McDonald 2010 criteria; [e]time CIS diagnosis to sampling.

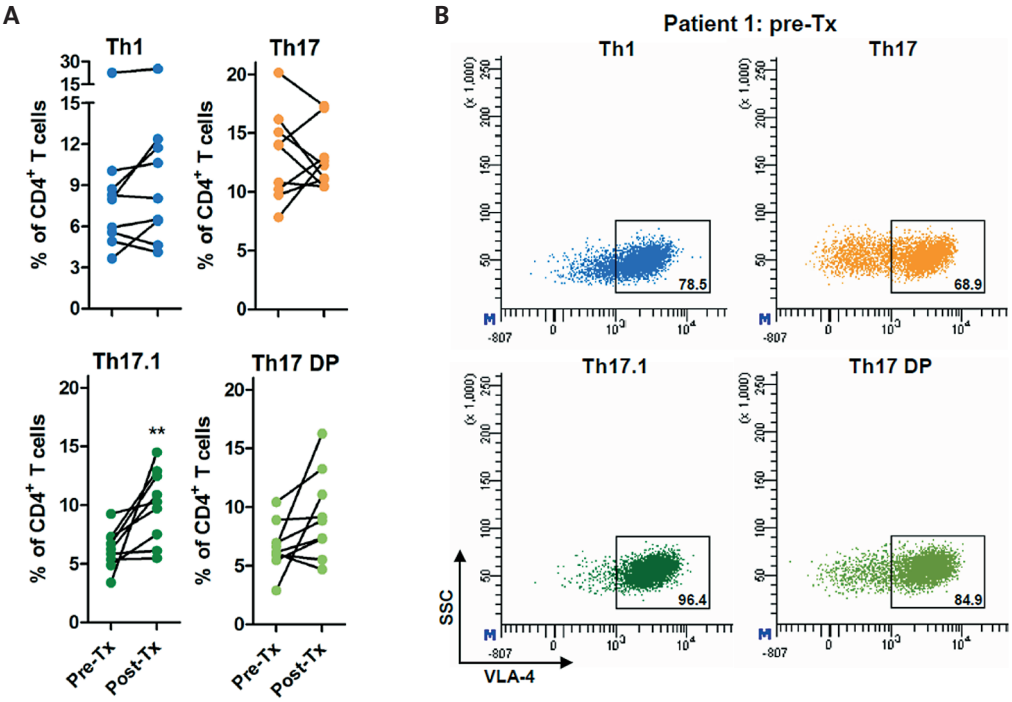
Abbreviations: CDMS, clinically definite MS; CIS, clinically isolated syndrome; IQR, interquartile range; na, not applicable or available; Nat, natalizumab; RRMS, relapsing-remitting MS; Tx, treatment

Gene	Forward primer	Reverse primer
ABCB1	GGAAATTTAGAAGATCTGATGTCAAAC	CACTGTAATAATAGGCATACCTGGTC
BATF	ACACAGAAGGCCGACACC	CTTGATCTCCTTGCGTAGAGC
CSF2	TCTCAGAAATGTTTGACCTCCA	GCCCTTGAGCTTGGTGAG
FCMR	GAACCTTCCTGCCATCCA	GAGCCATAGTCCAGTGCTCTC
FOXP3	ACCTACGCCACGCTCATC	TCATTAAGTGTCCGCTGCT
GZMB	CGGTGGCTTCCTGATACAA	CCCCAAGGTGACATTTATGG
IFNG	GGCATTTTGAAGAATTGGAAAG	TTTGGATGCTCTGGTCATCTT
IL17A	TGGGAAGACCTCATTGGTGT	GGATTTCGTGGGATTGTGAT
IL23R	CCTGGCTCTGAAGTGGAATTA	GGCTATTACTGCATCCCATTG
RORC	AGAAGGACAGGGAGCCAAG	CAAGGGATCACTTCAATTTGTG
STAT4	CCAATGGGAGTCTCTCAGTAGAA	TGTGACAGCCCTCATTTCCT
TBX21	GTCCAACAATGTGACCCAGA	AAAGATATGCGTGTTGGAAGC
TNF	CAGCCTCTTCTCCTTCCTGAT	GCCAGAGGGCTGATTAGAGA

Supplementary Table 2. qPCR primer sequences

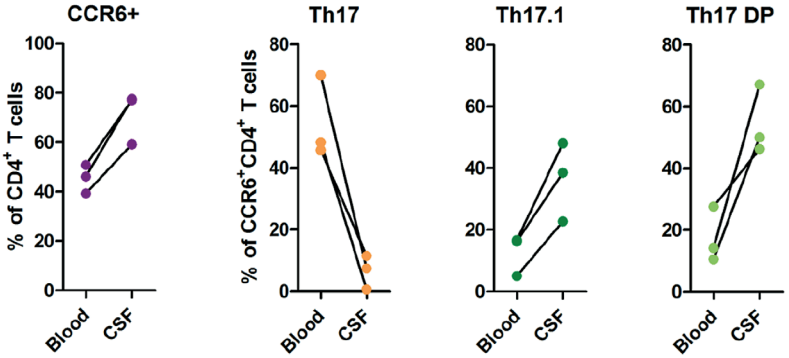
Surface marker	Cohort*	CD4+ Th subpopulation						
CD25	n=10	6^-3^+	6^+3^-	6^+3^+	$6^-3^+4^-$	$6^+3^-4^+$	$6^+3^+4^-$	$6^+3^+4^+$
% positive cells	HC	9.6 ± 0.8	28.1 ± 3.0	19.8 ± 1.7	6.9 ± 0.7	33.9 ± 3.1	13.9 ± 1.8	24.8 ± 2.0
	RRMS	10.3 ± 0.7	31.1 ± 1.7	20.3 ± 1.7	7.9 ± 1.0	36.6 ± 1.3	11.9 ± 1.0	26.1 ± 1.9
MFI positive cells	HC	1303 ± 23	1519 ± 41	1347 ± 83	1193 ± 30	1572 ± 49	1082 ± 17	1592 ± 120
	RRMS	1375 ± 55	1521 ± 84	1476 ± 199	1221 ± 46	1578 ± 96	1069 ± 19	1639 ± 238
CD161								
% positive cells	HC	20.8 ± 3.0	45.8 ± 2.4	53.7 ± 2.5	22.8 ± 3.6	46.4 ± 2.3	63.0 ± 2.7	47.7 ± 2.7
	RRMS	17.5 ± 2.9	44.4 ± 2.9	46.7 ± 3.5	20.3 ± 3.6	42.8 ± 3.0	56.1 ± 2.8	42.0 ± 3.5
MFI positive cells	HC	2074 ± 132	2695 ± 129	2764 ± 145	2153 ± 140	2694 ± 132	2931 ± 157	2517 ± 145
	RRMS	2023 ± 79	2595 ± 90	2638 ± 125	2094 ± 63	2504 ± 100	2820 ± 123	2356 ± 161
CD226								
% positive cells	HC	89.2 ± 1.4	91.3 ± 1.1	96.8 ± 0.6	87.3 ± 2.1	92.4 ± 1.0	98.5 ± 0.3	95.7 ± 0.7
	RRMS	89.2 ± 1.7	92.1 ± 1.0	96.4 ± 0.7	88.0 ± 2.7	92.2 ± 1.0	98.1 ± 0.5	95.4 ± 0.8
MFI positive cells	HC	3411 ± 157	3504 ± 200	4464 ± 200	3014 ± 137	3839 ± 184	4291 ± 181	4674 ± 229
	RRMS	3080 ± 156	3396 ± 164	4086 ± 130	2743 ± 202	3570 ± 156	4036 ± 147	4138 ± 122
VLA-4								
% positive cells	HC	83.4 ± 2.1	75.1 ± 1.9	90.8 ± 1.3	86.6 ± 2.0	70.4 ± 2.4	96.2 ± 0.7	85.5 ± 1.8
	RRMS	83.9 ± 1.4	76.3 ± 2.0	90.6 ± 1.2	87.4 ± 1.8	71.8 ± 2.1	96.2 ± 0.7	85.3 ± 1.8
MFI positive cells	HC	2268 ± 158	2309 ± 144	3597 ± 176	2092 ± 168	2548 ± 117	3622 ± 156	3586 ± 195
	RRMS	2108 ± 205	2409 ± 232	3283 ± 327	1959 ± 220	2644 ± 273	3342 ± 317	3192 ± 356
MCAM								
% positive cells	HC	0.9 ± 0.2	8.6 ± 0.9	4.6 ± 0.7	0.3 ± 0.1	9.8 ± 0.9	3.9 ± 0.6	4.9 ± 0.5
	RRMS	1.0 ± 0.1	8.6 ± 0.3	4.2 ± 0.3	0.4 ± 0.1	9.6 ± 0.5	3.0 ± 0.4	4.7 ± 0.4
MFI positive cells	HC	504 ± 20	555 ± 13	528 ± 11	525 ± 38	552 ± 11	519 ± 15	537 ± 16
	RRMS	506 ± 10	548 ± 10	507 ± 7	650 ± 63	545 ± 9	513 ± 13	508 ± 9
PSGL-1								
% positive cells	HC	92.0 ± 2.0	95.8 ± 0.9	97.3 ± 0.6	90.6 ± 2.3	97.0 ± 0.6	97.2 ± 0.5	97.6 ± 0.7
	RRMS	95.8 ± 0.5	97.6 ± 0.3	98.4 ± 0.2	95.1 ± 0.6	98.2 ± 0.2	98.1 ± 0.2	98.7 ± 0.3
MFI positive cells	HC	4758 ± 326	5863 ± 386	6163 ± 288	4960 ± 482	6432 ± 388	6126 ± 272	6216 ± 301
	RRMS	4682 ± 231	6322 ± 200	6219 ± 224	4817 ± 381	6741 ± 219	5990 ± 156	6332 ± 261

Supplementary Table 3. Expression of surface markers on distinct Th subpopulations
**Patients and controls were treatment-naive, age-/gender-matched and part of the cohorts described in Supplementary Table 1.*
None of these markers showed differences in expression between patients and controls

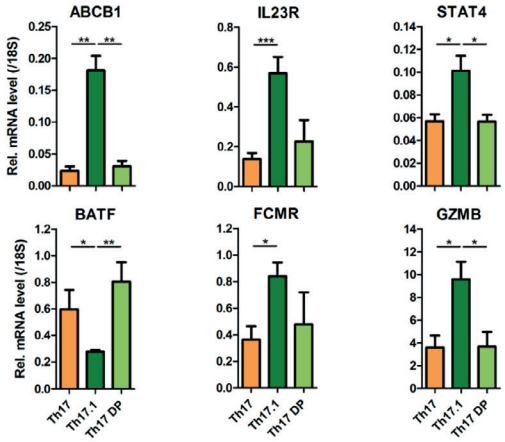


Supplementary Figure 1. (A) Validation of Th17.1 accumulation in the blood of 9 MS patients after natalizumab treatment.

*Frequencies of Th1 (CCR6$^-$CXCR3$^+$CCR4$^-$), Th17 (CCR6$^+$CXCR3$^-$CCR4$^+$), Th17.1 (CCR6$^+$CXCR3$^+$CCR4$^-$) and Th17 DP (CCR6$^+$CXCR3$^+$CCR4$^+$) cells within the CD4$^+$ T-cell pool were compared pre- and 12m post-treatment using multicolor flow cytometry. (**B**) Representative gating and percentages of VLA-4+ cells within Th1, Th17, Th17.1 and Th17 DP subpopulations in pre-treatment MS blood*

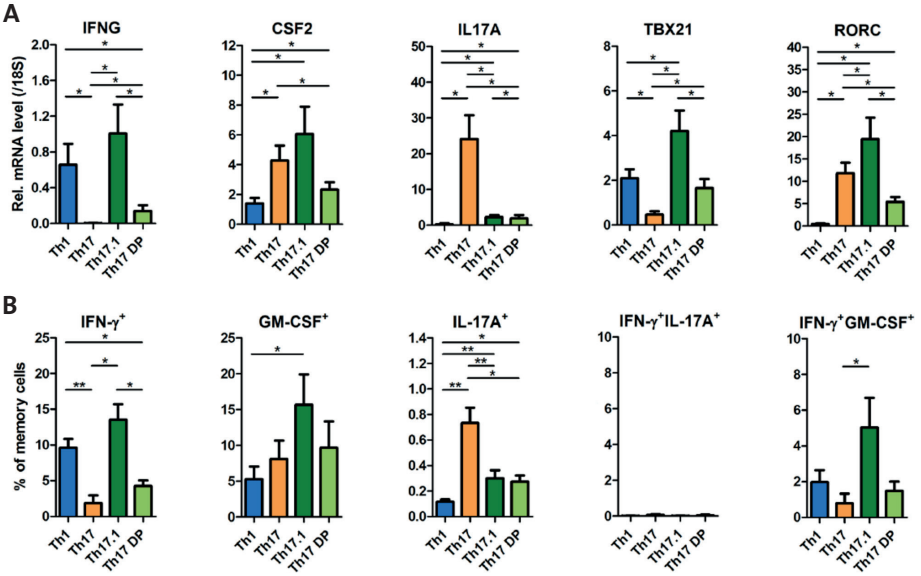


Supplementary Figure 2. Flow cytometric analysis of Th17 subpopulations in paired ex vivo CSF and blood samples of 3 late-stage MS patients.



Supplementary Figure 3. Validation of Th17.1 as a distinct Th17 subset based on the expression of key genes involved in Th17 differentiation and pathogenicity.

We sorted memory Th17 (CCR6$^+$CXCR3$^-$CCR4$^+$), Th17.1 (CCR6$^+$CXCR3$^+$CCR4$^-$) and Th17 DP (CCR6$^+$CXCR3$^+$CCR4$^+$) cells of 7 healthy blood donors and compared the relative expression levels of ABCB1 (MDR1), IL23R, STAT4, BATF, FCMR (TOSO) and GZMB (granzyme B). For FCMR and GZMB expression analyses, Th subsets were activated with anti-CD3/CD28 abs for 24 h.



Supplementary Figure 4. Validation of Th17.1 as a distinct Th17 subset based the expression of Th1- and Th17-associated pro-inflammatory cytokines and transcription factors.

*Th1 (CCR6$^-$CXCR3$^+$CCR4$^-$), Th17 (CCR6$^+$CXCR3$^-$CCR4$^+$), Th17.1 (CCR6$^+$CXCR3$^+$CCR4$^-$) and Th17 DP (CCR6$^+$CXCR3$^+$CCR4$^+$) cells were sorted from 7 healthy blood donors and compared for mRNA (**A**) and protein (**B**) expression of IFN-γ (IFNG), GM-CSF (CSF2), IL-17A (IL17A), T-bet (TBX21) and RORγt (RORC).*

Chapter 6

Smoking at time of CIS increases the risk of clinically definite multiple sclerosis

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ABSTRACT

Background: Cigarette smoking is a modifiable risk factor that influences the disease course of patients with multiple sclerosis (MS). However, in patients with a clinically isolated syndrome (CIS) there are conflicting results about the association between smoking and the risk of a subsequent MS diagnosis. The aim of this study was to determine the risk of clinically definite MS (CDMS) in smoking and non-smoking patients at time of a first demyelinating event.

Methods: Two hundred and fifty patients, aged 18-50 years, were included in our prospective CIS cohort. At time of the first neurological symptoms, patients completed a questionnaire about smoking habits. Cox regression analyses were performed to calculate univariate and multivariable hazard ratios for CDMS diagnosis in smoking and non-smoking CIS patients.

Results: One hundred and fourteen (46%) CIS patients were diagnosed with CDMS during a mean follow-up of 58 months. In total, 79 (32%) patients smoked at time of CIS. Sixty-seven % of the smoking CIS patients were diagnosed with CDMS during follow-up compared to 36% of the non-smoking CIS patients ($p<0.001$). Smoking at time of CIS was an independent predictor for CDMS diagnosis (HR: 2.3; $p=0.002$). Non-smoking CIS patients who had a history of smoking did not have a higher risk for CDMS than those who had never smoked.

Conclusions: Smoking at time of CIS was an independent risk factor for a future CDMS diagnosis. This is an additional argument to quit smoking at time of the first attack of suspected MS.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease, influenced by environmental factors in genetically susceptible individuals.[1] This results in demyelination, axonal loss and neurodegeneration.[1-3] The course of MS is heterogeneous in severity and prognosis.[1]
One of the environmental factors influencing the course of MS is cigarette smoking.[4] Studies in MS patients and healthy controls consistently provide evidence that both active and passive smoking result in an increased risk of MS and disease progression.[4-7] Smoking does not only increase MS risk, it also shortens the time to the secondary progressive phase of MS (SPMS).[8, 9] It has been shown that after cessation of smoking, negative effects slowly decrease, independent of the cumulative dose of smoking.[8, 10] Studies show that the risk of MS associated with HLA genotypes is influenced by smoking. This interaction leads to a much stronger effect on MS risk than the cumulative effect of genetic risk factors and smoking together.[11, 12]
In the majority of cases (85-90%) MS starts as a clinically isolated syndrome (CIS), followed by novel episodes of neurological symptoms resulting from inflammation of the central nervous system (CNS). However, a CIS attack can also remain a single event.[13] Only a few studies are available in CIS patients that investigated the effect of smoking on subsequent MS risk. Firm conclusions in these studies are hampered by several methodologic issues such as low patient numbers, retrospective designs,[14-16] or considerable numbers of CIS patients treated with interferon-beta.[17, 18] On the other hand, several studies in MS patients do suggest a link between smoking and MS progression.[7] Therefore, we determined the effect of smoking on clinically definite MS (CDMS) risk in a large prospective cohort of predominantly untreated CIS patients. It is important to know if smoking is associated with a future MS diagnosis, as smoking is up to now, one of the few modifiable risk factors for disease progression in MS.

METHODS

Patients

Data were collected prospectively from patients with CIS at the Neurology department of Erasmus MC University Hospital in Rotterdam, a tertiary referral centre for patients with MS. Data collection was in collaboration with several regional hospitals in The Netherlands. Included patients had their first symptoms between May 2006 and June 2017. Patients were aged between 18 and 50 years, with no history of previous neurological symptoms suggestive of CNS demyelination. CIS patients were included within 6 months following the first neurological symptoms. Patients with alternative diagnoses were excluded from the analyses. At baseline, a magnetic resonance imaging (MRI) scan and routine laboratory tests were utilized to rule out alternative diagnoses.[19] Following inclusion, patients were reassessed at least annually at the Neurological Outpatient Department.

Questionnaire

At baseline, CIS patients completed a questionnaire to gather information about smoking habits, including when they first started smoking, non- or reduced-smoking periods and how many cigarettes were smoked within these periods. Using results of the questionnaire, we were able to calculate the pack-years per patient.

Standard protocol approvals, and patient consent

This study was approved by the Medical Ethics Committee of Erasmus MC Rotterdam. Written informed consent was obtained from all patients.

Definitions

A relapse was defined as new symptoms or subacute worsening of existing symptoms after 30 days of improvement, or stable disease and no evidence of alternative diagnosis. Symptoms had to exist longer than 24 hours and not to be preceded by fever.[20] All exacerbations were confirmed by neurological examination. CDMS was defined as clinical dissemination in space and time with two exacerbations and (para) clinical evidence of two separate lesions, as described by Poser et al.[21] Patients who were diagnosed with CDMS during follow-up are referred to as CIS-CDMS and patients who remained CIS are referred to as CIS-CIS. Expanded Disability Status Scale (EDSS) scores were performed annually when patients were diagnosed with CDMS.[22] EDSS performed within 3 months after a relapse were not used in the analyses. Follow-up was calculated by subtracting CIS date from the last visit date. Patients were defined as smokers when they were smoking regularly at time of CIS. Non-smokers were those who did not smoke at time of CIS. Patients were defined as ex-smokers when they were not smoking at time of CIS, but did have a history of smoking in the years prior to CIS. To calculate pack-years, the number of years smoked was multiplied by the number of cigarettes smoked per day/20 in that period.

Statistical analysis

Statistical analyses were done using SPSS, version 21.0 (SPSS Inc) for Windows and GraphPad Prism5 (GraphPad) for Windows. Nominal data comparison between groups was done using chi-square or Fisher's exact test (gender, type of clinical onset, oligoclonal bands (OCB), ≥9 T2 lesions on baseline MRI, disease modifying therapy (DMT) at time of CIS, smoking at time of CIS, SPMS and alcohol use). The Kolmogorov-Smirnov test was performed to assess normality of data distribution. To compare continuous data we applied a two-tailed t-test (age at onset and follow-up time) or, when the data were non-parametric, a Mann-Whitney U-test (time from CIS to CDMS and pack-years). Time to second attack was calculated from onset of the first symptoms. Cox proportional hazard regression analyses were used to calculate univariate and multivariable hazard ratios (HR). Patients who did not have a second attack during follow-up were considered as censored observations. Hazard ratios were also obtained for time to EDSS 4.0 and time to EDSS 6.0. p values less than 0.05 were considered significant.

RESULTS

Patient characteristics

We included 250 patients who completed the baseline questionnaire about smoking at time of CIS. Of these 250 CIS patients, 114 (46%) patients had a second relapse and were diagnosed with CDMS during a mean follow-up time of 58.1 months (SD: 35.9).
The median time (interquartile range; IQR) from CIS to CDMS was 23.3 months (8.9-44.3). The median time (IQR) between the first neurological symptoms and inclusion in the study was 1.2 months (0.3-2.9 months).
Fifty-seven (23%) patients who were not yet diagnosed with CDMS were treated with DMT. The patient characteristics are shown in Table 1.

	CIS-patients (n=250)	CIS-CDMS (n=114)	CIS-CIS (n=136)	p-value [a]
Gender, female, n (%)	189 (75.6)	92 (80.7)	97 (71.3)	0.09
Age (years), mean (SD)	33.6 (8.3)	32.6 (7.9)	34.5 (8.5)	0.07
Follow-up time (months), mean (SD)	58.1 (35.9)	72.3 (30.4)	46.3 (36.0)	<0.01
Type of clinical onset, n (%)				
-Optic nerve	88 (35.2)	33 (28.9)	55 (40.4)	0.06
-Spinal cord	90 (36.0)	45 (39.5)	45 (33.1)	0.30
-Other localization	72 (28.8)	36 (31.6)	36 (26.5)	0.37
OCB, (> 1 band), (%)	113 (73.9)	63 (81.8)	50 (65.8)	0.02
≥9 lesions on T2-weighted images, n (%)	96 (38.6)	53 (46.9)	43 (31.6)	0.01
DMT at time of CIS, n (%)	57 (22.8)	26 (22.8)	31 (22.8)	1.00
Smoking at time of CIS, n (%)	79 (31.6)	53 (46.5)	26 (19.1)	<0.01
Pack-years at time of CIS, median (IQR)	1.0 (0.0-5.9)	2.4 (0.0-9.5)	0.0 (0.0-2.7)	<0.01

Table 1. Patient Characteristics (CIS-CDMS vs CIS-CIS patients)

[a] p-value calculated between CIS-CDMS and CIS-CIS

Abbreviations: CIS, Clinically isolated syndrome; CIS-CDMS, patients who are diagnosed with CDMS during follow-up after CIS defined by Poser criteria; CIS-CIS, not diagnosed with CDMS; na, not applicable; OCB, oligoclonal bands

Smokers versus non-smokers

In total, 79 of 250 (32%) patients smoked at time of CIS. Fifty-three of 79 (67%) smoking CIS patients were diagnosed with CDMS during follow-up compared to 61 of 171 (36%) in the non-smoking CIS patients (p<0.001). The number of pack-years was higher in the group that was diagnosed with CDMS (CIS-CDMS) during follow-up than in the group that remained CIS (CIS-CIS) (median (IQR) CIS-CDMS vs CIS-CIS: 2.4 (0.0-11.9) vs 0.0 (0.0-2.7) p=0.004). (Figure 1)

There were no differences between smokers and non-smokers in gender, localisation of first symptoms, age, OCB in CSF or MRI characteristics at baseline. Table 2 shows patient characteristics for smokers and non-smokers.

