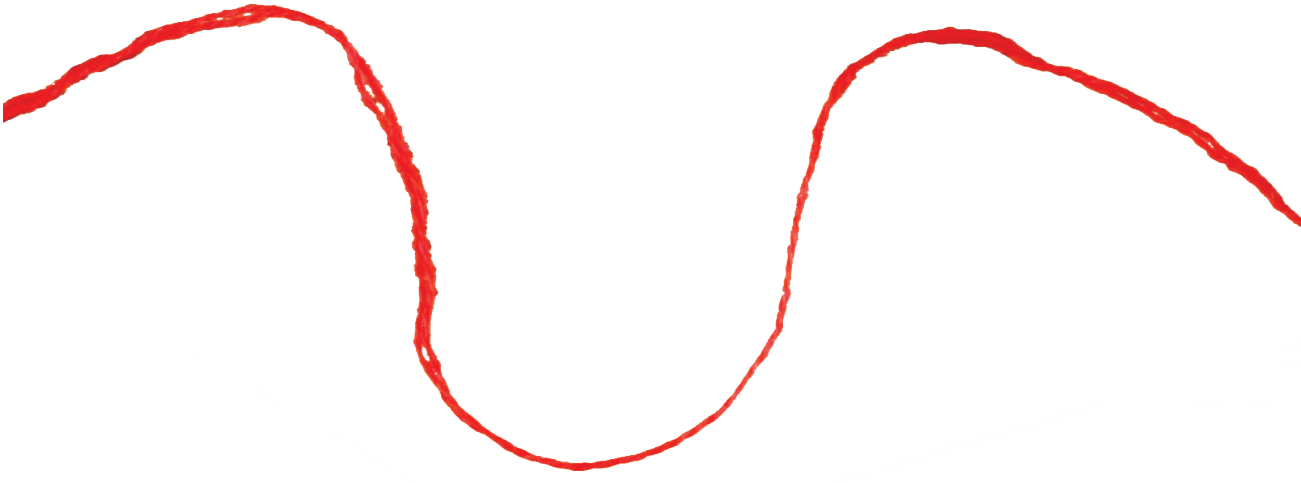
Multiple Sclerosis – Predicting the Next Attack



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Multiple Sclerosis - Predicting The Next Attack

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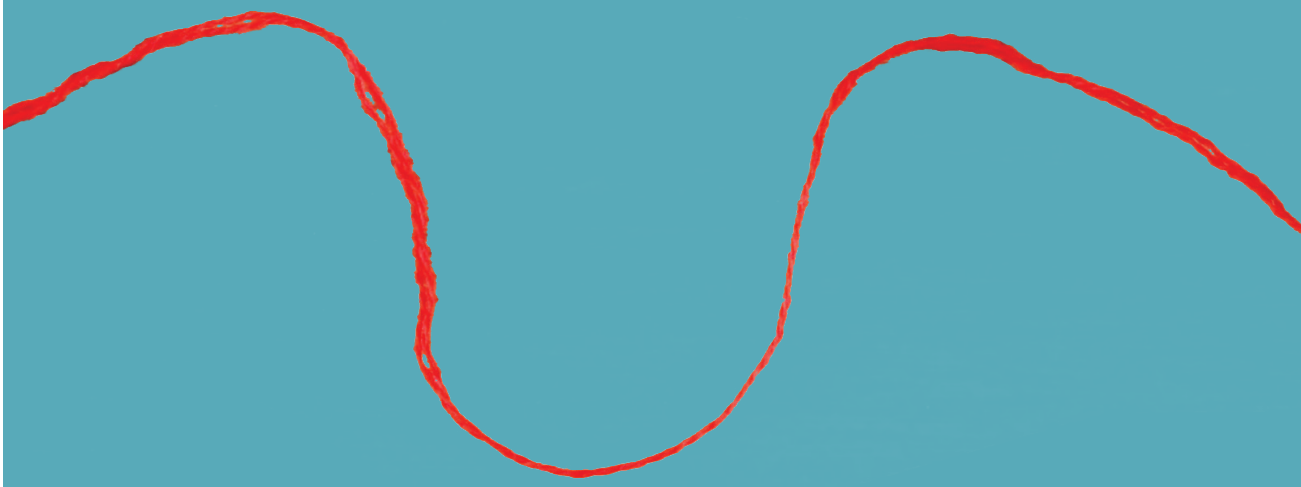