	Smoking CIS patients (n=79)	Non-smoking Cis patients (n=171)	p-value
Gender, female, n (%)	58 (73.4)	131 (76.6)	0.59
Age (years), mean (SD)	33.9 (7.7)	33.5 (8.5)	0.67
Follow-up time (months), mean (SD)	60.7 (30.7)	57.0 (38.1)	0.45
Type of clinical onset, n (%)			
-Optic nerve	25 (31.6)	63 (36.8)	0.42
-Spinal cord	29 (36.7)	61 (35.7)	0.87
-Other localization	25 (31.6)	47 (26.3)	0.50
OCB, (> 1 band), (%)	43 (75.4)	70 (72.9)	0.73
≥9 lesions on T2-weighted images, n (%)	33 (42.3)	63 (36.8)	0.41
DMT at time of CIS, n (%)	24 (30.4)	33 (19.3)	0.05
CDMS, n (%)	53 (67.1)	61 (35.7)	<0.01
SPMS, n (%)	3 (3.8)	5 (2.9)	0.72
Alcohol use, no. of patients (%)	55 (69.6)	79 (46.2)	<0.01

Table 2. Patient Characteristics (smoking vs non-smoking CIS patients)
Abbreviations: CIS, Clinically isolated syndrome; OCB, oligoclonal bands

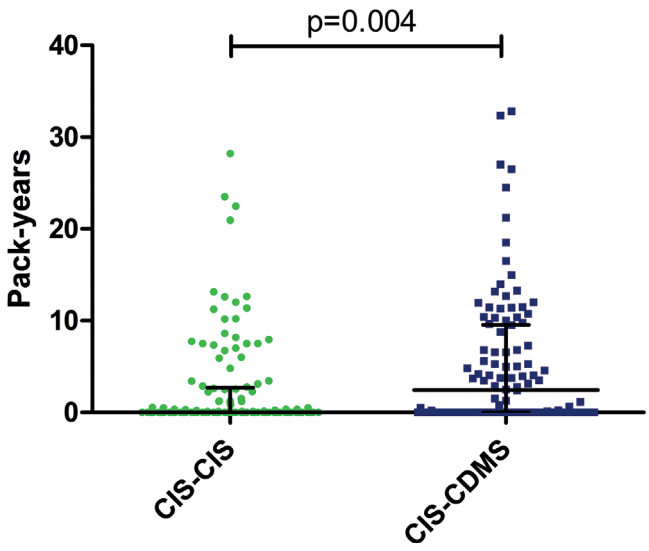

Figure 1. Pack-years in CIS patients
Comparison of pack-years between CIS-CIS and CIS-CDMS patients. Horizontal lines and error bars indicate median and IQR

Association of smoking at time of CIS with a shorter time to CDMS

Patients who smoked at time of CIS had a shorter time to CDMS diagnosis than patients who were not active smokers (univariate hazard ratio; HR: 2.1 p<0.001). (Figure 2) Corrections were applied for multiple variables that are associated with a second attack (OCB in CSF, more than 9 T2 lesions, gadolinium enhancing lesions on baseline MRI, and optic neuritis as first symptom, no DMT before CDMS). After these adjustments, multivariable COX regression analysis showed smoking as an independent predictor for a second attack. The HR was 2.3 (p=0.002).
In a sub-analysis we excluded the 50 CIS patients who had less than 2 years of follow-up. After exclusion, the results remained the same, HR: 2.0 (p<0.001).
Fifty-seven (23%) of the patients received DMT before CDMS diagnosis. When we excluded these patients the HR was unchanged 2.0 (p=0.001).

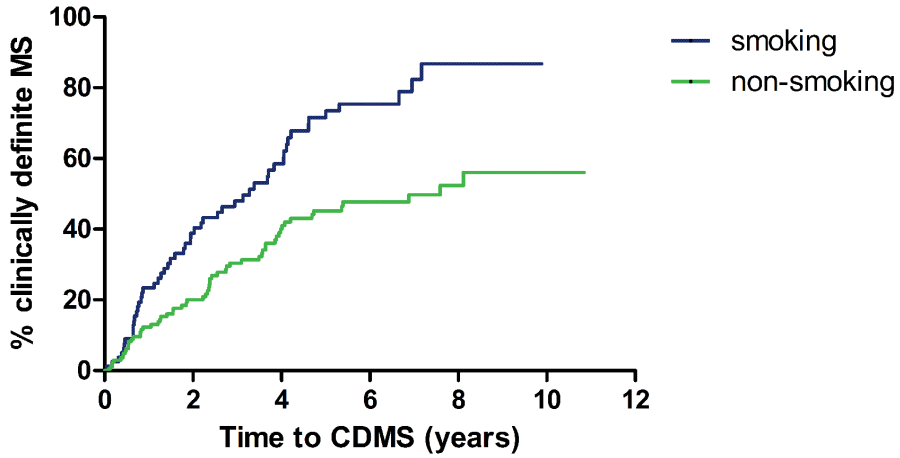

Figure 2. Time from CIS to CDMS in smoking and non-smoking patients
Kaplan-Meier curve for time from CIS to CDMS for smoking and non-smoking patients at time of CIS (log-rank test p<0.001)

Smoking in the past

In the group of patients that did not smoke at time of CIS (n=171), 63 (37%) patients had a history of smoking in the past (ex-smokers). Smoking in the past did not predict CDMS diagnosis in the group of non-smoking CIS patients (HR: 0.64 (p=0.12). Furthermore, in this non-smoking group (n=171), the number of pack-years was not correlated with time to CDMS (HR per pack-year: 0.96 (p=0.31))

Smoking at time of CIS and disability later in the disease

In this cohort we collected EDSS data from 96 of 114 (84%) patients who were diagnosed with CDMS. Nineteen patients reached an EDSS of 4.0 and eight patients an EDSS of 6.0 during follow-up. Six of these eight patients who reached an EDSS score of 6.0 or more were smoking at time of CIS. The HRs for both EDSS scores were not significant (HR for EDSS 4.0: 1.9 (p=0.18) and HR for EDSS 6.0: 4.1 (p=0.09)). However, there was a trend towards faster disability progression in CDMS patients who were smoking at time of CIS.

DISCUSSION

In this prospective study of patients included after a first attack of suspected MS, we determined the risk of CDMS in a cohort of 250, mostly untreated smoking and non-smoking CIS patients. We demonstrated that smoking at time of CIS is associated with a shorter time to a second clinical attack, and therefore an earlier diagnosis of CDMS.
Smoking is a well-established risk factor for MS and disease progression after MS diagnosis.[4, 5] This association is also found in other auto-inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).[23, 24]
Studies investigating the influence of smoking on MS risk in first attack patients have remained inconclusive, as described recently in a systematic review.[25] Three studies were relatively small and used retrospective data.[14-16] Other studies were large, but patients were treated with interferon beta immediately after CIS or two years after CIS,[17, 18] Interferon beta treatment could have postponed MS diagnosis.[26] Therefore, the potential correlation between smoking and MS risk may be overshadowed by this disease-postponing therapy.
Compared to these studies, the present study has less confounding factors, it has a prospective design and only a small proportion of patients was treated with DMT before CDMS diagnosis.
In the multivariable analysis, we corrected our results for currently known predictors for CDMS diagnosis (large number of T2 lesions, contrast enhancing lesions on baseline MRI, unique OCB in CSF and localization of CIS) and for DMT before CDMS diagnosis.[27] After these corrections, smoking remained clearly predictive for CDMS diagnosis. Therefore, smoking status can potentially improve prediction of a future CDMS diagnosis in CIS patients. Accurately predicting CDMS diagnosis is important to prevent unnecessary treatment of patients with low disease activity.[28]
The fact that smoking in the past in current non-smoking CIS patients was not associated with CDMS suggests that the harmful effects of smoking are reversible. This supports results of earlier studies, showing that after cessation of smoking, the negative effect on disability progression slowly decreases, independent to the cumulative dose of smoking.[8, 10]
Our study has some limitations. Although the mean follow-up time was long (almost 5 years), there was a wide range. To overcome this, we used a COX regression model to correct for follow-up time. We also performed a sub-analysis, where CIS patients with less than 2 years of follow-up were excluded. Excluding these patients left 200 patients for analysis and did not change our results. Yet, for demonstration of an association

between smoking at time of CIS and later disability (EDSS) a longer follow-up would be needed.
Second, there is a possibility that our results are explained by potential confounding lifestyle factors such as body mass index (BMI) or alcohol use. It has been shown that obesity is a risk factor for MS.[29] However, it is not likely that a high BMI explained the effect seen here, as obesity is more common in non-smokers.[30] A Swedish study showed an inverse association of alcohol consumption with MS.[31] We did not observe an effect of alcohol use on CDMS diagnosis in the regression analysis (data not shown).
Third, a follow-up MRI scan was not performed according to a fixed protocol. Instead, we used the classic Poser criteria that are based on clinical manifestations to define CDMS. Thus, we can only claim an association with clinical disease activity but not with lesion accrual on MRI scan.
It is not likely that CIS patients with a second attack during follow-up had over-reported smoking at time of CIS. Even in case recall would play a serious role here, recall of smoking would be expectedly more strong for the question of past smoking.[32] Yet, it was only recent smoking, more plausibly related to concurrent biological processes just before, during and after the first demyelinating attack, that showed an association.
The exact influence of ongoing smoking on the progression of the auto-inflammatory process around a first clinical attack of demyelination remains to be determined. It may involve several pathways, including both direct and indirect influences of tobacco toxins and smoke particles on T cells and antigen presenting cells.[33]
To conclude, we show in a large prospective cohort of CIS patients that smoking at time of CIS is an independent risk factor for a future CDMS diagnosis. Smoking status could even be a relevant parameter in predictive models on a possible MS disease course after CIS. Since smoking is a modifiable risk factor, our study draws attention to the relevance of counselling patients about smoking. Though intervention studies will be difficult to execute, this study may provide evidence for the argument to quit smoking for patients with a first attack of suspected MS.

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Part II

Clinically isolated syndrome in children versus adults

Chapter 7

Disease course after clinically isolated syndrome in children versus adults: a prospective cohort study

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ABSTRACT

Background: Clinically Isolated Syndrome (CIS) is a first demyelinating event of the central nervous system (CNS) and can be a single event. After CIS a chronic disease course with ongoing inflammation and relapses might occur, resulting in a diagnosis of multiple sclerosis (MS). As yet, there has been no prospective exploration of whether children and adults with CIS have the same disease course.

Methods: Patients with CIS, whose age ranged from 1-50 years, were prospectively followed. We divided the patients in three different age groups: patients 1-10, 11-17, and 18-50 years old. Among these groups demographic data, disease course, time to MS diagnosis and annualised relapse rates (ARR) were compared.

Results: We included 383 CIS patients, of whom 218 (56.9%) were diagnosed with MS. Children of between 11 and 17 years old had the highest rate of MS conversion (83.5% versus 50.0% in the other age groups together, $p<0.01$) and the shortest time to MS diagnosis (median time 2.6 months (IQR: 0.6-6.0) vs 8.2 months (IQR: 1.9-28.2) in the other age groups together, $p<0.01$). ARR corrected for follow-up was higher in children <18 years old than in adults ≥18 years old with MS (mean ARR 0.65 vs 0.43, $p<0.01$).

Conclusion: Children with CIS tend to have a more inflammatory disease course appearing from the high relapse rate in all children, and the highest rate of MS conversion in 11-17 year-old children. This supports early initiation of first-line disease modifying therapy (DMT) in children, perhaps even at first event in children at high risk for MS in line with current clinical practice in adults.

INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that can cause a broad spectrum of neurological deficits.[1] Worldwide over 2.5 million people suffer from MS, mostly young women.[1] Although rare, MS can also occur in children.[2] MS can be diagnosed when a first event of CNS demyelination, a so-called clinically isolated syndrome (CIS), is followed by a new clinical event.[3-5] In addition, MS can be diagnosed after CIS when new lesions, which fulfil MS diagnostic criteria for dissemination in time and space, are detected on Magnetic Resonance Imaging (MRI).[4,5] However, CIS can be a single event. It is unknown whether childhood-onset and adulthood-onset CIS and MS reflect the same disease.[6] Previous studies focussed on MS and did not compare disease course between children and adults after onset of CIS.[7-13] Despite existing parallels in childhood-onset and adulthood-onset MS, there are fundamental differences in presentation and disease course. Several studies have reported a higher relapse rate and MRI lesion load in children than in adults with MS.[8,10,11,14,15] On the other hand, progression of disability is slower in children with MS.[12,13] It should be noted that some of the previous studies included retrospective cohorts,[9,12,13,15] and some were conducted before the immunomodulatory treatment era in children.[7-9,12,13] Current recommendation and use of DMT in children with MS likely influenced their disease course and relapse rate.[16] Several studies have shown initiation of first-line DMT in adults with CIS delays MS diagnosis and disability accumulation.[17,18] In children with CIS it is not common practice yet to offer first-line DMT prior to MS diagnosis. Here, we performed a clinical prospective follow-up study of 383 patients with CIS, whose age ranged from 1 - 50 years, in order to report a comparison of childhood-onset versus adulthood-onset CIS and MS. The aim of our study was to compare clinical features at onset, time to MS diagnosis, relapse rate and disability.

METHODS

Patients and definitions

Patients with CIS were included between April 2006 and August 2015, either in our prospective cohort of adults with CIS (PRedicting the Outcome of a Demyelinating event, PROUD study),[19] or in our prospective cohort study of children with a first episode of acquired demyelination of the CNS (PROUDkids).[20] Both studies are ongoing multicentre observational studies conducted at Erasmus MC in Rotterdam, the Netherlands, and study protocols have been described previously.[19,20] CIS is defined as an acute or subacute episode of neurological dysfunction that lasts more than 24 hours in absence of fever or encephalopathy.[3,4,21] Patients <51 years old were included within 6 months after first onset. At baseline, an MRI scan was performed and routine laboratory tests were done. Patients with alternative diagnoses other than CIS were excluded. After baseline, patients were reassessed at least annually.
MS was diagnosed in adults according to the 2010 McDonald criteria.[5] Children were diagnosed with CIS and MS according to the diagnostic criteria from the International Paediatric Multiple Sclerosis Study Group (IPMSSG), which are based on the 2010

Mc Donald criteria.[4,5] Clinically definite multiple sclerosis (CDMS) was defined as two nonencephalopathic attacks with (para)clinical evidence of two separate lesions as described by Poser.[4,22] The Expanded Disability Status Scale (EDSS) was used to define disability.[23] EDSS scores were obtained at least 2 months after a relapse occurred and did not include standard cognitive assessments. Secondary progressive MS (SPMS) is defined as a history of gradual worsening after an initial relapsing-remitting disease course (RRMS), with or without exacerbations during the progressive course.[24] This study was approved by the medical ethical committees of Erasmus MC in Rotterdam and of the other participating centres. Written informed consent was provided for all patients.

Data analysis

Statistical analysis was performed using SPSS v20 (SPSS Inc., Chicago, Illinois, USA) and GraphPad Prism 5 (GraphPad., San Diego, USA). We divided the patients in three different age groups of clinical interest, based on evidence that puberty enhances CNS autoimmunity in females 25: i.e. pre-puberty, puberty, and adults (patients 1-10, 11-17 and 18-50 years old). Among these groups we compared demographic data, disease course and time to MS diagnosis. ARR were compared between children and adults with MS. Because of the small number of MS patients in age group 1-10 years old (n=6), age groups 1-10 and 11-17 were combined for this analysis. For the comparison of continuous data between two groups we applied a two-tailed t-test, or when not normally distributed a Mann-Whitney U test. For analysis of multiple groups, we used one-way-ANOVA for normally distributed data, and Kruskal-Wallis test for not normally distributed data. Categorical data were analysed using the Chi-square or Fisher's exact test. Kaplan-Meier survival analyses were used to analyse the time to MS diagnosis. Univariate and multivariable Cox proportional hazard regression models were used to obtain hazard ratios (HR) for MS diagnosis and EDSS 4.0 (onset of walking disability) respectively. Negative binomial regression, with the natural logarithm of follow-up years after MS diagnosis as offset, was used to analyse ARR after CIS. This offset was used to correct for the different follow-up times between patients. We used a generalised estimating equations (GEE) model to compare ARR with and without DMT. This GEE model includes the effect and interaction of treatment and age group and accounts for the correlation within patients.

RESULTS

Clinical features at CIS

We included 383 patients. Patient characteristics for the three different age groups are shown in table 1. Children more often presented with polyfocal clinical symptoms (31.8% <18 yrs vs 12.0% >18 yrs, p<0.01). Adults more often presented with isolated transverse myelitis (12.1% <18 yrs vs 28.6% >18 yrs, p<0.01). Children with CIS in age group 11-17 years had more lesions on their MRI (58.2% ≥9 T2 lesions). Lumbar puncture was performed less often in patients presenting with optic neuritis (ON) than in the other clinical presentations of CIS (55.9% vs 71.5% p<0.01). CSF OCB were less often present in 1-10 year-old children with CIS (27.3%).

Age groups	1-10 years (n=28)	11-17 years (n=79)	18-50 years (n=276)	Total (n=383)	p-value
Gender, female, n (%)	16 (57.1%)	48 (60.8%)	204 (73,9%)	268 (70.0%)	0.02
Age (years), mean (SD)	6.6 (3.05)	15.2 (1.69)	34.0 (7.88)	28.1 (11.8)	na
Caucasian ethnicity, n (%)	19 (67.9%)	42 (53.2%)	209 (75.7%)	270 (70.5%)	0.01
Type of clinical onset, n (%)					
-ON	10 (35.7%)	25 (30.3%)	108 (39.1%)	143 (37.3%)	0.44
-Spinal cord	2 (7.1%)	11 (13.9%)	79 (28.6%)	92 (24.0%)	<0.01
-Brainstem	0 (0.0%)	11 (13.9%)	33 (12.0%)	44 (11.5%)	0.13
-Other monofocal symptoms	6 (21.4%)	8 (10.1%)	23 (8.3%)	37 (9.7%)	0.08
-Polyfocal symptoms	10 (35.7%)	24 (30.4%)	33 (12.0%)	67 (17.5%)	<0.01
Features first MRI					
≥9 lesions on T2-weighted images, n (%)	6 (22.2%)	46 (58.2%)	99 (36.1%)	151 (39.7%)	<0.01
Dissemination in space[a], n (%)	8 (28.6%)	58 (73.4%)	125 (45.8%)	191 (50.3%)	<0.01
Gadolinium-enhancing lesions, n (%) (n=280)	4 (17.4%)	33 (54.1%)	85 (43.4%)	122 (43.6%)	0.02
Spinal cord MRI, n (%)	8 (28.6%)	43 (54.4%)	121 (43.8%)	172 (44.9%	0.17
Time CIS to MRI (months), median (IQR)	0.3 (0.1-0.9)	0.6 (0.2-1.6)	1.2 (0.5-2.2)	0.9 (0.3-2.0)	<0.01
CSF findings					
WBC count (·10^6/L), median (IQR) (n=216)	5.0 (2.0-16.0)	8.0 (4.3-18.8)	7.0 (3.3-12.0)	7.0 (3.0-14.8)	0.136
Positive OCB, n (%) (n=252)	6/22 (27.3%)	49/59 (83.1%)	125/171 (73.1%	180/252 (71.4%)	<0.01
IgG index, median (IQR) (n=242)	0.64 (0.49-0.79)	0.95 (0.68-1.42)	0.78 (0.57-1.29)	0.81 (0.57-1.30)	0.02
Follow-up					
CDMS, n (%)	6 (21.4%)	53 (67.1%)	103 (37.3%)	162 (42.3%)	<0.01
MS[b], n (%)	6 (21.4%)	66 (83.5%)	146 (52.9%)	218 (56.9%)	<0.01
Time CIS to CDMS (months), median (IQR)	16.5 (4.0-32.4)	9.0 (3.0-18.9)	19.0 (8.5-39.3)	15.2 (6.5-33.8)	<0.01
Time CIS to MS[b] (months), median (IQR)	16.4 (3.1-32.7)	2.6 (0.6-6.0)	8.2 (2.0-25.5)	4.8 (1.6-21.1)	<0.01
Follow-up (months), mean (SD)	38.3 (16.5)	38.1 (21.5)	48.5 (29.8)	45.6 (27.8)	<0.01
Initiation of DMT before MS diagnosis, n (%)	1 (3.6%)	2 (2.5%)	35 (12.7%)	38 (9.9%)	0.02
Time CIS to DMT (months) median (IQR)	21.4 (7.1-33.3) (n=6)	6.8 (3.6-14.0) (n=59)	7.5 (3.8-24.5) (n=111)	7.2 (3.8-21.4) (n=176)	0.26

Table 1. patient characteristics

[a]DIS according to McDonald 2010 criteria [b]MS diagnosis according to McDonald 2010 criteria.[5]
P-values describe the comparison between all age groups.
Abbreviations: CIS, clinically isolated syndrome; CDMS, clinically definite multiple sclerosis; DMT, disease modifying therapies; IgG, immunoglobulin G; MS multiple sclerosis; na, not applicable; ON, optic neuritis; OCB, oligoclonal bands; WBC, white blood cell count

Disease course and MS diagnosis

A total of 218 patients (56.9%) were diagnosed with MS during follow-up according to the 2010 McDonald criteria in adults and according to the IPMSSG criteria in children.[4,5] Children with CIS in age group 11-17 had the highest rate of MS conversion (83.5% versus 50.0% in the other age groups together p<0.01) and the shortest time to MS diagnosis (median time 2.6 months (IQR: 0.6-6.0) versus 8.2 months (IQR: 1.9-28.2) in the other age groups together, p<0.01). Children in the 11-17 year-old group also had the highest rate of CDMS and shortest time to CDMS diagnosis (Table 1). The time to MS diagnosis is presented in Figure 1. HR for future MS and CDMS diagnosis are shown in Table 2. HR did not change after adjustment for DMT initiation before MS diagnosis, lumbar puncture, or gadolinium administration.

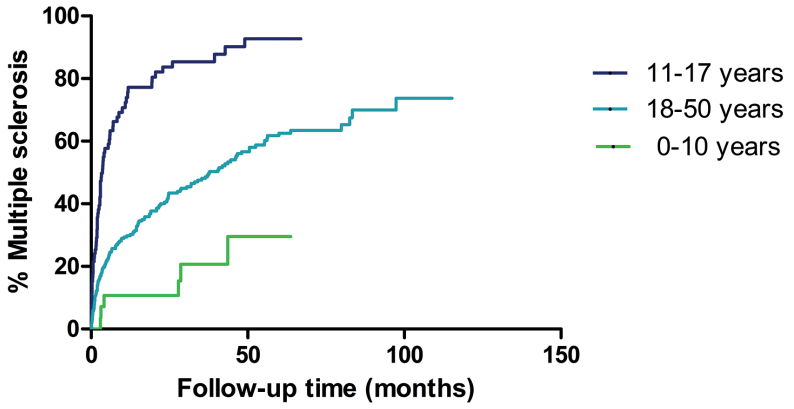

Figure 1. Time from CIS to MS diagnosis in different age categories

Kaplan-Meier curves for time to MS diagnosis for patients in different age categories. (log-rank test p<0.01).

Age group (years)		No. of patients	No. of events	Hazard ratio (95% CI)	p-value
1-10	MS	28	6	0.4 (0.2-0.8)	0.01
	CDMS	28	6	0.6 (0.3-1.4)	0.24
11-17	MS	79	66	3.2 (2.4-4.3)	<0.01
	CDMS	79	53	3.1 (2.2-4.4)	<0.01
18-50	MS	276	146	1 (ref)	1 (ref)
	CDMS	276	103	1 (ref)	1 (ref)

Table 2. hazard ratios for MS and CDMS diagnosis, univariate cox regression analysis

Annualized relapse rates corrected for follow-up were higher in children <18 years than in adults ≥18 years old with MS (mean ARR 0.65 vs 0.43, p<0.01). ARR between children and adults with MS with and without DMT are presented in Table 3. Highest ARR were found in 1-10 year-old children with MS (n=6): ARR without DMT 5.30 and with DMT 0.43 (p<0.01). We found similar associations between ARR and non-Caucasian ethnicity and lumbar puncture in children and adults.

Age group (years)	Total years at risk after MS diagnosis	Total no. of relapses	Overall ARR (95% CI)	ARR without DMT (95% CI)	ARR with DMT (95% CI)	p-value[a]	Interaction coefficient of DMT (95% CI)	p-value[b]
1-17 (n=72)	208	134	0.65 (0.44-0.96)	2.22 (1.04-4.81)	0.48 (0.36-0.65)	<0.01	0.50 (0.28-0.92)	0.03
18-50 (n=146)	451	193	0.43 (0.37-0.50)	0.92 (0.52-1.67)	0.40 (0.32-0.49)	<0.01	Ref.	Ref.

Table 3. ARR corrected for follow-up in MS patients per age group

a Presented p-values for the comparison of ARR with and without DMT within the different age groups.

b Presented p-values for the interaction coefficient of DMT for the different age groups.

Abbreviations: ARR, annualised relapse rate; DMT, disease modifying therapies; CI, confidence interval

Disability

Age at onset of CIS was associated with a shorter time to EDSS 4.0, HR 1.3 per 5 years increase in age at onset (P=0.03). After correction for type of clinical onset using a multivariable COX regression model, this HR did not change. Eight patients were diagnosed with SPMS, who had a higher age at time of CIS (Mean age of SPMS patients at CIS 42.1 (±4.6) vs 27.8 (±11.7) in RRMS p<0.01).

DISCUSSION

The purpose of our study was to report a comparison of childhood-onset versus adulthood-onset CIS and MS in a large prospective cohort during the current immunomodulatory treatment era. The results of our study suggest a more inflammatory disease course, rather than a neurodegenerative disease course, in 11-17 year-old children with CIS, as this group had the highest rate of MS conversion and the shortest time to MS diagnosis, higher ARR, higher MRI lesion load and a more inflammatory CSF profile. Interestingly, the youngest children (1-10 years) with CIS had a relatively lower rate of MS conversion, lower MRI lesion load and a less inflammatory CSF profile. The latter two findings could be explained by our inclusion criteria since we did not include children with acute disseminated encephalomyelitis (ADEM). SPMS, which reflects a more neurodegenerative phase of MS, was found in eight patients >37 years old at onset of CIS. However, secondary progression in MS is age dependant and probably therefore has not been observed yet in our younger patients with the current follow-up.[26] Furthermore,

we found higher EDSS progression rates in adults than in children. Previous studies on childhood-onset versus adulthood-onset MS, already have reported a higher ARR in children.[8,10,11] In our unique prospective study we followed children and adults already from onset of CIS. Overall, we found a female predominance both in patients with CIS and MS, except in young children diagnosed with MS before puberty, of whom four of six MS patients were boys. The difference in sex distribution in children diagnosed with MS before and after puberty and the higher rates of MS diagnoses after puberty suggests that sex hormones contribute to the onset of MS.[2,23,25] A remarkable high rate of non-Caucasian ethnicities was observed in 11-17 year-old children with CIS. Higher rates of non-Caucasian ethnicities have been reported in childhood-onset MS and might reflect a higher vulnerability of developing MS at a younger age.[20,27] A possible explanation for this might be that non-Caucasians miss certain protective genes since their ancestors were born in countries with a low prevalence of MS.[20]

The shorter time to MS diagnosis in 11-17 versus 1-10 year-old children is partly explained by the current diagnostic MRI criteria which allow for an early MS diagnosis in a subgroup of patients at first MRI, however, not in children younger than 12 years of age.[4,5] Still, we observed a high rate of CDMS and short time to CDMS diagnosis in 11-17 year-old children.

ARR in 1-10 year-old children with MS (n=6) was remarkable high, but the number of patients is too small to draw reliable conclusions. In a recently published large study ARR did not differ in children <12 years and ≥12 years.[28] The relative high rate of non-Caucasians (5/6) in 1-10 year-old children with MS in our cohort might contribute to this high ARR.[29]

Early initiation of DMT in adults with CIS might have influenced their disease course. However, a relatively small proportion of adults (12.5%) started DMT before MS diagnosis. In a sub-analysis (data not shown) we found similar results when we excluded these patients. It is unlikely observed differences in ARR are caused by early initiation of DMT, since time to DMT initiation after MS was similar in children and adults, and pre-treatment ARR differed significantly. DMT is already offered to adults with CIS who are at high risk for a future MS diagnosis, but not to children with CIS. However, DMT especially reduces MS relapse rates and has been shown beneficial in children with MS.[16] Therefore early initiation of first-line DMT in children with CIS at high risk for MS seems logical and might be considered in line with current clinical practice in adults. International collaboration is needed in order to define children with CIS at high risk for MS, and to investigate whether early initiation of DMT in 11-17 year old children is safe and beneficial.

A limitation of our study is that follow-up is limited and shorter in children since our prospective study in adults started prior to the study in children. With current follow-up, however, we could already demonstrate important differences in rates of MS diagnosis and relapses. It would be interesting to follow our cohort in order to compare clinical outcome and disease progression over several decades. Another limitation of our study is a selection bias since we only included patients with CIS, while it is known that MS can present with a spectrum of acquired demyelinating events, including ADEM.[4] However, it is not expected that this influences our results much, since a diagnosis of MS after ADEM is uncommon with the current diagnostics criteria.[4] In addition, there

could have been a selection in patients who were reported by physicians from other hospitals. We did not find a centre effect regarding demographics, MRI, CSF features or MS diagnosis. However, we did find a higher rate of CIS patients presenting with ON, included at Erasmus MC. This could be explained by referrals from the nearby Rotterdam Eye Hospital. Patients who were diagnosed with CDMS within 6 months after CIS who were not yet included in our prospective studies, could have been missed. However, we do not expect this includes many patients since it has been reported in another study that up to 30% of CIS patient were diagnosed with CDMS within 6 months30 and in our cohort 23% of CIS patients had CDMS within 6 months after onset of CIS. EDSS scores were assessed annually, therefore disability progression was not always reassessed. Furthermore, we do not have a standard MRI protocol, gadolinium was not administered to all patients at first event, and a follow-up MRI was not performed regularly, nor a lumbar puncture was performed in all patients. A follow-up MRI was more often performed in children than in adults, which influences the time to MS diagnosis. However, the short time to MS diagnosis in 11-17 year-old children was confirmed by the higher rate and shorter time to CDMS. Nevertheless, our data resemble clinical practice.
In summary, we found a more inflammatory disease course of CIS and MS in children. This supports the early initiation of first-line DMT in children with MS, and perhaps could argue for initiation of DMT in 11-17 year-old children with CIS who are at high risk for a future MS diagnosis in line with current clinical practice in adults.

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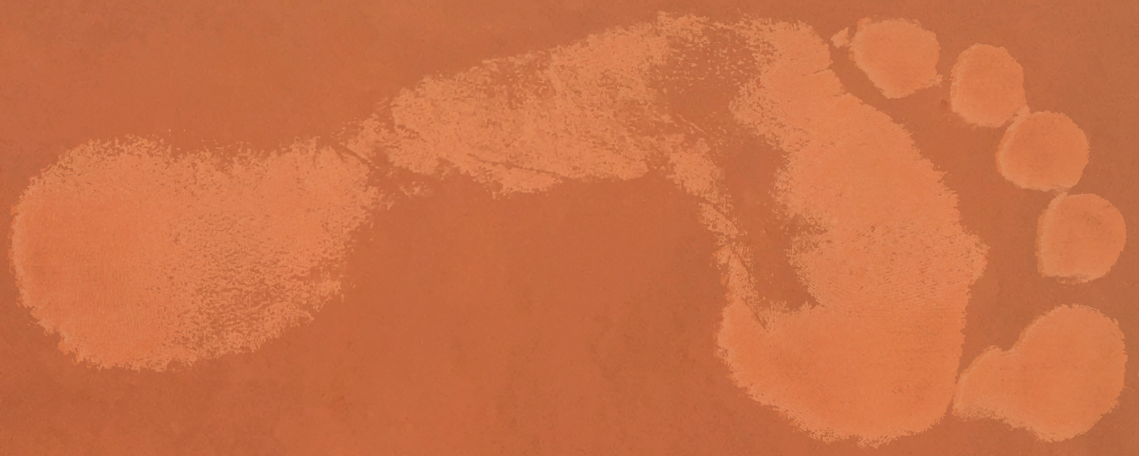

Chapter 8

T-cell activation marker sCD27 is associated with clinically definite multiple sclerosis in childhood acquired demyelinating syndromes

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ABSTRACT

Background: CSF levels of T-cell activation marker soluble CD27 (sCD27) are associated with subsequent disease activity after a first attack of suspected MS in adults. The predictive value for disease course in children with acquired demyelinating syndromes (ADS) is unknown.
Objectives: to assess the predictive value of sCD27 levels for clinically definite MS (CDMS) diagnosis in childhood ADS.

Methods: Children <18years with a first demyelinating event were prospectively included and followed. Soluble CD27 was determined in CSF using an ELISA. Cox regression analyses were used to calculate hazard ratios (HR) for CDMS.

Results: A total of 94 ADS children were included (ADS with encephalopathy (ADS+) n=33 and ADS without encephalopathy (ADS-) n=61). Twenty-nine of 61 ADS- children (48%) were diagnosed with CDMS during follow-up. At baseline, sCD27 levels were higher in patients with a future CDMS diagnosis (n=29) than in monophasic ADS+ (n=30), monophasic ADS- (n=28) and relapsing non-MS patients (n=7) ($p<0.001$). In ADS- patients, sCD27 was associated with CDMS (HR 1.8 per 100 U/mL increase in sCD27 levels, $p=0.031$), after adjustments for age, oligoclonal bands and presence of dissemination in space on baseline MRI.

Conclusion: CSF sCD27 levels at first attack of demyelination is associated with CDMS diagnosis in children. This makes sCD27 a potential clinically relevant quantitative marker when performing routine CSF diagnostics.

INTRODUCTION

Clinical manifestations of acute onset inflammatory demyelinating disease of the central nervous system (CNS) in children are termed acquired demyelinating syndromes (ADS).[1,2] ADS encompasses for example optic neuritis (ON), transverse myelitis (TM) as well as other presentations that localise to monofocal or polyfocal locations in the CNS, such as acute disseminated encephalomyelitis (ADEM). Up to one third of the children with ADS receive a later diagnosis of multiple sclerosis (MS).[1,3,4,5] At the time of a first attack, it can be a challenge to determine the disease course of these patients. Early identification of children who will have an active disease course is important and can have therapeutic implications.[6]

Soluble CD27 (sCD27) is a soluble form of CD27 secreted by activated T-cells after activation via the T-cell receptor, and is introduced as a potential biomarker for T-cell mediated inflammation.[7] CD27 and sCD27 have a role in maturation, activation and proliferation of T and B cells.[8,9] High sCD27 levels are reported in autoimmune diseases like rheumatoid arthritis, systemic lupus erythematosus and MS.[10-12] Bielekova et al, validated CSF sCD27 as a biomarker for intrathecal T-cell activation in MS, using an extensive and validated battery of biomarkers for CNS inflammation.[13] Serum sCD27 does not discriminate between healthy individuals and MS patients.[14] In a recent study, high sCD27 levels in CSF associate with MS diagnosis and disease course in adult patients with clinically isolated syndromes.[15]

These observations in adults have not yet been validated in the paediatric population with CNS demyelination. It is shown that children with MS tend to have a more inflammatory disease course than adults.[16,17] Therefore,we hypothesized that the predictive value of sCD27 levels for a second attack of MS will be equal or even higher in children than in adults. Furthermore, ADS with encephalopathy (ADEM) are known to have extensive intracerebral inflammation on MRI scans and may have severe clinical presentations.[18] The levels of sCD27 might therefore differ between ADS subtypes.

Here we examined whether sCD27 levels at first attack in children differ between ADS subtypes and assessed the predictive value of sCD27 for a second attack of MS in paediatric ADS patients.

METHODS

Study participants

Patients <18 years were included in the Dutch prospective and multicentre study for children with acquired demyelinating syndromes (ADS) (PROUD-kids study).[2] All patients with a lumbar puncture and baseline MRI, performed for routine diagnostics <6 months after onset of first symptoms, were included between June 2006 and February 2017. Patients with alternative diagnosis were excluded. Patients were assessed at baseline and reassessed regularly. Patients were instructed to contact the hospital in case of suspected exacerbation.

Definitions

Acquired demyelinating syndromes in children encompass the first attack of demyelination in the central nervous system, including patients presenting with encephalopathy (ADEM, defined as ADS+) and ADS without encephalopathy (defined as ADS-).[2,3] CDMS was defined as two non-encephalopathic attacks, based on the clinical criteria proposed by the International Paediatric MS study group for paediatric MS diagnosis.[3] Relapsing patients who have a distinct clinical phenotype other than CDMS were also included in this study, such as ADEM followed by relapsing optic neuritis (ADEM-ON),[19] anti-aquaporin 4 antibody (AQP4-ab) positive and negative relapsing disease.[3,20,21]
A relapse was defined as acute worsening of existing symptoms or new symptoms after 30 days of improvement or stable disease and no evidence of an alternative diagnosis. The symptoms should exist for more than 24 hours and not be preceded by fever.[22] Exacerbations were confirmed by neurological examination.
Follow-up duration was calculated by subtracting the date of first symptoms from the last visit date. Disability was expressed by the Expanded Disability Status Scale (EDSS).[23]

CSF samples and sCD27 ELISA

CSF samples were centrifuged for 10 minutes at 3000 rpm to separate the supernatant from cells and cellular components. After centrifugation, all samples were stored in -80 degrees Celsius until use.[24] Routine diagnostics of CSF included oligoclonal bands (OCBs), IgG index, cell count and total protein. Soluble CD27 levels were measured in duplo using the available commercial ELISA kit (Pelikine compact human sCD27 kit) manufactured by Sanquin in Amsterdam, the Netherlands.[7] The manufacturer's instructions were followed when performing the sCD27 ELISA. Levels of sCD27 were expressed by U/mL by reference to a standard curve supplied with the ELISA kit. The clinical diagnosis was blinded for the analysts who performed the ELISA. The detection limit of the ELISA was 6 U/mL.

Standard protocol approvals, registrations and patient consents

The PROUD-kids study was approved by the Erasmus MC ethical committee and by the ethical committees of the other participating centres. Written informed consent was obtained from patients and/or their families.

Statistical analysis

Statistical analyses were performed using SPSS 24.0. Kolmogorov-Smirnov test was used to assess the normality of the data. Figures are made in Graphpad Prism5. Soluble CD27 levels were not normally distributed, and were therefore log transformed to attain normally distributed data. Due to log-transformation, geometric means were calculated. For group comparisons, Student's t-test and Mann-Whitney U test were used for continuous variables when appropriate. Student's t-test was performed to compare the

sCD27 levels in different ADS subgroups. Chi-square and Fisher exact test were used for categorical data. Correlation analyses were done for two continuous variables.
Cox proportional hazard regression models were used to calculate univariate and multivariable hazard ratios (HR) in the ADS- group, with CDMS set as endpoint. The Cox proportional hazard assumption was tested by including a time dependent covariate in the model. Known predictors for MS diagnosis such as age of onset, OCB and fulfilling dissemination in space (DIS) at baseline MRI are used for adjustments in the multivariable analysis for sCD27 levels.
Annualised relapse rate (ARR) for CDMS patients was compared between groups with high and low levels of sCD27 using a binomial regression model with the natural logarithm of number of follow-up years after a second clinical attack as offset. This offset corrects for the difference in follow-up duration between patients. The data were over-dispersed and therefore the Poisson regression model was not suitable for our data set. P-value of <0.05 was considered significant.

RESULTS

Patients

A total of 94 children with a first attack of ADS were included in this study. Of these children, 33 presented with ADS+ and 61 with ADS-. The median age for ADS+ was 4.5 years (IQR 2.6-6.3) and for ADS- patients 14.5 years (IQR 11.3-16.0). During follow-up, 30/33 (91%) of the ADS+ patients remained monophasic. Three ADS+ patients (9%) had a relapsing disease and fulfilled the criteria for ADEM-ON. No patient presenting with ADS+ was diagnosed with CDMS. Within the ADS- patients, 33/61 (54%) had a second attack. Of these 33 relapsing patients, 29/33 (88%) children were diagnosed with a second attack fulfilling the criteria for CDMS and the other 4/33 (12%) were diagnosed with a relapsing demyelinating disorder other than MS (2 AQP4-ab positive and 2 AQP4-ab negative patients). The median time to CDMS was 10.3 months (IQR 4.3-15.7 months). The median follow-up duration for all included patients was 2.5 years (IQR 1.4-4.9 years). The following flowchart (figure 1) illustrates the presenting phenotypes (ADS+ and ADS-) and diagnoses during follow-up.

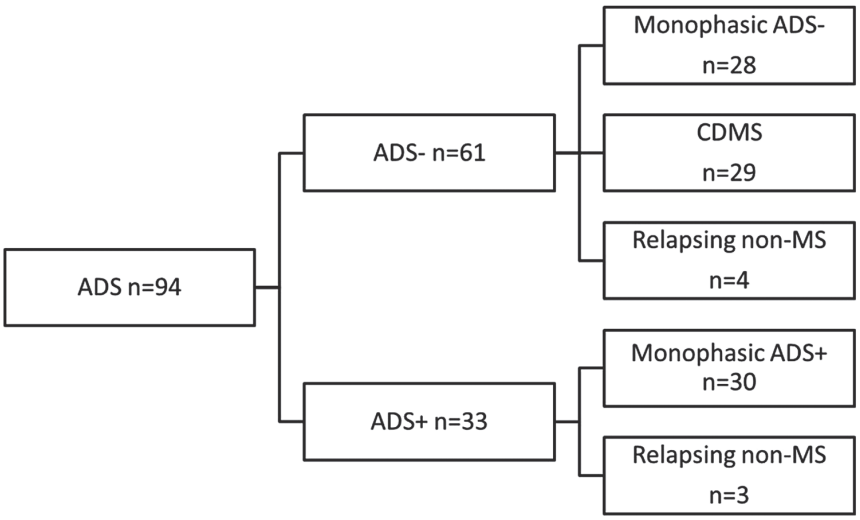

Figure 1. Flowchart of included ADS patients

Patients presented as ADS without encephalopathy (ADS-) and ADS with encephalopathy (ADS+; ADEM). The disease course during follow-up are shown dividing patients in monophasic ADS-, monophasic ADS+, CDMS and relapsing non-MS. The relapsing non-MS patients included 3 ADEM followed by optic neuritis, 2 AQP4 positive NMOSD and 2 patients with AQP4 negative NMOSD.

Abbreviations: ADS, acquired demyelinating syndromes; ADS-, ADS without encephalopathy, ADS+, ADS with encephalopathy; ADEM, acute disseminated encephalomyelitis; AQP4, anti-aquaporin 4 antibodies; CDMS, clinically definite MS

The median time between onset of symptoms and CSF sampling was 2.2 weeks (IQR 0.7-5.9). No correlation was found between the levels of sCD27 and time between first symptoms and CSF sampling in the total group and in all groups separately. Twenty-four of 94 patients (26%) received acute treatment (intravenous corticosteroids) before CSF sampling. No difference in sCD27 was found in patients who did or did not receive intravenous corticosteroids. No patients were on disease modifying therapy (DMT) or oral steroids before CSF sampling. Patient characteristics are shown in Table 1.

Characteristic	Mono- ADS+ (n=30)	Mono-ADS- (n=28)	CDMS (n=29)	Relapsing non-MS (n=7)	All (n=94)	p-value[a]
Female sex, n (%)	20 (67)	13 (46)	19 (66)	4 (57)	56 (60)	0.380
Age (years), mean (SD)	4.1 (2.5-6.1)	11.7 (6.2-16.0)	15.1 (13.8-16.0)	10,7 (6.0-16.3)	11.3 (5.1-15.2)	<.001
Follow-up time (years), median (IQR)	3.7 (1.3-6.1)	2.1 (1.1-3.9)	2.6 (1.8-4.6)	2.3 (1.2-4.3)	2.6 (1.4-4.9)	0.343
Clinical presentation, n (%)						
Isolated optic neuritis, n (%) Isolated bilateral ON, n (%)	0 na	7 (25) 2/7 (22)	7 (24) 0	0 na	14 (15) 2/14 (14)	0.940[b]
Isolated transverse myelitis, n (%) Isolated LETM, n(%)	0 na	8 (29) 4/8 (50)	7 (24) 0	1 (14) 1/1 (100)	16 (17) 5/16 (31)	0.704[b]
Other CIS, n (%)	0	6 (21)	3 (10)	1 (14)	10 (11)	0.251[b]
Polyfocal CIS, n (%) LETM and ON, n (%)	0	7 (25) 3/7 (60)	12 (41) 0	2 (29) 2/2 (100)	21 (22) 5 (5)	0.190[b]
Polyfocal CIS with encephalopathy, no.(%)	30 (100)	0	0	3 (43)	26 (37)	N/A[b]
CSF OCB, (≥ 2 bands), n (%) (n=78)	0/21	14/24 (58)	25/27 (93)	0/6	39/78 (48)	<0.001
IgG index, median (IQR) (n=67)	0.57 (0.53-0.74)	0.57 (0.53-0.77)	1.11 (0.88-1.84)	0.63 (0.52-0.66)	0.72 (0.56-1.02)	<0.001
CSF WBC count, median (IQR) (n=90)	22 (7-48)	5 (3-10)	14 (7-28)	43 (9-77)	10 (5-35)	0.491
% of CSF mononuclear WBC, median (IQR) (46/90)	77 (55-95)	75 (45-90)	100 (90-100)	90 (70-95)	90 (65-100)	0.004
Time from symptom onset to CSF sampling (weeks), median (IQR)	1.6 (0.6-2.8)	1.3 (0.4-5.9)	5.0 (2.4-12.9)	2.6 (1.3-10.6)	2.3 (0.7-5.9)	<.001
CSF sampling prior to acute treatment, n (%)	23 (77)	21 (75)	23 (79)	3 (43)	70 (75)	0.249

Table 1. Patient characteristics

a Comparison between all subgroups.

b Comparison between monophasic ADS- and CDMS.

Abbreviations: mono-ADS+, monophasic acquired demyelinating syndromes with encephalopathy; mono-ADS-, monophasic acquired demyelinating syndromes without encephalopathy; CDMS, clinically definite multiple sclerosis; relapsing non-MS, relapsing ADS not diagnosed as MS; ON, optic neuritis; CIS, clinically isolated syndrome; LETM, longitudinally extended transverse myelitis (more than 2 segments involved); OCB, oligoclonal bands; IQR, interquartile range; WBC, white blood cell; na, not applicable

Soluble CD27 levels in subgroups of ADS

Soluble CD27 levels at first attack of ADS were higher in patients with a future second attack of MS (n=29) than in ADS-patients who remained monophasic (n=28) (geometric means 65 U/mL; 95% CI 47-89 vs 13 U/mL; 95% CI 9-18, p<0.001). Patients with monophasic ADS+ (n=30) did not differ in sCD27 levels from monophasic ADS- patients (n=28) (geometric mean 18 U/mL; 95% CI 11-30 vs 13 U/mL; 95% CI 9-18), but did differ from ADS- patients with future CDMS diagnosis (geometric mean 13 U/mL; 95% CI 9-18 vs 65 U/mL; 95% CI 48-89; p<0.001). Patients with a relapsing non-MS disease course (n=7; 4 patients with ADS- onset and 3 with ADS+ onset) had lower sCD27 levels at onset than patients with a future CDMS diagnosis (geometric mean 18 U/mL; 95% CI 8-42 vs 65 U/mL; 95% CI 48-89 U/mL; p=0.001), but did not differ from monophasic ADS+ and monophasic ADS- patients.

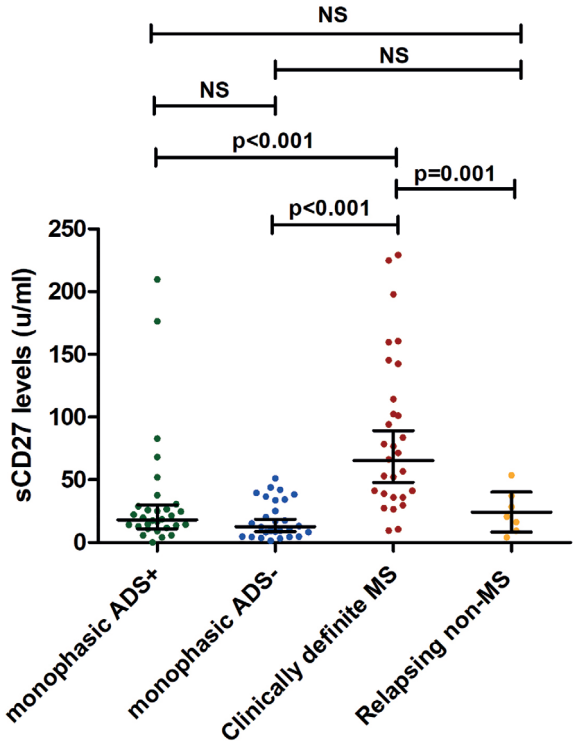

Figure 2. Comparison of CSF sCD27 levels in ADS at time of first demyelinating event

Comparison of CSF soluble CD27 levels between patients with ADS+ (ADEM), monophasic ADS-, CDMS and relapsing non-MS patients. The relapsing non-MS patients included 3 ADEM followed by optic neuritis, 2 AQP4 positive NMOSD and 2 patients with AQP4 negative NMOSD.

Horizontal lines with error bars indicate geometric means with 95% CI.

Abbreviations: ADS, acquired demyelinating syndromes; ADS-, ADS without encephalopathy; ADS+, ADS with encephalopathy; ADEM, acute disseminated encephalomyelitis; CDMS, clinically definite MS

These results are displayed in Figure 2. No differences were found in sCD27 levels between anti-MOG positive (n=13) and anti-MOG negative (n=81) patients (data not shown).
Fourteen of the 59 ADS- patients (24%) (after excluding the 2 AQP4 positive patients) fulfilled MS diagnosis at baseline by fulfilling the IPMSSG 2012 criteria for MS on first MRI.[3] The sCD27 levels of these 14 children are significantly higher than ADS- patients who did not fulfil the criteria at baseline (geometric mean 78 U/mL; 95% CI 53-115 vs 20 U/mL; 95% CI 15-30, p<0.001).
Of the 28 monophasic ADS– patients, 10 patients showed sCD27 levels that exceed the upper bound of the 95% confidence interval of the geometric mean (13 U/mL; 95% CI 9-18), as shown in Figure 2. Of these patients, 8/10 fulfilled the diagnosis of MS by follow-up MRI scans, but did not experience a second attack during follow-up. The sCD27 geometric mean of these 8 patients was higher than the other 20 monophasic ADS- patients (geometric mean 35 U/mL; 95% CI 27-45 vs 9 U/mL; 95% CI 6-12; p<0.001), but lower than the patients with future CDMS diagnosis (geometric mean 35 U/mL; 95% CI 27-45 vs 65 U/mL; 95% CI 48-89 U/mL; p=0.04).
A subgroup analysis (n=59) was performed after excluding patients with <2 years of follow-up (patients with CDMS n=20). This did not change our observation that sCD27 levels are elevated in patients with a future second attack of MS compared to the three other subgroups (geometric mean 61 U/mL, 95% CI 42-89 vs 15 U/mL, 95% CI 10-22; p<0.001).

Soluble CD27 correlates with CSF and MRI parameters

Patients with unique OCBs in CSF had higher sCD27 levels compared to patients without OCB (geometric means 42 U/mL; 95% CI 30-60 vs 17 U/mL; 95% CI 12-23, p<0.001).
Soluble CD27 levels were positively correlated with IgG index (Spearman rho 0.695, p<0.001) as well as white blood cell count (Spearman rho 0.444, p<0.001).
Patients fulfilling DIS on baseline MRI (n=50) showed higher sCD27 levels than patients without DIS (geometric mean 35 U/mL; 95% CI 26-46 vs 16 U/mL; 95% CI 11-24, p=0.003).
A total of 72 patients (77%) received gadolinium when the first MRI was performed. The sCD27 levels were significantly higher in children who showed contrast enhancement (n=31, 58%) than patients without contrast enhancement (geometric mean 49 U/mL; 95% CI 34-71 vs 19 U/mL; 95% CI 12-28, p=0.001).
For the analyses depicted above, all patients were analysed. Exclusion of patients with ADS+ (ADEM) and relapsing non-MS from the analysis (thus only including patients with monophasic ADS- and CDMS), did not alter the results. Results are shown in Figure 3.

High levels of sCD27 at first attack in ADS- patients are independently associated with a shorter time to CDMS.

As described above, all patients with a second attack of MS had an ADS- presentation. No patient with ADS+ was diagnosed with CDMS. Therefore, in the following analyses ADS+ patients were excluded as well as the relapsing non-MS patients with AQP4-ab.

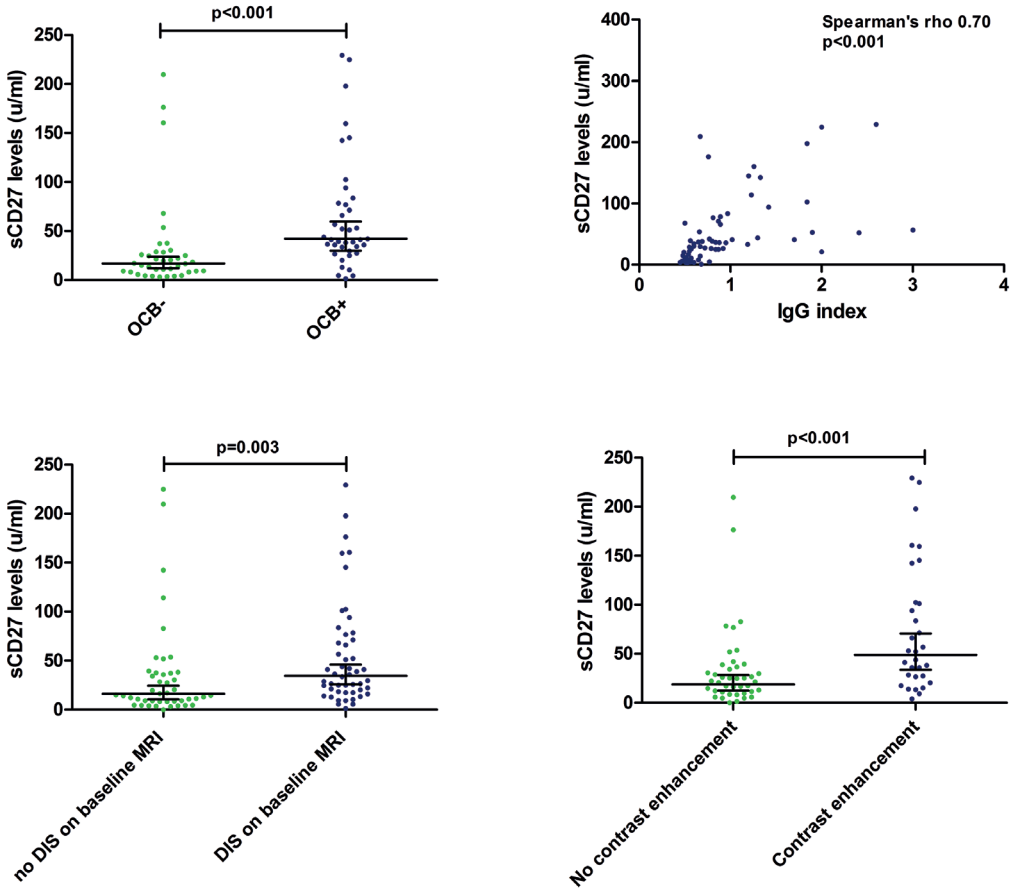

Figure 3. Comparison of sCD27 levels with CSF and MRI parameters

Comparing CSF and MRI parameters with the levels of sCD27 in ADS patients.
Horizontal lines with error bars indicate geometric means with 95% CI.
Abbreviations: ADS, acquired demyelinating syndromes; OCB, oligoclonal bands; DIS, dissemination in space

A Kaplan Meier curve was obtained after dichotomizing the sCD27 levels, using the median of the included ADS- patients (n=59); median sCD27 36.0 U/ml. Six of 29 patients in the low sCD27 group were diagnosed with CDMS (21%) versus 23/30 (79%) in the high level group. Figure 4 shows the Kaplan Meier curve for time to CDMS diagnosis (log-rank test, p=0.006).
The univariate HR for CDMS was 2.8 (95% CI 1.7-4.6) per 100 U/mL increase in sCD27 levels (p<0.001). In the multivariable COX regression analyses, we corrected for age of onset, OCB and dissemination in space (DIS). After these corrections, sCD27 was independently associated with time to CDMS diagnosis with an HR of 1.8 (95% CI 1.0-3.3) per 100 U/mL increase in sCD27 levels (p=0.031).

Eight of 29 patients (28%) who received MS diagnosis based on MRI received DMT before CDMS and this might have postponed the second attack. We performed a sub-analysis using the COX-regression analysis where we excluded these patients, resulting in the same HR for the univariate (2.8, 95% CI 1.7-4.9; p<0.001) and multivariable analyses after adjusting for age of onset, OCB and DIS (1.8, 95% CI 0.96-3.4; p=0.061).

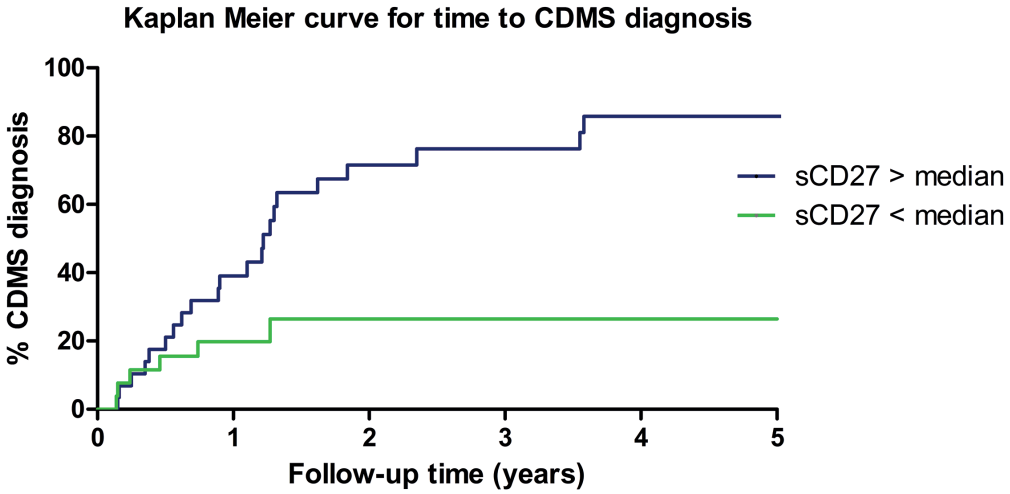

Figure 4. Kaplan Meier curve for time to CDMS diagnosis in ADS without encephalopathy

Kaplan Meier curve for time to CDMS diagnosis. All ADS- patients were stratified into two groups by the median CSF sCD27 level of all CIS patients; median 36 U/mL.

Abbreviations: ADS, acquired demyelinating syndromes; ADS-, ADS without encephalopathy; CDMS, clinically definite MS

Annualised relapse rate and disability

In patients who were diagnosed with CDMS, we used a negative binomial regression model for analyzing the ARR. The median of sCD27 levels in CDMS patients (n=29; 71 U/mL) was used to stratify patients into two groups with either high or low sCD27 levels. There was no difference found in ARR between high and low level group after correcting for follow-up duration. No correlation was found between sCD27 levels and EDSS score during follow-up.

DISCUSSION

Here we show that the T-cell activation marker sCD27 in CSF at first attack in paediatric ADS differs among ADS subtypes. Soluble CD27 levels in patients with a future diagnosis of CDMS were higher than in monophasic ADS+, monophasic ADS- and relapsing non-MS patients. There was no difference between the latter three groups. We analyzed ADS+ patients separately from ADS- patients as MS diagnoses after ADS+ is extremely low and also in our cohort no patients with ADS+ were diagnosed with CDMS during follow-up. Soluble CD27 was associated with a shorter time to CDMS diagnosis independently of known relevant clinical parameters such as MRI and CSF characteristics.
An interesting observation was that 80% of the monophasic ADS- patients, whose sCD27 levels exceeded the 95% CI intervals of the whole monophasic ADS- group, fulfilled the IPMSSG diagnostic criteria for MS based on follow-up MRI. The sCD27 levels of this group were higher than monophasic ADS- and somewhat lower than patients with CDMS diagnosis. This may be related to the fact that these patients (who are diagnosed with MS by MRI alone) have a less active clinical disease course, compared to CDMS patients with higher levels of sCD27.
In line with van der Vuurst de Vries et al, we observed higher soluble CD27 levels in patients with a future CDMS diagnosis than in monophasic ADS patients without encephalopathy (including clinically isolated syndromes).[15] The levels were even higher in children than in adults with MS (geometric mean 65 U/ml; 95% CI 48-89 versus 42 U/mL; 95% CI 29-51 respectively).[15] Our data not only validates the conclusion of van der Vuurst de Vries et al, but is also congruent with previous observations that paediatric MS patients have a more inflammatory disease course compared to adults. [16, 25-30]
The higher levels in patients with a future CDMS diagnosis most likely correspond to a higher intrathecal T-cell activation and higher inflammatory activity. The correlation we found between sCD27, OCB and IgG index is in line with previous adult studies. [13-15] In vitro, a functional role of sCD27 on stimulation and differentiation of B-cells is described earlier.[31, 32] However, the exact role for sCD27 on IgG production remains to be investigated.
In adult MS patients, a higher ARR was found in patients with high sCD27 levels at time of clinically isolated syndrome.[15] One would expect also a higher relapse rate in paediatric MS patients with high sCD27 levels at time of the first attack, however, no association with ARR was found. This finding might be explained by a ceiling effect, as the overall relapse rate in our paediatric MS cohort is high.[16]
No association was found with EDSS, which is little surprising given the slower disease progression in paediatric MS.[27, 33] Longer follow-up duration will be needed to investigate a possible relationship between sCD27 levels and chronic disease progression.
There were a few limitations of this study. First, the follow-up duration varied between patients. We addressed this problem by correcting for follow-up duration in the survival analyses. We also performed a sub-analysis in which we excluded patients with less than two years of follow-up. This did not alter the conclusions. Second, we did not perform a follow-up MRI on a regular basis. However, we aimed to assess the value of sCD27 levels on the clinical disease course and therefore chose CDMS as a study endpoint instead of the McDonald criteria. In addition, the use of DMTs may have delayed a second attack and could have influenced the results of the survival analyses. Therefore we per-

formed sub-analyses after excluding patients with ADS- who used DMT before CDMS diagnosis. After excluding these patients, the follow-up analysis was not significant (p=0.061), however, there was still a clear trend and the univariate analysis remained significant. Lastly, patients who remained monophasic had a shorter time to CSF sampling compared to patients who were diagnosed with CDMS during follow-up. This may have been caused by the difference in severity of the presenting symptoms and the differential diagnosis at onset, for example in ADEM, where acute non-demyelinating pathology needs to be ruled out. Yet, we found no correlation between the time to CSF sampling and the levels of sCD27, making it unlikely that this influenced our results.
In summary, we show that CSF sCD27 in children with ADS at time of the first attack is associated with a future CDMS diagnosis, independently of MRI and CSF parameters. This result is in line with the earlier finding in adult CIS patients that higher sCD27 was associated with subsequent MS diagnosis.[14] Therefore we can conclude that sCD27 is a potential clinically relevant quantitative marker when performing routine CSF diagnostics not only in adults but also in children with ADS. The next step will be validation of these findings in international cohorts.

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Chapter 9

High neurofilament levels are associated with clinically definite multiple sclerosis in children and adults with clinically isolated syndrome

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ABSTRACT

Background: A promising biomarker for axonal damage early in the disease course of multiple sclerosis (MS) is neurofilament light chain (NfL). It is unknown whether NfL has the same predictive value for MS diagnosis in children as in adults.

Objective: To explore the predictive value of NfL levels in CSF for MS diagnosis in paediatric and adult CIS patients.

Methods: A total of 88 adult and 65 paediatric patients with a first attack of demyelination were included and followed (mean follow-up time in adults: 62.8 months (SD±38.7) and 43.8 months (SD±27.1) in children). Thirty control patients were also included. Lumbar puncture was done within 6 months after onset of symptoms. NfL was determined in CSF using ELISA. COX regression analyses were used to calculate hazard ratios (HR) for clinically definite MS (CDMS) diagnosis.

Results: After adjustments for age, oligoclonal bands, and asymptomatic T2-lesions on baseline MRI, increased NfL levels in both paediatric and adult CIS patients were associated with a shorter time to CDMS diagnosis (children HR 3.7; p=0.007, adults HR 2.1; p=0.032). For CIS patients with a future CDMS diagnosis, children showed higher NfL levels than adults (geometric mean 4888 vs 2156 pg/mL; p=0.007).

Conclusion: CSF NfL levels are associated with CDMS diagnosis in children and adults with CIS. This makes NfL a promising predictive marker for disease course with potential value in clinical practice.

INTRODUCTION

Childhood-onset multiple sclerosis (MS) occurs in 3-5% of all MS patients.[1,2] Although children with MS have a more inflammatory disease course with a higher relapse rate than adult patients,[3-5] clinical follow-up studies suggest that disability progression is slower in children than in adults.[4,6,7] However, impairment of age-expected brain growth was seen early in the disease course of paediatric MS patients.[8] This indicates that not only neuroinflammation but also neurodegeneration occurs early in childhood-onset MS.

Axonal damage is considered one of the major causes for persisting neurological disability in MS.[9] A promising biomarker for axonal damage is neurofilament light chain (NfL). Neurofilament light chain is an element of the neuron cytoskeleton, and is released in the extracellular space after neuronal cell death.[10] In healthy individuals, NfL levels increase with age, which reflects neurodegeneration and is part of the physiological aging process.[11]

High NfL levels in CSF in adults with clinically isolated syndrome (CIS) have been reported as an independent risk factor for MS diagnosis.[12] Furthermore, NfL levels have been associated with brain volume changes in adult CIS patients.[12] However, whether NfL is also increased at disease onset in children is still unknown.

The primary purpose of this prospective study was to investigate whether NfL levels can predict diagnosis of clinically definite MS (CDMS) in children with CIS. Our second aim was to compare NfL levels in CSF at time of a first demyelinating event between children and adults. Finally, we examined the association between NfL and signs of axonal loss on magnetic resonance imaging (MRI).

METHODS

Study participants

Children and adults were included in either our prospective cohort of adult patients with CIS (PRedicting the OUtcome of a Demyelinating event, PROUD study) or in our prospective cohort of children with acquired demyelinating syndromes (ADS) (PROUD-kids study).[13,14] Both studies are ongoing multicentre studies initiated by Erasmus MC in Rotterdam, The Netherlands, which is a tertiary referral centre for adult and paediatric MS patients (MS Centre ErasMS, and National Paediatric MS Centre).

All patients were included between February 2002 and December 2015 within 6 months after a first event of demyelination of the central nervous system (CNS). Adult patients were younger than 50 years of age, and paediatric patients were younger than 18 years. No patients had a history of previous neurological symptoms suggestive for CNS demyelination. Patients with alternative diagnoses were excluded from analyses.

The included patients underwent a baseline brain MRI and routine laboratory tests to rule out other possible diagnoses. A lumbar puncture (LP) was performed and extra CSF was collected and stored at −80°C until use.

Patients were assessed at baseline and were reassessed regularly. At baseline instructions were given to the patients to contact the hospital in case of suspected exacerbation.

Cerebrospinal fluid of adult control samples (n=30) was obtained in the Erasmus MC from patients with neurological symptoms but no objective clinical or paraclinical findings to define a specific neurological disease (symptomatic controls). [15]

Standard protocol approvals and patient consents

The study protocol was approved by the Medical Ethics Committee of Erasmus MC Rotterdam and of the other participating centres. Written informed consent was obtained from all patients and/or their families.

Definitions

CIS was defined as a first attack of demyelination in the CNS without encephalopathy.[16] Clinically definite MS (CDMS) was defined by the Poser criteria as two non-encephalopathic attacks with clinical evidence of two separate lesions.[17] ADS in children encompass the first attack of demyelination, including CIS and acute disseminated encephalomyelitis (ADEM).[14] Patients presenting with other ADS subtypes than CIS or ADEM were excluded from the analyses. Children were diagnosed with CIS, ADEM and CDMS according to the diagnostic criteria proposed by the International Paediatric Multiple Sclerosis Study Group.[13] CDMS was used as the primary outcome. Patients who remained CIS during follow-up are referred to as CIS-CIS and patients who were diagnosed with CDMS during follow-up are referred to as CIS-CDMS. In both children and adults, an exacerbation is defined as sub-acute worsening of existing symptoms, or new symptoms after at least 30 days of improvement or stable disease. Symptoms should exist for more than 24 hours, not be preceded by fever, and not be caused by an alternative diagnosis.[18] All exacerbations were confirmed by neurological examination.
Expanded Disability Status Scale (EDSS) was used to assess disability.[19] When patients were diagnosed with CDMS an EDSS was done annually. EDSS scores performed within 3 months after an exacerbation were not considered. Follow-up was calculated by subtracting the date of first symptoms from the last visit date. Baseline MRI scans were performed at 1.5 Tesla scanners and reviewed blindly. Available T1-, axial T2-, axial and/or sagittal fluid attenuated inversion recovery (FLAIR)- images were used. The MRIs were scored on ≥9 T2 lesions, dissemination in space and time, and asymptomatic T2 lesions. The presence of T1-hypointense lesions on baseline MRI were assessed in CDMS patients. T1-hypointense lesions were defined as non-enhancing lesions being hypointense relative to cortical grey matter.[20] Patients who did not receive gadolinium were excluded for the analysis of T1-hypointense lesions.

CSF sampling and NfL ELISA

Routine CSF diagnostics including IgG index, oligoclonal bands (OCB), cell count and total protein were performed. The remaining CSF was immediately centrifuged for 10 minutes at 3000 rpm to separate the supernatant from cells and cellular elements. After centrifugation, samples were aliquoted and stored at –80°C until use.
CSF analyses for OCBs were performed in local laboratories using isoelectric focusing.[21]

OCB status was regarded as positive if there were ≥2 unique bands in CSF compared to serum. IgG index above 0.66 was considered as elevated.
Neurofilament light chain levels in CSF were measured batch-wise in two rounds, according to the manufacturer's instructions, using a stable commercially available solid phase sandwich ELISA (UmanDiagnostics, Umea, Sweden).[22] NfL concentrations (picogram per millilitre (pg/mL)) were calculated using a standard curve according to manufacturer's instructions. All samples were tested double blind and measured in duplicate. The detection limit of the ELISA was 150 pg/mL.

Data analysis

We used SPSS software, version 21.0 (SPSS Inc) and GraphPad Prism5 to perform statistical analyses. After log transformation, NfL levels showed a parametric distribution in both children and adult samples. Therefore we calculated geometric means for NFL levels. Group comparisons for continuous data were performed using 2-tailed t test for normally distributed variables (NfL, age at onset, follow-up time) and Mann-Whitney U test was used for non-parametric data (time between CIS and LP). We used one-way ANOVA and post-hoc Bonferroni correction for parametric data to analyse differences between multiple groups. Chi-square or Fisher exact test were performed for categorical variables (gender, type of clinical onset, OCB, elevated IgG index, asymptomatic T2 lesions, ≥9 T2-lesions, and disease modifying therapies (DMT) after CIS and before CDMS). Spearman rank correlation was used for correlation analyses between non-parametric continues variables. Time to CDMS diagnosis was determined by subtracting the date of the first symptoms from the date of diagnosis. COX proportional hazard regression analyses were used to calculate univariate and multivariable hazard ratios (HR) for time to CDMS diagnosis. Known predictors for MS diagnosis were used in the multivariable analyses (OCB, asymptomatic T2-lesions). Patients who were not diagnosed with CDMS during follow-up were considered as censored observations. We used the median of NfL levels to establish cut-off values for high and low levels of NfL in children and adults separately. p-values less than 0.05 were considered significant.

RESULTS

Patients characteristics

A total of 65 children with a first demyelinating event of the CNS, 88 adult patients with CIS, and 30 age and gender matched adult control individuals were included in this study.
Of the 65 children, 24 presented with ADEM and 41 with CIS. Twenty-five of 41 (61%) children with CIS were diagnosed with CDMS during a mean follow-up time of 38.4 months (SD: 21.0). The mean follow-up time in adult CIS patients was 68.2 months (SD: 39.3) in this period 43 (49%) patients were diagnosed with CDMS.
The median time (interquartile range; IQR) from CIS to CDMS in adult patients was 36.4 months (14.4-48.9) and in children 10.8 months (5.0-15.7).

The time between CIS and LP was not significantly different between children and adult patients. No patients were receiving DMT at time of LP.
Sixteen adult patients (18%) and 14 (34%) children who were not yet diagnosed with CDMS received DMT (glatiramer acetate (n=11), interferon (n=19), natalizumab (n=1)).
The patient characteristics for adults and children are shown in Table 1 and Table 2.

Adults	Controls (n=30)	CIS-patients (n=88)	CIS-CDMS (n=43)	CIS-CIS (n=45)	p-value[a]
Female sex, n (%)	20 (66.7)	59 (67.0)	34 (79.1)	25 (55.6)	0.02
Age[b] (years), mean (SD)	33.4 (±9.5)	31.2 (±7.2)	31.9 (±7.1)	33.6 (±7.3)	0.28
Follow-up time (months), mean (SD)	na	62.8 (±38.7)	89.0 (±36.8)	48.3 (±30.7)	<0.01
Type of clinical onset, n (%)					
-Optic nerve	na	41 (46.6)	21 (48.8)	20 (44.4)	0.68
-Spinal cord	na	23 (26.1)	11 (25.6)	12 (26.7)	0.91
-Other localization	na	24 (27.3)	11 (25.6)	13 (28.9)	0.73
OCB, (≥2 bands), n (%)	na	63/83 (75.9)	36/41 (87.8)	27/42 (64.3)	0.01
Elevated IgG index (cut-off: 0.66), n (%)	na	44/85 (51.8)	22/41 (53.7)	22/44 (50.0)	0.74
Time CIS to LP (weeks), median (IQR)	na	6.1 (2.7-13.2)	6.0 (2.9-12.6)	6.7 (2.6-14.1)	0.97
≥9 lesions on T2-weighted images, n (%)	na	27 (30.7)	18 (41.9	9 (20.0)	0.03
Asymptomatic T2-lesions, n (%)	na	76 (86.4)	38 (88.4)	38 (84.4)	0.59
MS based on first MRI[c], n (%)	na	16 (18.2)	11 (25.6)	5 (11.1)	0.08
DMT before CDMS diagnosis, n (%)	na	16 (18.2)	12 (27.9)	4 (8.9)	0.02
Time between CIS and start DMT (months), median (IQR)	na	27.8 (9.7-43.1)	29.5 (9.9-46.6)	14.4 (5.9-29.0)	0.25

Table 1. Patient characteristics (adults)

[a] p value calculated between CIS-CDMS and CIS-CIS

[b] For patients with CIS: age at CIS, for Controls: age at lumbar puncture

[c] Dissemination in space and time at baseline based on McDonald 2010 criteria

Abbreviations: CIS, Clinically isolated syndrome; CIS-CDMS, patients who are diagnosed with CDMS during follow-up after CIS defined by Poser criteria; CIS-CIS, not diagnosed with CDMS; na, not applicable; DMT, disease modifying therapy; OCB, oligoclonal bands; Ig, Immunoglobulin; LP, lumbar puncture.

Children	ADS-patients (n=65)	ADEM (n=24)	CIS-CDMS (n=25)	CIS-CIS (n=16)	p-value [a]
Female sex, n (%)	38 (58.8)	16 (66.7)	16 (64.0)	6 (37.5)	0.10
Age (years), median (IQR)	12.5 (5.4-15.5)	4.1 (2.6-7.2)	15.0 (13.8-16.0)	14.2 (9.0-16.4)	0.52
Follow-up time (months), mean (SD)	43.8 (±27.1)	53.1 (±33.7)	44.1 (±22.3)	29.5 (±15.5)	0.03
Type of clinical onset, n (%)					
-Optic nerve	11 (16.9)	0 (0.0)	5 (20.0)	6 (37.5)	0.22
-Spinal cord	11 (16.9)	0 (0.0)	6 (24.0)	5 (31.2)	0.61
-Other localization	7 (10.8)	0 (0.0)	4 (16.0)	3 (18.8)	1.00
-Polyfocal without encephalopathy	12 (18.4)	0 (0.0)	10 (40.0)	2 (12.5)	0.08
-Polyfocal with encephalopathy	24 (36.9)	24 (100.0)	0 (0.0)	0 (0.0)	1.00
OCB, (≥2 bands), (%)	28/54 (51.9)	0/17 (0.0)	21/23 (91.3)	7/14 (50.0)	0.01
Elevated IgG index (cut-off: 0.66), n (%)	33/50 (66.0)	4/12 (33.3)	22/23 (95.7)	7/15 (46.7)	<0.01
Time first symptoms to LP (weeks), median (IQR)	2.3 (0.8-7.6)	1.6 (0.6-2.6)	6.0 (1.9-12.8)	1.8 (0.6-7.8)	0.10
≥9 lesions on T2-weighted images, n (%)	27 (41.5)	6 (25.0)	6 (64.0)	5 (31.2)	0.04
Asymptomatic T2-lesions, n (%)	55 (8.6)	22 (91.7)	24 (96.0)	9 (56.2)	0.003
MS based on first MRI[b], n (%)	10 (15.4)	na	9 (36.0)	1 (6.2)	0.03
DMT before CDMS diagnosis, n (%)	14/41 (34.1)	na	9 (36.0)	5 (31.3)	0.75
Time between CIS and start DMT (months), median (IQR)	6.4 (3.3-12.1)	na	6.3 (1.7-14.8)	6.5 (3.5-10.4)	0.72

Table 2. Patient characteristics (children)

[a] p value calculated between CIS-CDMS and CIS-CIS

[b] Dissemination in space and time at baseline based on McDonald 2010 criteria

Abbreviations: CIS, Clinically isolated syndrome; CIS-CDMS, patients who are diagnosed with CDMS during follow-up after CIS defined by Poser criteria; CIS-CIS, not diagnosed with CDMS; na, not applicable; DMT, disease modifying therapy; OCB, oligoclonal bands; Ig, Immunoglobulin; LP, lumbar puncture.

NfL levels at time of the first demyelinating event per clinical subgroup

Patients (children and adults) at time of a first attack of demyelination showed higher NfL levels than control individuals; geometric mean 2040 vs 444 pg/mL; p<0.001. In Figure 1, NfL levels from controls, adult, and paediatric patients are shown.
In adult patients, NfL levels at time of CIS were higher in the group that was diagnosed with CDMS (CIS-CDMS, n=43 (49%)) compared to the group that remained CIS during follow-up (CIS-CIS); geometric mean 2156 vs 1342 pg/mL; p=0.012. (Figure 2)
In children, we compared NfL levels between CIS-CIS, CIS-CDMS, and ADEM patients. NfL levels at time of CIS in paediatric CIS-CDMS patients (n=25; 61%) were higher than in paediatric CIS-CIS patients; geometric mean 4888 vs 967 pg/mL; p=0.01. Children with ADEM did not differ in NfL levels from CIS-CIS and CIS-CDMS children; geometric mean 2683 pg/mL (Figure 2). There was no correlation between time from onset of symptoms to LP and NfL levels in both children and adults.

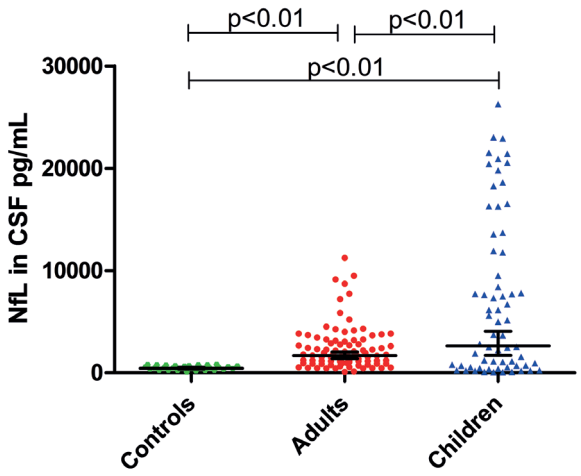

Figure 1. CSF NfL levels in Controls vs Adults vs Children
CIS and ADEM patients are included in children.
Horizontal lines with error bars indicate geometric mean with 95% CI.
Abbreviations: NfL, Neurofilament light chain; pg/mL, picogram/millilitre

NfL levels compared between children and adult

Next, we compared NfL levels between children and adult patients at time of CIS. In the CIS-CDMS group, NfL levels were higher in children compared to adults; geometric mean 4888 vs 2156 pg/mL; p=0.007. NfL levels were not different between adults and children in the CIS-CIS groups (Figure 2).

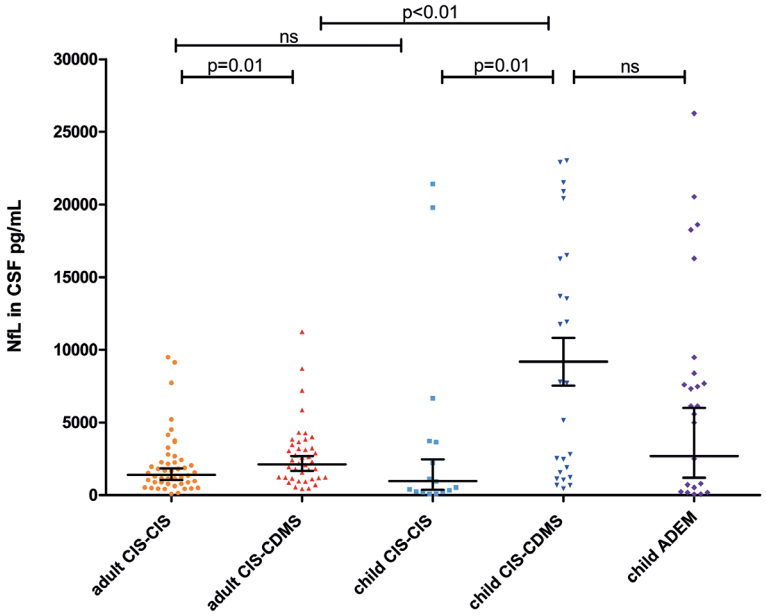

Figure 2. NfL levels in clinical subgroups of adults and children
Horizontal lines with error bars indicate geometric mean with 95% CI.
Abbreviations: NfL, Neurofilament light chain; ns, not significant; pg/mL, picogram/millilitre

Association of NfL levels with time to CDMS diagnosis in children and adults with CIS

To analyse time to CDMS diagnosis, we used median CSF NfL levels in CIS patients as cut-off. This resulted in a cut-off of 1802 pg/mL for adults and 2537 pg/mL for children. These cut-offs were used to divide CIS patients (ADEM excluded) into groups with high and low NfL levels, and were subsequently used in the COX regression analysis.
The univariate COX regression analysis showed a HR for CDMS diagnosis of 2.1; p=0.024 in adults and 3.8 in children with CIS; p=0.003. Kaplan-Meier curves are shown in Figure 3A/B.
In a multivariable COX regression analysis, we corrected for clinically relevant parameters; the presence of asymptomatic T2 lesions on the baseline MRI and OCB. We also corrected for age at onset, based on correlation with NfL levels in control individuals (spearman rho 0.59, p=0.001). The HR in the multivariable COX regression analysis for high NfL levels was 2.1 in adults (p=0.032) and 3.7 in children (p=0.007). (Table 3)

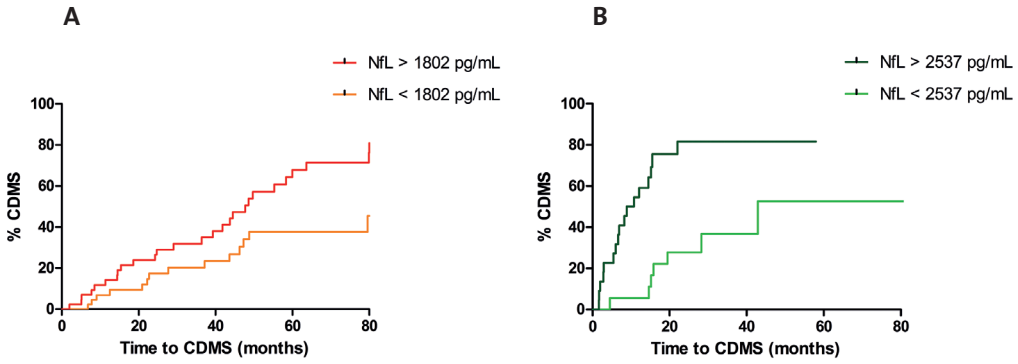

Figure 3. Time from CIS to CDMS in CIS patients with high and low CSF NfL levels
A. *Adults (log-rank test p=0.02).*
B. *Children (log-rank test p=0.001). Kaplan-Meier curves showing time to CDMS diagnosis for CIS patients (ADEM excluded) with either high or low CSF NfL levels.*
Abbreviations: CDMS, Clinically definite multiple sclerosis; NfL, Neurofilament light chain; pg/mL, picogram/millilitre

Sixteen (18%) adult CIS patients and 14 (34%) children received DMT before CDMS diagnosis. This could have postponed the second attack. The HR in children increased after excluding patients who received DMT before CDMS diagnosis. In adults the univariate HR did not change, and the HR in the multivariable analysis showed a trend towards significance.
When we add DMT before CDMS diagnosis into the COX regression model, the HRs did not change.
Thirteen (11%) adults and nine (22%) children were treated with methylprednisolone

within three months before LP. When we corrected for this in the COX regression model, the results did not change.
In another sub-analysis, we excluded CIS-CIS patients who had less than 2 years follow-up (children: n=5, adults: n=10). After this exclusion, HRs were not altered in adults and increased in children. Table 3 shows the univariate and multivariable HRs including those of the subanalyses.

	Univariate analysis HR (95% CI)	P-value	Multivariable analysis HR (95% CI)	P-value
Adults				
Total group (n=88)	2.1 (1.1-3.9)	0.024	2.1 (1.1-4.1)	0.032
After excluding patients with DMT before CDMS diagnosis (n=72)	2.2 (1.1-4.7)	0.035	2.2 (1.0-4.9)	0.061
After excluding CIS-CIS patients with FU <2 years (n=78)	2.0 (1.1-3.8)	0.027	2.1 (1.1-4.1)	0.034
Children				
Total group (n=41)	3.8 (1.6-9.2)	0.003	3.7 (1.4-9.3)	0.007
After excluding patients with DMT before CDMS diagnosis (n=27)	19.8 (2.5-155.5)	0.005	13.7 (1.6-114.3)	0.015
After excluding CIS-CIS patients with FU <2 years (n=36)	3.8 (1.6-9.2)	0.003	4.2 (1.6-11.3)	0.004

Table 3. Cox regression (univariate and multivariable) hazard ratios for CDMS diagnosis in adults and children with CIS
Hazard ratios for CDMS diagnosis in subgroups for adults and children with CIS (ADEM excluded)
Multivariable analyses: corrected for presence of asymptomatic T2 lesions on baseline MRI, presence of OCB and age of onset
Abbreviations: HR, hazard ratio; CI, confidence interval; DMT, disease modifying treatment; CDMS, clinically definite multiple sclerosis; FU, follow-up; CIS, clinically isolated syndrome.

Association of CSF NfL levels with disability

We did not find a correlation between CSF NfL levels and EDSS scores after CDMS diagnosis. We collected EDSS data from 55/68 (81%) patients who were diagnosed with CDMS. Only 6 patients (4 adults and 2 children) reached an EDSS of 3.0 or more.

Association of CSF NfL levels with signs of axonal damage on MRI

CSF NfL levels were increased in CDMS patients showing T1-hypointense lesions on baseline MRI (adults 20/39, 51%; children 15/23, 65%). We found this in adults (geometric mean 3188 vs 1588 pg/mL; p=0.001) and in children (geometric mean 8920 vs 1668; p=0.001). (Figure 4A/B)

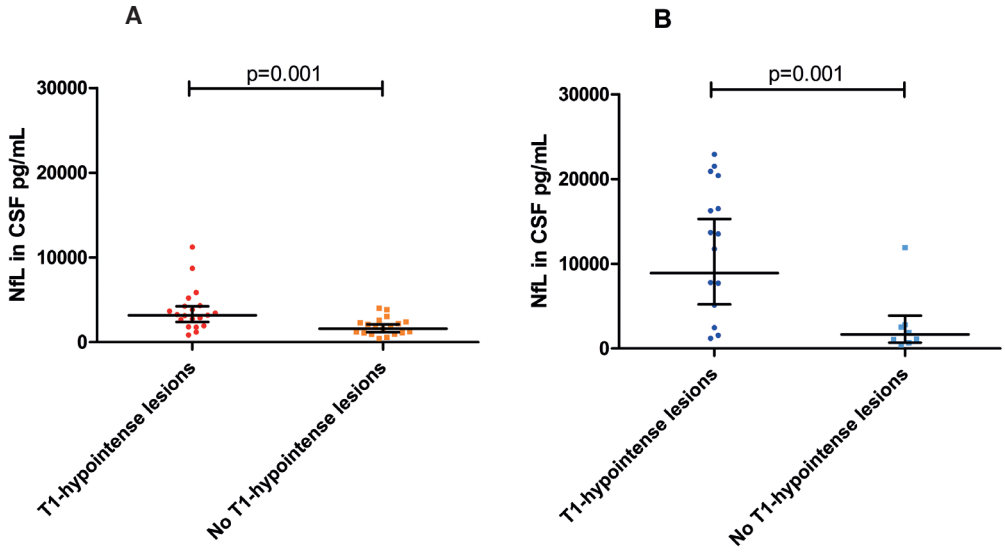

Figure 4. CSF NfL levels in CIS-CDMS adults and children with and without T1-hypointense lesions on baseline MRI
A. *Adults: T1-hypointense lesions vs no T1-hypointense lesions on baseline MRI.*
B. *Children: T1-hypointense lesions vs no T1-hypointense lesions on baseline MRI.*
Horizontal lines with error bars indicate geometric mean with 95% CI.
Abbreviations: NfL, Neurofilament light chain; pg/mL, picogram/millilitre; CDMS, Clinically definite multiple sclerosis

DISCUSSION

In this prospective study, we demonstrate that CSF NfL levels in children and adults with a first attack of suspected MS are predictive for CDMS diagnosis. Furthermore at time of CIS, CSF NfL levels in patients with a future CDMS diagnosis are higher in children than in adult patients. This underlines that not only inflammation is more severe in children[3-5] but that children also have more axonal damage early in the disease course of MS than adult patients.[8]
To our knowledge, we are the first to show that CSF NfL levels are associated with a subsequent diagnosis of CDMS in children with CIS. In addition, the results validate the predictive value of CSF NfL levels for CDMS diagnosis in adult CIS patients.[12, 23] Both in adults and children, these findings were independent of known predictive factors for CDMS, i.e. asymptomatic T2 lesions on baseline MRI and unique OCBs in CSF. Moreover, CSF NfL levels predicted a second attack even better than these currently used markers. It is essential to improve currently available routes to prediction to prevent unnecessary treatment of patients with low clinical disease activity, especially because these immunomodulatory therapies can have serious side effects. Recently, other potential CSF biomarkers for a future MS diagnosis have been identified.[24, 25] Our findings draw further attention to the relevance of including CSF analyses as part of routine diagnostics.

Since NfL is considered a biomarker for axonal damage, neurodegeneration, and brain atrophy,[26, 27] we reasoned that its presence in CSF could be associated with T1-hypointense lesions on MRI, which are signs of axonal loss.[28] In children with CIS, these T1-hypointense lesions have been reported to be highly predictive for MS diagnosis.[20] Here, we demonstrate that children and adult CIS patients with T1-hypointense lesions on baseline MRI have higher NfL levels than patients without these lesions.
CSF NfL levels in children with ADEM were not significantly different from levels in patients who remained CIS or who were eventually diagnosed with CDMS. Nevertheless, these levels were high in ADEM patients (geometric mean 2683 pg/mL), indicating considerable axonal damage. Studies have reported cognitive impairment and persistent motor dysfunction in children with ADEM.[29, 30] Moreover, it has been shown that subsequent white matter maturation and age-expected brain growth is disturbed not only in paediatric MS, but also in monophasic ADS, including ADEM.[31, 32] These findings support the occurrence of damage during the acute phase with a lasting impact. Which stresses the importance of adequate follow-up and support after the acute event.
NfL levels have been reported to be also increased in serum of adult CIS and MS patients,[33, 34] but the predictive value in CIS patients for MS diagnosis of this marker seems limited to CSF.[12] As we here aimed to assess and compare prediction in both children and adults with CIS, we restricted in this study to CSF samples.

There are some limitations in this study. First, the range of follow-up is rather wide. We did correct for this in the COX regression analyses, and we also performed a sub-analysis after excluding CIS-CIS patients with a follow-up less than 2 years, which did not change our findings. Second, in both the adult and paediatric study, we did not perform a follow-up MRI on a regular basis. Therefore, the Poser criteria were used instead of the McDonald 2010 criteria. In this way, we could show an effect on clinical disease activity (second attack) instead of disease activity measured with MRI. Third, in order to prove an association of CSF NfL levels with EDSS, we will need a longer follow-up period since disability occurs later in the disease course especially in children.[6] Fourth, we did not have access to advanced imaging techniques for quantification of neurodegeneration (e.g. T1-hypointense lesion volumes, total brain volume and brain tissue integrity). However, we used the presence of T1-hypointense lesions as an MRI marker for axonal damage,[28] because it is easily assessable. Furthermore, we did not include paediatric controls since we did not receive ethical permission to collect paediatric control CSF samples, which made the collection of this rare material not possible. Yet, control groups in other paediatric studies indicate low physiological levels of NfL, in the same range as the adult controls in the present study.[35, 36]
Last, although our sample size was relatively small due to limited availability of CSF, we were still able to correct our results for other predictive factors for MS diagnosis. Our findings in children were also compatible to those in adults, further stressing the robustness of the observations.
In conclusion, we show that high levels of CSF NfL are associated with CDMS diagnosis independently of known predictive factors (i.e. asymptomatic T2 lesions and OCB) in both children and adults. CSF NfL levels at time of a first demyelinating event are higher in children than in adults with a future CDMS diagnosis. In addition, this marker

for axonal damage, is associated with MRI signs of neurodegeneration in both groups. Given that therapeutic interventions might delay disease progression and accumulation of disability,[37, 38] it is essential to accurately predict MS diagnosis, not the least in the paediatric population. Hence, NfL in CSF is a promising predictive marker for the disease course in both adults and children with a first demyelinating event, with a potential value in future clinical practice.

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Chapter 10

General discussion

Patients who have a first attack of multiple sclerosis (MS), also called clinically isolated syndrome or CIS, face a precarious future. Not all patients will be diagnosed with MS and the disease course after MS diagnosis is highly heterogeneous.[1] Being able to adequately counsel these patients about their long-term prognosis is important, especially since MS affects mostly young patients in the most productive years of their lives.[2]
This thesis focuses on the disease course after a first attack of demyelination, both in adults with CIS and children with childhood-onset acquired demyelinating syndromes (ADS), a rare variant within the spectrum of demyelination.[3] We focused on defining prognostic factors, both clinical markers and biomarkers, for a better prediction of disease course after a first attack of demyelination, not only for predicting MS diagnosis but also for predicting disease course after MS diagnosis. The studies in this paper are executed within two prospective patient cohorts. One cohort consists of adult CIS patients and the other cohort consists of paediatric ADS patients, giving the unique opportunity to compare the disease course of children and adults after a first attack of demyelination. All patients were included at time of the first attack of demyelination and after that followed prospectively. This chapter discusses the key findings of the studies described in this thesis and directions for future research.

Diagnosing MS at time of the first attack

The majority of CIS patients will experience a second relapse. However, more than one third remains monophasic.[4] Fifty percent of the patients in our cohort of adult CIS patients with a mean follow-up time of 5.5 years had a second relapse.
The first generally recognized criteria for MS were described by Allison and Millar in 1954,[5] followed by multiple revisions.[6] Poser et al. described criteria for clinically definite MS (CDMS) in 1983, these were purely based on two clinical manifestations of demyelination in two different neurological localizations at two different timepoints.[7] Since 2001, MRI findings are included in the diagnostic criteria.[8] Not only clinical dissemination in space (DIS) and time (DIT) but also DIS and DIT on the MRI scan can now lead to an MS diagnosis. After this first version of the McDonald MRI criteria, multiple revisions are implemented.[8-11] The criteria have been simplified over time and allow a faster MS diagnosis.
Since the 2010 criteria, a diagnosis of MS could be made based on a single CIS attack with no subsequent clinical relapses. Therefore this patient group comes even closer to the group with radiologically isolated syndrome (RIS). Because DMT can delay MS diagnosis in CIS patients, the discussion comes closer to whether high risk RIS patients should also be treated with immunomodulating therapies.
The latest revisions in 2017 include presence of unique oligoclonal bands (OCB) in cerebrospinal fluid (CSF) as a substitute for DIT on MRI.[11] This means that a CIS patient can be diagnosed with MS when the MRI meets criteria for DIS and OCB are present in CSF. Furthermore, not only asymptomatic but also symptomatic lesions can be used to meet criteria for DIS and DIT. One could imagine that these simplifications of the criteria could lead to a higher number of MS diagnosis in patients who will not have a future second attack. Accurate diagnostic criteria are essential to select patients for early treatment with immunomodulating therapies. Early treatment has been shown

beneficial for disease outcome.[12-14] However, these therapies have adverse effects, and treating patients who will remain clinically monophasic should be prevented.

In **chapter 2**, we applied the latest diagnostic criteria to a cohort of 229 CIS patients with a mean follow-up time of 5.4 years. We compared the performance of the new 2017 criteria to the former 2010 criteria in predicting CDMS diagnosis according to the Poser criteria.[7] The most important finding was that with the new criteria twice as many patients could be diagnosed with MS at time of the first attack (97 vs 46 patients). The 2017 criteria were more sensitive than the 2010 criteria (68% vs 36%), but specificity decreased from 85% to 61%. These results imply that the 2017 criteria will probably lead to a higher number of MS diagnoses in patients with a less active disease course. To allow earlier initiation of disease modifying therapies (DMT), a high sensitivity of diagnostic criteria is essential. But a decrease in specificity can lead to erroneous diagnoses and unnecessary treatment of patients with a benign disease course. Unnecessary treatment should be avoided, most importantly, because these therapies can have serious adverse effects.[15, 16] In 63% of the patients who were diagnosed with MS based on the 2017 criteria at baseline (and not based on the 2010 criteria) MS diagnosis was made based on DIS and OCB in CSF and no DIT on the baseline MRI. Forty-seven percent of this group was not diagnosed with CDMS during follow-up. The reduction of specificity seems to be mostly caused by the inclusion of OCB to the criteria.
In our CIS cohort, follow-up MRI scans were not performed uniformly according to a strict protocol, therefore we could not demonstrate if the new criteria at baseline were better in predicting MS diagnosis based on new lesions on a follow-up MRI scan. However, one could argue whether predicting activity on MRI or predicting clinical disease activity is more relevant for further treatment decisions.
Validation of these criteria in other cohorts of CIS patients is essential to test their accuracy in clinical practice.

Clinical prognostic factors in patients with CIS

Fatigue is a common symptom in MS patients, more than 75% of these patients report fatigue.[17, 18] Our study group showed earlier that fatigue was already present early in the disease course, at time of the first attack.[19] The same study showed that fatigue was an independent predictor for a subsequent CDMS diagnosis, unrelated to anatomical localization of the first attack. This implicates that fatigue in MS patients may not be related to the attack itself but probably to MS related pathophysiological processes as inflammation, demyelination and axonal loss.[20, 21] However, fatigue in MS patients can also be the result of MS-related symptoms such as sleep deprivation, MS-related neuro-psychiatric disorders, or effect of medication.[20, 21] Yet, several studies indicated an independent relation between inflammation and fatigue.[22, 23]
In **chapter 3**, we explored the long-term course of fatigue after CIS. First, we validated our earlier finding that patients with a future CDMS diagnosis had already a higher fatigue score (FSS)[24] at time of the first attack than patients who remained monophasic during a mean follow-up of 4.3 years. Next, we showed that after CDMS diagnosis the FSS increased even more. Fatigue seems to exist early in the disease course of MS,

probably will remain present during the further follow-up and increases after CDMS diagnosis. Other studies in MS patients also suggested a persistency of fatigue over time.[17, 25, 26] However, the follow-up time in these studies was short with a maximum of 3 years. Furthermore, these studies evaluated fatigue years after MS diagnosis. Our study shows that the majority of MS patients have to cope with disabling fatigue, which exists already very early in the disease course, therefore fatigue deserves proper attention in the standard care for MS patients already in the most early stages of the disease.

Cigarette smoking has been shown to influence the disease course of MS patients.[27] In **chapter 6**, we demonstrated that smoking at time of CIS was an independent predictive factor for a second attack during follow-up (HR: 2.3; p<0.01). We expected this outcome since earlier studies have shown the influence of smoking on disability and the time to secondary progressive MS (SPMS). In addition, there is an increased risk of MS for people who smoke in the general population.[27, 28] However, earlier studies remained inconclusive in respect to the predictive value of smoking in CIS patients.[29] These earlier studies had small sample sizes and used retrospective data.[30, 31] Other studies included patients who were treated with interferon beta immediately after CIS or two years after CIS.[32, 33] Treatment with interferon beta could have postponed a second attack and may have overshadowed the potential correlation between smoking and CDMS diagnosis. Interestingly, our data showed that smoking in the past in current non-smoking patients was not associated with CDMS. This finding suggests that the harmful effects of smoking are reversible. This is in line with earlier studies, showing that the negative effects on disability progression decrease after cessation of smoking.[34, 35] These results were independently of the cumulative dose of smoking. The pathophysiological mechanism of the influence of smoking on MS remains to be determined. Direct and indirect influences of tobacco toxins and smoke particles on T cells and antigen presenting cells may be part of this mechanism.[36]
Since smoking is a modifiable risk-factor for MS, counselling patients about smoking is of great importance, especially because the negative effects of smoking seem to reduce after cessation of smoking. Smoking status could also be a relevant parameter in predictive models for MS in CIS patients.

Children versus adults

Multiple sclerosis with a childhood-onset is rare. Around 3-5% of MS patients have their first attack during childhood, and only <1% under the age of 12.[3, 37] Diagnosing paediatric MS is challenging because of the rarity of this disease in children and the more extensive differential diagnosis for childhood-onset MS.[38]
Although children have a higher number of relapses, more severe relapses,[39] and more lesions on brain MRI,[40, 41] time to reach disability endpoints is longer in children than in adult patients with MS.[42, 43] Yet, because the start of the disease in children is at a younger age, paediatric patients reach these disability endpoints earlier in life than adult MS patients. These results are based on retrospective studies or studies conducted before the immunomodulating treatment era in children. The second part of this thesis focusses on children versus adults.

In **chapter 7**, we compared disease course after CIS between three age groups (1-10 years, 11-17 years, and 18-50 years). Patients were included in two prospective patient cohorts with paediatric and adult patients followed after a first attack of demyelination. This study included a total of 383 CIS patients. We observed, in line with the studies mentioned before,[39-41] that children with CIS tend to have a more inflammatory disease course than adult patients with CIS. Children aged between 11 and 17 years were more often diagnosed with MS and had a shorter time between CIS and MS diagnosis than patients in the other age groups. Patients in this age group also had more T2 lesions on the baseline MRI scan and a more inflammatory CSF profile. The secondary progressive phase of MS was reached for eight patients who were all older than 37 years of age at onset of CIS.[44] Since SPMS is age dependent,[45, 46] the patients with a younger age at disease onset probably did not reach this progressive phase yet. For both children and adults, there was a female predominance, except for the youngest age group before puberty (1-10 years), in this young group more boys than girls were diagnosed with MS. This difference in sex distribution before and after puberty and the total increase in MS diagnoses after puberty implies a role for sex hormones in the onset of MS. This role for female and male sex hormones is also suggested in other studies.[47, 48]
Furthermore, we found, in line with other literature, a higher rate of non-Caucasian CIS patients in the paediatric group than in the adult group.[49, 50] Non-Caucasian ethnicity seems to lead to a higher vulnerability for developing MS at a younger age, this could be due to a lack of protective genes, since their ancestors were born in countries with a low MS prevalence.
After MS diagnosis, paediatric MS patients had a higher annualized relapse rate (ARR) than patients with adulthood-onset MS. However, after the start of DMT treatment, the ARR was comparable between adults and children. This change in ARR after administration of DMT and the more inflammatory disease course in children supports early initiation of DMT in children with MS, even at time of CIS in children who are at high risk for MS diagnosis in the age group 11-17 years. This is in line with current clinical practice in adult CIS and MS patients. However, data about long term adverse effects of DMT in children are limited. It is crucial to investigate whether early initiation of DMT in children is safe and beneficial, since childhood-onset MS is rare, international collaboration is needed.

As described above, disability progression is slower in paediatric than in adult patients with MS.[42, 43, 51] However, neurodegeneration plays also a role in early childhood-onset MS, It has been shown that age-expected brain growth was impaired early in the disease course in paediatric MS patients.[52] One of the major causes for persisting neurological disability in MS is axonal damage.[53] A well-established biomarker for this process is neurofilament light chain (NfL), an element of the neuron cytoskeleton.[54] NfL is released in the extracellular space after neuronal cell death. NfL increases with age in healthy individuals, reflecting neurodegeneration as part of the physiological aging process.[55] In adult CIS patients, NfL levels are shown to be associated with a future MS diagnosis.[56, 57] In **chapter 9**, we show that this is also the case for paediatric CIS patients. NfL levels in CSF at time of the first attack predicted a second attack (CDMS) both in children and adults with CIS. NfL levels were even higher in children than in adults, implying that early in the disease course, children have not only signs of more inflammation but also

seem to have more axonal damage than adults.[40, 41] NfL levels predicted a second attack even better than currently used markers such as asymptomatic T2 lesions on MRI and unique OCBs in CSF. These findings suggest an important role for CSF analyses as part of the routine diagnostic process. Furthermore, we found in children and adults with a future CDMS diagnosis, a correlation between NfL levels and T1 hypo-intense lesions on baseline MRI, another sign of axonal loss. These T1 hypo-intense lesions are highly predictive for MS diagnosis in children.[58] We did not find an association between NfL levels at time of the first attack and disability later in the disease course. In order to prove such an association, a longer follow-up time is probably needed, since disability occurs late in the disease course, especially in children.[42, 43]
The results of the above described studies show that the disease course after CIS in children is not only more inflammatory than in adults but they also show that signs of neurodegeneration are present already early in the disease course. Initiating treatment (DMT) to prevent further relapses with possible residual damage seems logical. Predicting who will have an active disease course is crucial, especially in this young patient group, since these children have a long time of treatment ahead and long-term damage of these therapies started in childhood is not studied very well. Markers such as NfL could possible help in selecting patients who will have an active disease course.

T cell activation in CIS and ADS

A biomarker that could possibly help in the early selection of patients with an active disease course is the immunological marker soluble CD27 (sCD27). Soluble CD27 is released by activated T cells, after activation via the T-cell receptor/CD3 complex,[59] as shown in figure 1.

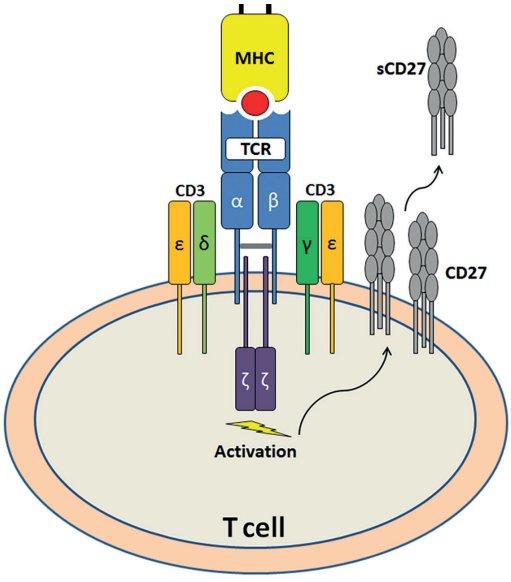

Figure 1. sCD27 release after T cell stimulation via the T-cell receptor/CD3 complex

A clear association has been found between sCD27 and the presence of intrathecal T cells.[60] This T-cell activation marker seems to be directly related to the core immunopathology of MS.[61, 62] It has been shown that sCD27 is higher in CSF from MS patients than from control individuals.[60, 63, 64]
In **chapter 4**, we show that high CSF levels of sCD27 in adults with CIS are independently associated with a future MS diagnosis. Furthermore, we found a higher attack frequency in patients with high sCD27 levels at baseline than patients with low sCD27 levels. CD27 and sCD27 induce activation and proliferation of T and B cells.[65, 66] There is some evidence that sCD27 could play a role in the IgG production by B cells. An in vitro study showed that memory B cells could be stimulated to differentiate into IgG-secreting plasma cells by binding of sCD27 to the CD27 ligand CD70.[67] This functional role of sCD27 could explain the strong correlation that we and others found between sCD27 and IgG index.[60, 63]

Our finding that sCD27 predicts a future MS diagnosis in adults with CIS is validated in a cohort of children with ADS in **chapter 8**. We show that children with a first attack of demyelination and a future CDMS diagnosis have higher sCD27 levels than patients with a first attack of demyelination with encephalopathy, relapsing non-MS patients and monophasic paediatric CIS patients. In accordance with our findings in adults, sCD27 levels are predictive for a second attack in paediatric CIS patients. This validation strengthens the conclusions from both studies. Notable is that sCD27 levels are higher in children than in adults with a future MS diagnosis, which reflects the more inflammatory disease course in children compared to adults, as is also shown in **chapter 7**.[39-41] Both in adults and children, we did not see an association between sCD27 and disability later in the disease. This is not surprising since most disability occurs late in the disease course this is not surprising. As mentioned before, disability progression in paediatric MS is slower than in adults.[42] In children we did not find a correlation between sCD27 and ARR, this is in contrast with our findings in adults where ARR did correlate with sCD27. An explanation for this inconsistent finding could be a ceiling effect, since the relapse rate in children is overall high.[39]
Soluble CD27 seems an activation marker directly related to the immunopathology of MS. Both in adults and paediatric patients with a first attack of demyelination, sCD27 could be a useful quantitative marker for disease activity when performing routine CSF diagnostics.

T helper 17 (Th17) cells (CCR6+) express IL-17 and are shown to be critical regulators of MS disease activity.[68] CCR6 and IL-17 are considered as key determinants for trans-migrating across the blood-brain barrier.[69] Th17 cells are functionally highly heterogeneous. Depending on the pro-inflammatory milieu, Th17 cells consist of sub-populations that express not only CCR6 and IL-17 but also other distinct surface markers (CXCR3, CCR4) and pro-inflammatory cytokines (IFN-γ, GM-CSF).[68, 70] Especially GM-CSF is considered as an important mediator in MS onset.[71]
In **chapter 5**, we showed that Th1-like Th17 cells (CCR6+CXCR3+), a subpopulation of Th17 cells expressing IL-17 but also both IFN-γ and GM-CSF,[72] were selectively associated with early disease activity in CIS and MS patients. Effector memory Th1-like Th17 cells were

more abundant in blood from CIS patients who remained CIS for at least 5 years (CIS-CIS) than in CIS patients who converted to CDMS within 1 year (CIS-CDMS). This is not the case for Th17 (CCR6+, CXCR3-) cells. Th1-like Th17 frequencies in CIS-CDMS patients at time of CIS are comparable with the frequencies in blood from untreated MS patients. Interestingly, this same subset was more abundant in CSF of CIS and early MS patients and had a higher production of the pro-inflammatory cytokines IFN-Y and GM-CSF than their paired peripheral blood equivalents. This implies a local recruitment of this highly activated subset in the CSF of early MS patients. Furthermore, Th17.1 cells (a subpopulation of Th1-like Th17 cells, CCR6+CXCR3+CCR4-) express the highest VLA-4 levels and selectively accumulate in blood from patients who were relapse free while treated with natalizumab. There was no accumulation of Th17.1 cells in blood from MS patients treated with natalizumab who experienced relapses.
In summary, we show evidence for the recruitment of highly pro-inflammatory specific Th17 subsets into the CSF during disease onset. Th1-like Th17 subpopulations, in particular Th17.1 cells, probably play an important role in clinical disease activity in patients with MS. This data could be of value for developing more specific T cell-targeted therapies. However, to determine their local impact in the MS brain, localization and revealing the antigen specificity of these subsets in brain lesions is the first step.

Main findings

- The revised 2017 McDonald criteria for MS double the number of MS diagnoses at baseline
- Including OCB as a substitute for 'dissemination in time' in the McDonald 2017 criteria has the highest impact on the decrease in specificity

- Fatigue is validated as an independent predictor for CDMS diagnosis in CIS patients
- Fatigue, present at time of CIS, will probably remain present during the further follow-up and increases after CDMS diagnosis

- Cigarette smoking at time of CIS is an independent predictor for CDMS diagnosis
- Past smoking in currently non-smoking CIS patients is not associated with CDMS diagnosis

- Children with CIS have a more inflammatory disease course, with a higher relapse rate than adult CIS patients
- Children with CIS between 11-17 years of age are more often and earlier diagnosed with MS, have more T2 lesions on baseline MRI and have a more inflammatory CSF profile than patients in other age groups
- The female predominance is not seen in the youngest age group, before puberty (1-10 years of age)

- High CSF NfL levels predict a future MS diagnosis both in adult and paediatric CIS patients
- NfL levels correlate with T1 hypo-intense lesions on baseline MRI, a sign of axonal loss, in children and adults with a future CDMS diagnosis

- sCD27 is an independent predictor for MS diagnosis in adult and paediatric CIS patients
- Adult CIS patients with high sCD27 levels at baseline have a higher attack frequency during follow-up than patients with low sCD27 levels

- Th1-like Th17 cells are more abundant in peripheral blood of CIS patients who remain monophasic during follow-up than in CIS patients with a short time to CDMS diagnosis
- Th1-like Th17 subpopulations, in particular Th17.1 cells, are recruited to the CSF during disease onset of MS
- Th17.1 cells have the highest VLA-4 expression and selectively accumulate in blood from relapse free natalizumab treated patients

Clinical implications

- The McDonald 2017 criteria probably lead to a higher number of MS diagnoses in patients with a less active disease course

- Fatigue deserves proper attention throughout the disease course of MS

- Patients with CIS and MS should quit smoking

- Early initiation of DMT should be considered in paediatric CIS patients who are at high risk for developing MS

FUTURE PERSPECTIVES

Multiple markers are identified for predicting disease course after a first attack of demyelination. MRI is, up to now, the best tool to predict disease course. In the new diagnostic criteria, MRI remains the most important component.[11] However, the search for markers in blood and CSF to reveal new biomarkers for predicting disease course and providing a more individualized care is crucial and ongoing.[73] These markers could also be helpful in gaining more insight into the pathogenesis of MS.
Since the predictive value of NfL in CSF has been shown in multiple CIS cohort including ours, this marker may be a good candidate to be incorporated in following versions of the MS criteria.

Our findings on the recruitment of the highly pro-inflammatory Th1-like Th17 subsets in CSF of early MS patients and the association of these subsets and relapses while treated with Natalizumab could be of value for selecting patients for whom another therapy would be more effective. Furthermore these findings could help in developing more specific T cell-targeted therapies. However, first the local impact of these subsets on the MS brain and the antigen specificity of these subsets in brain lesions should be revealed.

The two prospective patient cohorts with adult CIS patients and paediatric ADS patients have been followed for up to 10 years now, and becoming more valuable as follow-up grows. Especially for revealing factors that predict disability and time to the secondary progressive phase of MS, a long follow-up time is required. Therefore it is of great importance to keep following the patients in these prospective cohorts in the future.

International collaboration is of great value to increase patient numbers, especially when studying such a rare entity as childhood-onset ADS. For genetic and proteomic studies, large sample sizes are required. These studies could help in finding new protein markers or genes which are associated with MS diagnosis in MS or ADS patients. It is crucial that the data collection is uniform, this is important to be able to pool the data and to draw reliable conclusions. There are guidelines available for standardised collecting samples and reporting results.[74, 75] Another reason why international collaboration is interesting is the possibility to investigating environmental factors, which may vary in different parts of the world.

A relatively new topic in MS research is the gut microbiome. The gut microbiome is defined as the complex balance between the microbial populations in the gastrointestinal tract and the immune system.[76] Alterations of this microbiome may lead to dysregulation of immune responses not only in the gut but also in the central nervous system. In experimental autoimmune encephalomyelitis, a mouse model for MS, certain intestinal bacterial populations led to a more pro inflammatory condition that may result in developing autoimmune diseases. Whereas other intestinal bacterial populations have been shown to protect against inflammation in the CNS.[77] Known risk factors for MS are smoking, vitamin D insufficiency, differences in diet and alcohol use. These risk factors also influence the gut microbiome.[76] There is growing evidence of differences in the

microbiota between MS patients and controls.[78] A recent study showed that transgenic mice transplanted with the microbiota derived from a monozygotic twin with MS had a higher incidence of MS-like autoimmunity than the mice who received a faeces transplant from the healthy twin.[79] It will be of great value to examine the microbiome early in the disease course, in therapy naïve CIS patients. This might lead to more diagnostic and prognostic markers. Exploring the microbiome in those untreated patients will help in understanding the pathogenesis of MS, and may contribute to develop lower risk therapeutic management in the future. In both the PROUD study in adults and the PROUD-kids study in paediatric patients, we started collecting faecal samples to study the microbiota in these two patient cohorts.

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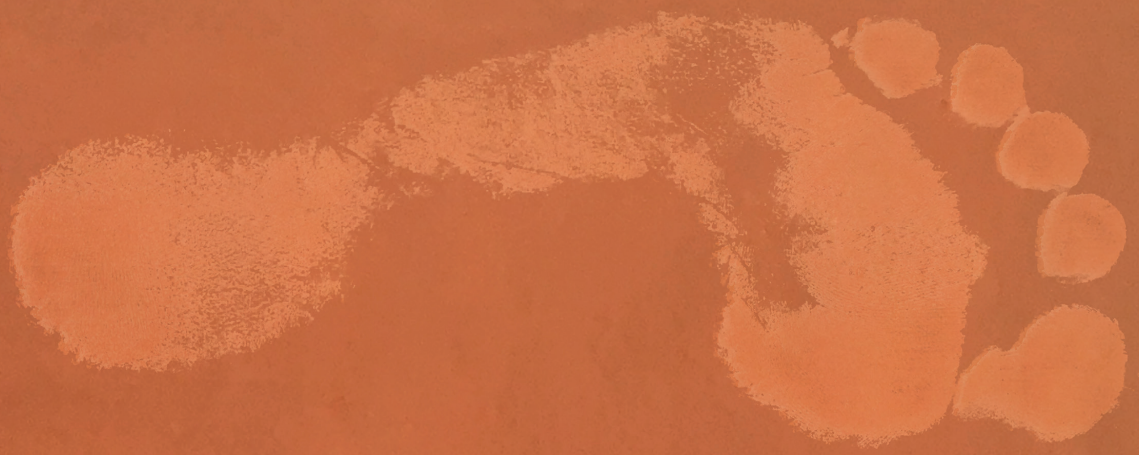


Chapter 11

Summary

Samenvatting

SUMMARY

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), affecting genetically susceptible individuals, influenced by environmental factors. In 85% of cases, the disease presents itself with clinical relapses as a result of demyelination in different parts of the CNS. Because the course of MS is highly heterogeneous, prediction factors at time of the first attack (CIS) are needed. The need for reliable prognostic factors is even more urgent since there is a growing number of disease modifying therapies (DMT) available for MS. Early initiation of these therapies is favourable for the prevention of future relapses and disability.
The studies described in this thesis are executed within two prospective cohorts of adult and paediatric patients followed after a first attack of demyelination. We aimed to find prognostic factors for the disease course after a first attack of demyelination both in children and adults.
Chapter 1 gives a short introduction on MS and describes currently known and used clinical, imaging and biological factors for the prediction of disease course.

The first part of this thesis focused on the disease course after CIS in adult patients. In 2017, the International Panel on Diagnosis of Multiple Sclerosis proposed new revisions to the McDonald criteria for MS. The most important change was that with the new criteria dissemination in time (DIT) could be realized when unique oligoclonal bands (OCB) were found in the cerebrospinal fluid (CSF), this could replace DIT on MRI. The other change was that not only asymptomatic but also symptomatic lesions on MRI could contribute to DIT and dissemination in space (DIS). In **chapter 2**, we validated these criteria in 229 adult CIS patients. With these new criteria twice as many MS diagnoses could be made at time of the first attack (97 (54%) vs 46 (26%); p<0.01). We found a higher sensitivity for a second attack using the new 2017 criteria than when using the former 2010 criteria (68% vs 36%; p<0.01). However, the specificity was lower for the new criteria (61% vs 85%; p<0.01). These data imply that the McDonald 2017 criteria will lead to a higher number of MS diagnoses in patients with a less active disease course.

Fatigue is one of the most reported and disabling symptoms for MS patients. **Chapter 3** evaluates the long-term course of fatigue after CIS. First, we validated our previous finding that fatigue at time of CIS predicts a future CDMS diagnosis after adjustments for sex, age, ethnicity, localization of symptoms, gadolinium enhancement at baseline MRI, and DMT before CDMS diagnosis in 235 CIS patients (hazard ratio (HR) 2.6; p<0.01). After that, we showed that the fatigue scores (FSS) increased by 0.86 units after CDMS diagnosis (p=0.01). We demonstrated that fatigue started early in the disease course, already at time of CIS, and increased after a second attack.

In **chapter 4**, we investigated whether the T-cell activation marker soluble CD27 (sCD27) measured in CSF from CIS patients predicts MS diagnosis and a high relapse rate. First we showed higher level of CSF sCD27 in 77 patients with CIS than in 30 control individuals. We showed that within the CIS group, high sCD27 levels were predictive for MS diagnosis independently of MRI and CSF measurements (HR 2.4 per 100 U/mL increase

of sCD27; p<0.01). Interestingly, within the group that experienced a second attack, we observed that patients with high sCD27 levels at time of CIS had a 5.5 times higher annualized relapse rate (ARR) during follow-up after the second attack compared to patients with low sCD27 levels at time of CIS (ARR 0.33 vs 0.06; p=0.02). Soluble CD27 levels were also associated with IgG index, unique OCB in CSF, ≥9 T2 lesions and subclinical T2 lesions on baseline MRI.

In **chapter 5**, we performed an extensive study, in different patient groups, on Th1-like Th17 cells ($CCR6^+CXCR3^+$), which is a subpopulation of T helper 17 cells ($CCR6^+$), a key mediator in MS disease activity. We found that Th1-like Th17 cells, expressing IL-17 but also IFN-γ and GM-CSF, were decreased in peripheral blood from CIS patients who converted to CDMS within one year (CIS-CDMS) compared to CIS patients who remained monophasic for at least 5 years (CIS-CIS) (6% vs 11%; p=0.01). For Th1 ($CCR6^-CXCR3^+$) and Th17 ($CCR6^+CXCR3^-$) cells this reduction was not seen. A reduced effector memory (EM) to central memory (CM) ratio was found for the Th1-like Th17 subset in CIS-CDMS patients (0.3 vs 0.5; p<0.01). This ratio correlated with anti-EBNA1 IgG titres (p=0.01) and fatigue scores (FSS) (p<0.01), markers that are shown to be predictive for an early MS diagnosis.
In blood from patients who were already diagnosed with MS, we found strongly reduced frequencies of Th1-like Th17 effector memory (EM) cells compared to CIS-CIS and healthy controls (0.7% vs 2.8% vs 3.3%; p<0.01). In MS patients, a higher proportion of Th1-like Th17 cells was positive for the activation markers CD38 and HLA-DR. This was not seen for Th1 cells. This pleads for a selective activation of Th1-like Th17 effector cells in blood from patients with MS.
After that, we analysed paired CSF and blood samples from CIS and early MS patients. We found that the Th1-like Th17 proportion in CSF was 2- to 3-fold higher and produced more IFN-γ and GM-CSF than the paired blood Th17-like Th17 cells. Ex vivo experiments confirmed the local enrichment of Th1-like Th17 cells in the CNS, this subset was enriched in brain tissue from late-stage MS patients and not in brain tissue from non-demented controls. Next, we studied the migration of peripheral T helper subpopulations into the CNS. VLA-4 was the most abundant adhesion molecule on Th1-like Th17 cells, especially on the Th17.1 subset ($CCR6^+CXCR3^+CCR4^-$). This specific subset accumulated selectively in blood of natalizumab (an anti-VLA-4 monoclonal antibody) treated patients. We saw this only in natalizumab-treated patients who remained free of relapses (relapse-free patients: pre-treatment 3.8% vs post-treatment 6.8%; p<0.01, patients with relapses: pre-treatment 3.2% vs post-treatment 4.0%). And downregulation of VLA-4 by natalizumab was most prominent on Th17.1 cells. In vitro trans-well migration assays confirmed that Th17.1 was the main Th17 subpopulation migrating across human brain endothelial layers towards the inflammatory mediator CXCL10. Furthermore, Th17.1 cells were enriched in CSF from CIS and early MS patients compared to paired blood samples. Also lower Th17.1 frequencies were seen in blood from CIS-CDMS vs CIS-CIS patients (p=0.02). These data demonstrate the ability of Th1-like Th17 cells, in particular Th17.1 cells to migrate to the CNS and influence disease activity in early MS.

Cigarette smoking is shown to be a risk factor that influences disease course of MS patients. However, there are conflicting results for the predictive value of smoking for

MS diagnosis in CIS patients. In **chapter 6**, we studied the association between smoking at time of CIS and a subsequent CDMS diagnosis in 250 CIS patients. We found a higher rate of CDMS diagnoses in CIS patients who smoked at time of CIS than in non-smoking CIS patients (67% vs 36%; p<0.01). And smoking at time of CIS was an independent predictor for CDMS diagnosis (HR: 2.3; p<0.01). Interestingly, non-smoking CIS patients who had a history of smoking did not have a higher risk of CDMS than CIS patients who had never smoked, suggesting that the harmful effects of smoking are reversible.

Part 2 focused on the disease course after CIS in paediatric versus adult patients. In **chapter 7**, we compared disease course and clinical features at disease onset between 107 children and 276 adults who were followed after CIS. We divided the total group in three age groups (1-10, 11-17, 18-50 years). Lesion load on MRI was highest in the 11-17-year-old children. Children presented more often with polyfocal clinical symptoms than adults (32% vs 12%; p<0.01). We saw the highest rate of MS and CDMS conversion in the 11-17-year-old CIS group (84% vs 50%; p<0.01). This group also had the shortest time to MS diagnosis (HR: 3.2, 95%; p<0.01). Annualized relapse rates were evaluated in patients who were diagnosed with MS using a negative binomial regression model with log link. Without DMT, paediatric patients showed a higher ARR than adults (2.22 vs 0.91; p<0.01). However, after DMT administration ARR was the same for paediatric and adult MS patients (0.48 vs 0.40). These data showed a more inflammatory disease course in children after CIS than in adults, this argues for early initiation of DMT in 11-17-year-old children in line with current clinical practice in adult CIS patients.

Based on our earlier finding in adult patients with CIS that high sCD27 levels were predictive for a future MS diagnosis, we evaluated this T-cell activation marker in 94 children with ADS in **chapter 8**. We found the same result as we found in adults described earlier in this thesis. High CSF sCD27 levels in paediatric ADS patients without encephalopathy were associated with a future CDMS diagnosis (HR 2.8 per 100U/mL increase in sCD27 levels; p<0.01). Children with ADS and encephalopathy did not differ in sCD27 levels from monophasic ADS patients without encephalopathy. However, ADS patients with encephalopathy had lower levels sCD27 than ADS patients without encephalopathy who were diagnosed with CDMS during follow-up. Also in children, sCD27 levels were associated with OCB and IgG index.

It has been shown that neurofilament light chain (NfL), a marker for axonal damage, in CSF is predictive for MS diagnosis in adult CIS patients. In children, this was not studied yet. Therefore we investigated the predictive value of NfL in CSF from 65 paediatric and 88 adult CIS patients. In **chapter 9**, we first validated that high NfL levels were associated with CDMS diagnosis in adult patients with CIS (HR: 2.1; p=0.03). After that we demonstrated that NfL was also an independent predictor for CDMS diagnosis in paediatric CIS patients (HR: 3.7; p<0.01). Moreover, in the group with a future CDMS diagnosis, children had higher NfL levels than adults at time of CIS (4888 vs 2156pg/mL; p<0.01). We also showed that patients with a future CDMS diagnosis and T1-hypointense lesions on baseline MRI, which is a sign for axonal damage, had higher NfL levels than patients without T1-hypointense lesions, in both adults and children (adults: 3188 vs 1588pg/mL;

p<0.01, children: 8920 vs 1668pg/mL; p<0.01). These results imply that children not only have a more inflammatory disease course than adults, as shown earlier in this thesis, but also that this young patient group has signs of neurodegeneration already early in the disease course.

Chapter 10 discusses the main findings of this thesis and recommendations for future research.

SAMENVATTING

Multiple sclerose (MS) is een chronische ontstekingsziekte van het centrale zenuwstelsel (CZS). Zowel genetische als omgevingsfactoren zijn van invloed op het ontstaan van MS. MS start bij 85% van de patiënten met klinische aanvallen als gevolg van demyelinisatie van de zenuwen in verschillende delen van het CZS. Omdat het ziektebeloop van MS-patiënten erg heterogeen in ernst en prognose is, zijn er op het moment van de eerste aanval (CIS) factoren nodig die het ziektebeloop kunnen voorspellen. Betrouwbare prognostische factoren zijn erg belangrijk vanwege het groeiende aantal beschikbare immunomodulerende therapieën (IMT) voor MS-patiënten. Vroeg in het ziekteproces starten met deze therapieën voorkomt latere MS aanvallen en invaliditeit.
De studies die beschreven worden in dit proefschrift zijn uitgevoerd binnen twee prospectieve cohorten van volwassenen en kinderen die gevolgd zijn na een eerste aanval van demyelinisatie. Het doel van deze studies is om prognostische factoren te vinden die het ziektebeloop na een eerste aanval van demyelinisatie in volwassenen en kinderen kunnen voorspellen.
Hoofdstuk 1 geeft een korte introductie over MS en beschrijft klinische en biologische factoren die nu bekend zijn en gebruikt worden voor het voorspellen van het ziektebeloop.

Het eerste deel van dit proefschrift gaat over het ziektebeloop na CIS bij volwassen patiënten. In 2017 heeft 'the International Panel on Diagnosis of Multiple Sclerosis' een voorstel gedaan voor nieuwe revisies van de McDonald criteria voor MS. De belangrijkste verandering in de nieuwe criteria is dat als er unieke oligoclonale banden (OCB) worden gevonden in de liquor er niet meer aan spreiding in tijd (DIT) hoeft te worden voldaan. De andere belangrijke verandering is dat niet alleen asymptomatische maar ook symptomatische afwijkingen op de MRI-scan kunnen bijdragen aan DIT en spreiding in plaats (DIS). In **hoofdstuk 2** hebben we deze nieuwe criteria toegepast op een cohort van 229 volwassen CIS-patiënten. Met deze nieuwe criteria konden twee keer zoveel MS-diagnoses worden gesteld op het moment van de eerste aanval (97 (54%) vs 46 (26%); p<0.01). Wanneer we de nieuwe 2017 criteria gebruikten vonden we een hogere sensitiviteit voor een tweede aanval dan wanneer we de oude 2010 criteria gebruikten (68% vs 36%; p<0.01). De specificiteit was echter lager voor de nieuwe criteria (61% vs 85%; p<0.01). Deze data laten zien dat de McDonald 2017 criteria leiden tot een hoger aantal MS-diagnoses bij patiënten met een minder actief ziektebeloop.

Vermoeidheid is een van de meest gerapporteerde en invaliderende symptomen voor MS-patiënten. **Hoofdstuk 3** evalueert het langetermijn beloop van vermoeidheid na CIS. Eerst hebben we onze eerdere bevinding bevestigd, namelijk dat vermoeidheid op het moment van CIS voorspellend is voor een toekomstige CDMS-diagnose. Dit was ook het geval na correctie voor geslacht, leeftijd, etniciteit, locatie van de symptomen, contrast-aankleuring op de eerste MRI en IMT voor CDMS-diagnose (hazard ratio (HR) 2.6; p<0.01). Hierna hebben we laten zien dat de vermoeidheids-score (FSS) 0.86 punten hoger was na CDMS-diagnose (p=0.01). Vermoeidheid was al vroeg in het ziektebeloop, op het moment van CIS, aanwezig en werd erger na een tweede aanval.

In **hoofdstuk 4** hebben we onderzocht of de T-cel activatie marker soluble CD27 (sCD27) gemeten in liquor van CIS-patiënten voorspellend is voor MS-diagnose en voor een verhoogde aanvalsfrequentie. We vonden dat binnen de CIS-groep, hoge sCD27 waarden voorspelden voor MS-diagnose, onafhankelijk van MRI en liquor metingen (HR 2.4 per 100 U/mL verhoging van sCD27; p<0.01). Interessant was, dat binnen de groep die een tweede aanval had gekregen, patiënten met hoge sCD27 waarden op het moment van CIS een 5.5 keer hogere jaarlijkse aanvalsfrequentie (ARR) hadden tijdens follow-up, vergeleken met patiënten met lage sCD27 waarden op het moment van CIS (ARR 0.33 vs 0.06; p=0.02). Soluble CD27 waarden waren ook geassocieerd met IgG index, unieke OCB in liquor, ≥9 T2 afwijkingen en subklinische T2 afwijkingen op de eerste MRI-scan.

In **hoofdstuk 5** hebben we een uitgebreide studie binnen verschillende patiëntengroepen gedaan naar Th1-like-Th17 cellen ($CCR6^+CXCR3^+$), een subpopulatie van T-helper 17 (Th17) cellen ($CCR6^+$). Deze cellen spelen een belangrijke rol in ziekteactiviteit bij MS-patiënten. We vonden dat Th1-like Th17 cellen, die niet alleen IL-17 maar ook IFN-γ en GM-CSF tot expressie brengen, minder aanwezig waren in bloed van CIS-patiënten die binnen een jaar gediagnostiseerd werden met CDMS (CIS-CDMS) dan in bloed van CIS-patiënten die tenminste 5 jaar lang CIS bleven (CIS-CIS) (6% vs 11%; p=0.01). Voor Th1 ($CCR6^-CXCR3^+$) en Th17 ($CCR6^+$ $CXCR3^-$) cellen zagen we deze verlaagde frequentie niet. Ook vonden we een verlaagde effector memory (EM) / central memory (CM) ratio voor de Th1-like Th17 subset in CIS-CDMS patiënten (0.3 vs 0.5; p<0.01). Deze ratio correleerde met de anti-EBNA1 IgG-titer (p=0.01) en de vermoeidheids-score (FSS) (p<0.01), dit zijn beide markers die geassocieerd zijn met een vroege MS-diagnose.
In bloed van patiënten die al met MS gediagnostiseerd waren, vonden we sterk verlaagde frequenties van Th1-like Th17 EM cellen vergeleken met CIS-CIS patiënten en gezonde controles (0.7% vs 2.8% vs 3.3%; p<0.01). Daarnaast was een groter deel van de Th1-like Th17 cellen positief voor de activatie markers CD38 en HLA-DR. Dit zagen we niet voor Th1 cellen. Dit pleit voor een selectieve activatie van Th1-like Th17 effector-cellen in bloed van patiënten met MS.
Hierna hebben we gepaarde liquor en bloed samples van CIS en vroege MS-patiënten geanalyseerd. Hierin vonden we dat het deel Th1-like Th17 cellen in liquor 2 tot 3 keer hoger was dan in bloed en dat deze cellen in liquor meer IFN-γ and GM-CSF produceerden dan gepaarde bloed Th1-like Th17 cellen. Ex vivo experimenten bevestigden de lokale verhoging van Th1-like Th17 cellen in het CZS: deze subset was verhoogd in hersenweefsel van MS-patiënten. Dit was niet het geval in hersenweefsel van controles.
Hierna bestudeerden we de migratie van perifere T-helper subpopulaties naar het CZS. VLA-4 was het adhesiemolecuul dat het meest tot expressie kwam op de Th1-like Th17 cellen, in het bijzonder op de Th17.1 subset ($CCR6^+CXCR3^+CCR4^-$). Deze specifieke subset hoopte selectief op in het bloed van met natalizumab (een anti-VLA-4 monoclonale antistof) behandelde patiënten. We zagen deze ophoping in bloed alleen in natalizumab-behandelde patiënten die vrij van aanvallen bleven tijdens de behandelperiode (aanvalsvrije patiënten: voor behandeling 3.8% vs na behandeling 6.8%; p<0.01, patiënten met aanvallen: voor behandeling 3.2% vs na behandeling 4.0%). Vermindering van VLA-4 expressie door natalizumab werd vooral gezien in de Th17.1 cellen.
In vitro trans-well migratie analyses lieten zien dat Th17.1 inderdaad de Th17 subpopulatie

was die het meest migreerde door menselijke brein endotheelcel-lagen richting de inflammatoire cytokine CXCL10. Daarnaast waren Th17.1 cellen verhoogd in liquor van CIS en vroege MS-patiënten in vergelijking met gepaard bloed. Ook zagen we verlaagde Th17.1 frequenties in bloed van CIS-CDMS vs CIS-CIS patiënten (p=0.02). Deze data laat het vermogen van Th1-like Th17 cellen, met name de Th17.1 subset, zien om op te hopen in het CZS en ziekteactiviteit te beïnvloeden in vroege MS-patiënten.

Het roken van sigaretten is in eerdere studies aangetoond als risicofactor die het ziektebeloop van MS-patiënten beïnvloedt. Er zijn echter tegenstrijdige resultaten wat betreft de voorspellende waarde van roken voor MS-diagnose in CIS-patiënten. In **hoofdstuk 6** is de associatie tussen roken op het moment van CIS en een toekomstige CDMS-diagnose onderzocht in 250 CIS-patiënten. Er was een hoger aantal CDMS-diagnoses in CIS-patiënten die rookten vergeleken met CIS-patiënten die niet rookten (67% vs 36%; p<0.01). Ook was roken op het moment van CIS een onafhankelijke voorspeller voor CDMS-diagnose (HR: 2.3; p<0.01). Daarbij was het interessant dat niet rokende CIS-patiënten die in het verleden wel gerookt hadden, geen hoger risico op CDMS hadden in vergelijking met CIS-patiënten die nog nooit gerookt hadden. Dit wijst erop dat de schadelijke effecten van roken reversibel lijken te zijn.

Het tweede deel van dit proefschrift gaat over het ziektebeloop na CIS bij kinderen versus volwassenen.
Hoofdstuk 7 vergelijkt het ziektebeloop en de klinische parameters op het moment van de eerste neurologische uitval tussen 107 kinderen en 276 volwassenen met CIS. De gehele groep is verdeeld in 3 leeftijdscategorieën (1-10, 11-17, 18-50 jaar). Het aantal witte stof afwijkingen op de MRI was het grootst in de kinderen van 11-17 jaar. Ook presenteerden kinderen zich vaker met polyfocale-symptomen vergeleken met volwassenen (32% vs 12%; p<0.01). Kinderen in de leeftijd 11-17 jaar werden het meest gediagnostiseerd met MS tijdens follow-up (84% vs 50%; p<0.01). Ook was de tijd tot MS-diagnose voor deze groep het kortst (HR: 3.2, 95%; p<0.01). We hebben de jaarlijkse aanvalsfrequenties binnen de patiënten die gediagnostiseerd waren met MS onderzocht. Binnen de groep patiënten zonder IMT hadden kinderen de hoogste aanvalsfrequentie vergeleken met volwassenen (2.22 vs 0.91; p<0.01). Maar in de groep die behandeld werd met IMT was de aanvalsfrequentie tussen kinderen en volwassenen gelijk (0.48 vs 0.40). Deze data wijzen erop dat kinderen een meer inflammatoir ziektebeloop hebben dan volwassenen, dit is een argument voor het vroeg starten met IMT bij kinderen van 11-17 jaar. Dit is in overeenstemming met het huidige beleid in volwassen patiënten met CIS.

Gebaseerd op onze eerdere bevinding in volwassen CIS-patiënten dat hoge sCD27 waarden voorspellend waren voor een toekomstige MS-diagnose, hebben we deze T-cel activatie marker ook bekeken bij 94 kinderen met een eerste aanval van demyelinisatie (ADS). In **hoofdstuk 8** beschrijven we dat hoge sCD27 waarden in liquor van kinderen met ADS zonder encefalopathie geassocieerd zijn met een toekomstige CDMS-diagnose (HR 2.8 per 100 U/mL verhoging van sCD27; p<0.01). Deze resultaten kwamen overeen met wat we ook in volwassenen vonden, zoals eerder beschreven in dit proefschrift. De sCD27 waarden van kinderen met ADS en encefalopathie waren hetzelfde als voor

monofasische ADS patiënten zonder encefalopathie. ADS patiënten met encefalopathie hadden lagere sCD27 waarden vergeleken met ADS patiënten zonder encefalopathie die tijdens follow-up gediagnostiseerd werden met CDMS. Ook bij kinderen waren sCD27 waarden geassocieerd met de IgG index en unieke OCB in liquor.

Uit eerdere studies bleek dat neurofilament light chain (NfL), een marker voor axonale schade, in liquor voorspellend is voor MS-diagnose bij volwassenen met CIS. In kinderen was dit nog niet eerder onderzocht. Daarom hebben we de voorspellende waarde van NfL in liquor van 65 kinderen en 88 volwassenen met CIS bekeken. In **hoofdstuk 9** hebben we eerst gevalideerd dat hoge NfL waarden in liquor geassocieerd zijn met CDMS-diagnose in volwassen CIS-patiënten (HR 2.1; p=0.03). Hierna hebben we laten zien dat NfL een onafhankelijke voorspeller voor CDMS-diagnose is in kinderen met CIS (HR: 3.7; p<0.01). Daarnaast zagen we dat binnen de groep die werd gediagnostiseerd met CDMS, kinderen op het moment van CIS hogere NfL waarden hadden dan volwassenen (4888 vs 2156pg/mL; p<0.01). Ook lieten we zien dat binnen de patiënten met een toekomstige CDMS-diagnose, de patiënten met T1-hypointense afwijkingen op de eerste MRI-scan (een teken voor axonale schade) bij zowel volwassenen als kinderen hogere NfL waarden hadden dan de patiënten zonder deze afwijkingen (volwassenen: 3188 vs 1588pg/mL; p<0.01, kinderen: 8920 vs 1668pg/mL; p<0.01). Dit wijst erop dat kinderen niet alleen een meer inflammatoir ziektebeloop hebben dan volwassenen, zoals eerder in dit proefschrift beschreven, maar dat deze jonge patiëntengroep ook al vroeg in het ziektebeloop tekenen van neurodegeneratie vertoont.

In **hoofdstuk 10** worden de belangrijkste bevindingen van dit proefschrift bediscussieerd en worden er aanbevelingen gedaan voor toekomstig onderzoek.

Appendix

Voorgeschiedenis vragenlijst
Hospital Anxiety and Depression Scale
Fatigue Severity Scale

Voorgeschiedenis vragenlijst

A: Ziektegeschiedenis	
1. Heeft u ooit de ziekte van Pfeiffer gehad?	O Ja *(ga naar vraag 1A)* O Nee *(ga naar vraag 2)* O Weet niet *(ga naar vraag 2)*
1A. Zo ja, op welke leeftijd heeft u het gehad?	
2. Heeft u ooit de bof gehad?	O Ja *(ga naar vraag 2A)* O Nee *(ga naar vraag 3)* O Weet niet *(ga naar vraag 3)*
2A. Zo ja, op welke leeftijd heeft u het gehad?	
3. Heeft u ooit de waterpokken gehad?	O Ja *(ga naar vraag 3A)* O Nee *(ga naar deel B)* O Weet niet *(ga naar deel B)*
3A. Zo ja, op welke leeftijd heeft u het gehad? ***(Na beantwoorden van deze vraag, ga naar deel B)***	

B: Alcohol	
Drinkt u alcohol? ***(Na beantwoorden van deze vraag, ga naar deel C)***	O Ja Zoja, hoeveel glazen/eenheden per week drinkt u? O Nee

C: Roken	
1. Rookt u?	O Ja *(ga naar vraag 1A)* O Nee *(ga naar deel D)*
1A. Zo ja, hoeveel rookt u gemiddeld per dag? ***(Na beantwoorden van deze vraag, ga naar deel D)***	_____ sigaretten /dag _____ sigaren /dag _____ pakjes shag /week _____ pijp /dag

D: Roken		
1. Heeft u meer dan 100 sigaretten in uw hele leven gerookt?	O Ja	O Nee
2. Zijn er wel eens periodes in uw leven geweest waarin u minstens 1 sigaret per week rookte?	O Ja	O Nee
Als u beide voorgaande vragen met "Ja" beantwoord heeft, ga door naar deel E. ***Als u één van de voorgaande 2 vragen met "Nee" beantwoord heeft, dan kunt u stoppen. U hoeft het formulier niet verder in te vullen. Bedankt voor uw medewerking.***		

E: Roken	
1. In welk jaar bent u gestart met roken?	
2. Heeft u, sinds u gestart bent met roken, tussentijds gedurende minimaal één jaar niet gerookt?	O Ja ***(ga naar vraag 2A)*** O Nee ***(U bent klaar. Bedankt voor uw medewerking)***
2A. Zo ja, geef hieronder globaal aan vanaf welk jaar tot welk jaar u gerookt heeft en gemiddeld hoeveel sigaretten per dag of pakjes shag per week u in de betreffende periode gerookt heeft.	
Ik heb gerookt: vanaf_____(jaar)tot_____(jaar) en heb gemiddeld _____sigaretten per dag/pakjes shag per week* gerookt. vanaf_____(jaar)tot_____(jaar) en heb gemiddeld _____sigaretten per dag/pakjes shag per week* gerookt. vanaf_____(jaar)tot_____(jaar) en heb gemiddeld _____sigaretten per dag/pakjes shag per week* gerookt. vanaf_____(jaar)tot_____(jaar) en heb gemiddeld _____sigaretten per dag/pakjes shag per week* gerookt. Zo nodig kunt u op de achterkant van dit vel nog meer perioden aangeven. * streep weg wat niet van toepassing is ***(Dit was de laatste vraag. Bedankt voor uw medewerking.)***	

Hospital Anxiety and Depression Scale

In deze vragenlijst wordt gevraagd hoe u zich in de afgelopen week gevoeld heeft. Geef uw antwoord aan met een kruisje in het hokje dat het beste weergeeft hoe u zich **gedurende de afgelopen week** gevoeld heeft. Denk niet te lang na over uw antwoord. Er bestaan geen foute antwoorden, elk antwoord is goed, zolang het maar uw eigen (eerste) indruk weergeeft.

1. Ik voel me gespannen:
   - ❑ Meestal
   - ❑ Vaak
   - ❑ Af en toe, soms
   - ❑ Helemaal niet

2. Ik geniet nog steeds van de dingen waar ik vroeger van genoot:
   - ❑ Zeker zo veel
   - ❑ Wel wat minder
   - ❑ Duidelijk minder
   - ❑ Eigenlijk nauwelijks nog

3. Ik heb een soort angstgevoel alsof er iets vreselijks zal gebeuren
   - ❑ Jazeker, en vrij erg
   - ❑ Ja, maar niet zo erg
   - ❑ Een beetje, maar het hindert me niet
   - ❑ Helemaal niet

4. Ik kan best lachen en de dingen van de vrolijke kant zien:
   - ❑ Net zoveel als vroeger
   - ❑ Nu wel wat minder
   - ❑ Duidelijk wat minder
   - ❑ Helemaal niet

5. Ik maak me ongerust:
   - ❑ Heel erg vaak
   - ❑ Vaak
   - ❑ Af en toe, maar niet zo vaak
   - ❑ Heel soms

6. Ik voel me opgewekt:
   - ❑ Helemaal niet
   - ❑ Heel af en toe
   - ❑ Soms
   - ❑ Meestal

7. Ik kan best rustig zitten en me ontspannen:
   - ❑ Jazeker
   - ❑ Meestal
   - ❑ Af en toe
   - ❑ Helemaal niet

8. Ik heb het gevoel dat alles moeizamer gaat:
   - ❑ Bijna altijd
   - ❑ Heel vaak
   - ❑ Soms
   - ❑ Helemaal niet

9. Ik heb een soort angstig, gespannen gevoel:
   - ❑ Helemaal niet
   - ❑ Soms
   - ❑ Vrij vaak
   - ❑ Heel vaak

10. Het intersseert me niet meer hoe ik er uit zie:
    - ❑ Inderdaad, helemaal niet meer
    - ❑ Niet meer zoveel als eigenlijk zou moeten
    - ❑ Het interesseert me wel, maar iets minder dan vroeger
    - ❑ Het interesseert me nog net zoveel als vroeger

194

11. Ik ben onrustig en voel dat ik iets te doen moet hebben:
    - ❑ Inderdaad, heel duidelijk
    - ❑ Duidelijk
    - ❑ Enigszins
    - ❑ Helemaal niet

12. Ik verheug me van tevoren op dingen die komen gaan:
    - ❑ Net zoveel als vroeger
    - ❑ Een beetje minder dan vroeger
    - ❑ Veel minder dan vroeger
    - ❑ Bijna nooit

13. Ik raak plotseling in paniek:
    - ❑ Inderdaad, zeer vaak
    - ❑ Tamelijk vaak
    - ❑ Soms
    - ❑ Helemaal nooit

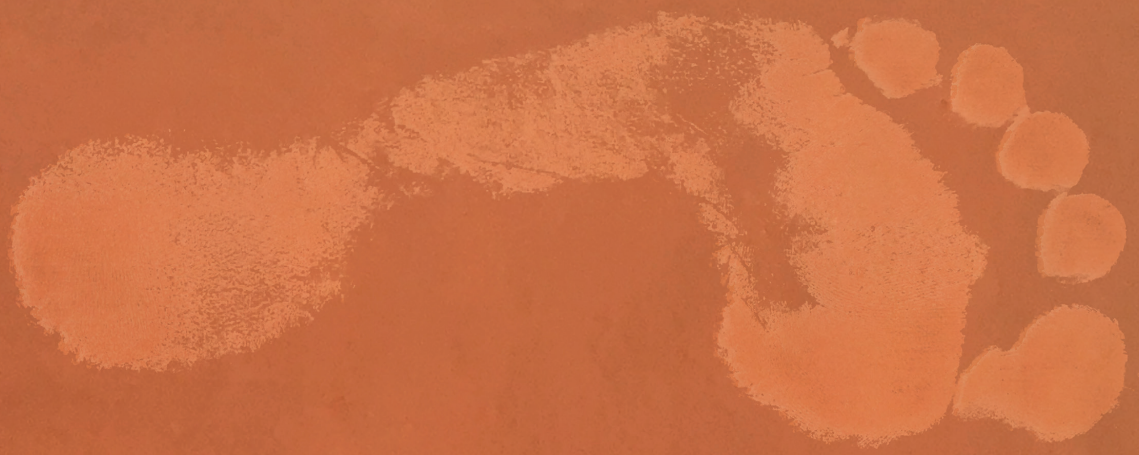
14. Ik kan van een goed boek genieten, of van zoiets als een radio- of televisieprogramma:
    - ❑ Vaak
    - ❑ Tamelijk vaak
    - ❑ Af en toe
    - ❑ Heel zelden

195

Vermoeidheid (Fatigue Severity Scale)

Lees de volgende uitspraken één voor één aandachtig door en omcirkel per uitspraak een cijfer tussen 1 en 7, afhankelijk van hoe goed de uitspraak volgens u aansluit bij hoe u zich de afgelopen week gevoeld heeft.

Gedurende afgelopen week:	**volledig oneens**	**grotendeels oneens**	**gedeeltelijk oneens**	**niet oneens /niet eens**	**gedeeltelijk eens**	**grotendeels eens**	**volledig eens**
1. was ik minder gemotiveerd om dingen te doen als ik vermoeid was.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
2. leidde lichamelijke inspanning tot vermoeidheid.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
3. was ik snel moe/vermoeid.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
4. beïnvloedde moeheid/vermoeidheid mijn lichamelijk functioneren.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
5. leidde moeheid/vermoeidheid voor mij vaak tot problemen.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
6. verhinderde moeheid/vermoeidheid langdurige lichamelijke inspanning.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
7. beïnvloedde moeheid/vermoeidheid de uitvoering van bepaalde taken en verplichtingen.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
8. behoorde moeheid/vermoeidheid tot mijn 3 voornaamste belemmerende klachten.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
9. beïnvloedde moeheid/vermoeidheid mijn werk, gezinsleven of sociale activiteiten.	**1**	**2**	**3**	**4**	**5**	**6**	**7**



Epilogue

ABOUT THE AUTHOR

Roos van der Vuurst de Vries was born on November 22nd, 1988 in Rotterdam, the Netherlands. In 2001 she attended secondary school at Montessori Lyceum Rotterdam and graduated in 2007 (Gymnasium). She studied medicine at the Erasmus University in Rotterdam. After obtaining her Medical degree in June 2014, Roos started her PhD research on the project described in this thesis at the MS Centre ErasMS in Rotterdam under supervision of Prof. dr. R.Q. Hintzen (department of Neurology). She succeeded Dr. Tessel F. Runia and Dr. Naghmeh Jafari as coordinator of the multicentre prospective PROUD study (Predicting the OUtcome of a Demyelinating event). From June 2017 onwards she works as a resident at the department of Neurology at Erasmus Medical Centre in Rotterdam (head: Prof. dr. P.A.E. Sillevis Smitt).

PHD PORTFOLIO

1. PhD training	*Year*	*Workload (ECTS)*
Courses		
EDSS training	2014	0.2
ACCES course basic and advanced	2014	0.7
Basic genetics (MolMed)	2014	0.6
Basic introduction course SPSS	2014	1.0
Biostatistics for clinicians	2015	2.0
Research Integrity	2015	0.3
Introduction in GraphPad Prism	2015	0.3
Basiscursus Regelgeving Klinisch onderzoek (BROK)	2015	1.4
Survival analysis (Nihes)	2016	1.9
Advanced immunology	2017	1.5
Biomedical English writing and communication	2017	4.0
Oral presentations		
Regionale neurologen avond, Erasmus MC, Rotterdam	2015	1.2
Meeting of the Dutch MS Research Foundation (2 presentations)	2016	2.4
Wetenschappelijke vergadering NVN (2 presentations)	2016, 2017	2.0
Referaat afdeling neurologie, Erasmus MC (2 presentations)	2017, 2018	2.4
ECTRIMS, Berlin	2018	1.2
Poster presentations		
ECTRIMS (4 posters)	2016-2018	4.0
Wetenschappelijke vergadering NVN	2016	1.0
Meeting of the Dutch MS Research foundation	2016	1.0
(Inter)national conferences		
Congress of the European Committee of Treatment and Research in MS (ECTRIMS)	2014-2018	5.0
Nubin symposium VUMC, Amsterdam	2014	0.5
Meeting of the Dutch MS Research foundation	2015,2016	1.0
Wetenschappelijke vergadering NVN	2016,2017	1.0
MS symposium VUMC, Amsterdam	2016,2017	1.0
TN2 Conference, Amsterdam	2017	0.5
2. Teaching		
medical student	2015	3.0
Total ECTS		41.1

LIST OF PUBLICATIONS

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Application of the 2017 revised McDonald criteria for multiple sclerosis to patients with a typical clinically isolated syndrome
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Wong YYM, **van der Vuurst de Vries RM**, van Pelt ED, Ketelslegers IA, Melief MJ, Wierenga-Wolf AF, Catsman-Berrevoets CE, Neuteboom RF, Hintzen RQ.
T-cell activation marker sCD27 is associated with clinically definite multiple sclerosis in childhood acquired demyelinating syndromes
Multiple Sclerosis Journal, 2018

van der Vuurst de Vries RM*, Wong YYM*, Mescheriakova JY, van Pelt ED, Runia TF, Jafari N, Siepman TAM, Melief MJ, Wierenga-Wolf AF, van Luijn MM, Samijn JP, Neuteboom RF, Hintzen RQ.
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Fatigue after a first attack of suspected multiple sclerosis.
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202

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203

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LIST OF ABBREVIATIONS

ADEM	Acute disseminated encephalomyelitis
ADEM-ON	ADEM followed by relapsing optic neuritis
ADS	Acquired demyelinating syndromes
ADS+	ADS with encephalopathy
ADS-	ADS without encephalopathy
AID	Autoimmune disease
AIDS	Acquired immunodeficiency syndrome
AUC	Area under the curve
AQP4	Aquaporin-4
ARR	Annualized relapse rate
BMI	Body mass index
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CDMS	Clinically definite multiple sclerosis
CHI3L1	Chitinase 3 like 1
CI	Confidence interval
CIS	Clinically isolated syndrome
CM	Central memory
CNS	Central nervous system
CSF	Cerebrospinal fluid
CXCR	CXC chemokine receptor
DIS	Dissemination in Space
DIT	Dissemination in Time
DMT	Disease modifying therapy
DNA	Deoxyribonucleic acid
DP	Double positive
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded disability severity scale
EBV	Epstein-Barr virus
ELISA	Enzyme-Linked Immuno Sorbent Assay
EM	Effector memory
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FLAIR	Fluid-attenuated inversion recovery
FN	False negative
FP	False positive
FSS	Fatigue severity scale
FU	Follow-up
GEE	Generalized estimating equations
GM-CSF	granulocyte macrophage colony-stimulating factor
GWAS	Genomic-wide association study
HADS	Hospital Anxiety and Depression Scale
HC	Healthy control
HR	Hazard ratio
HLA	Human leukocyte antigen
IFN	Interferon
IgG	Immunoglobulin G

IL	Interleukin
IPMSSG	International Pediatric Multiple Sclerosis Study Group
IQR	Inter quartile range
IRIS	Immune reconstitution inflammatory syndrome
KFLC	Kappa free light chains
LFLC	Lambda free light chains
LP	Lumbar puncture
MC	Medical centre
MFI	Median fluorescent intensity
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
N	Number of patients
NA	Not applicable
NfL	Neurofilament light chain
NS	Not significant
NMO	Neuromyelitis optica
NMOSD	Neuromyelitis optica spectrum disorders
NPV	Negative predictive value
OCB	Oligoclonal bands
OCT	Optical coherence tomography
ON	Optic neuritis
OND	Other neurological diseases
OR	Odds ratio
pg	picogram
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive multiple sclerosis
PPV	Positive predictive value
PDW	Proton-density-weighted
PROUD	PRedicting the OUtcome of a Demyelinating event
PROUDkids	PRedicting the OUtcome of a Demyelinating event in children
RA	Rheumatoid arthritis
RIS	Radiologically isolated syndrome
RNFL	Retinal nerve fibre layer
ROC	Receiver operating curve
RR	Relative risk
RRMS	Relapsing remitting multiple sclerosis
SC	Symptomatic control
sCD27	Soluble CD27
SD	Standard deviation
SE	Spin-echo
SLE	Systemic lupus erythematosus
Th	T helper
TM	Transverse myelitis
TN	True negative
TP	True positive
SPMS	Secundary progressive multiple sclerosis
VEP	Visual evoked potential
VLA-4	Very late antigen-4
WBC	White blood cell count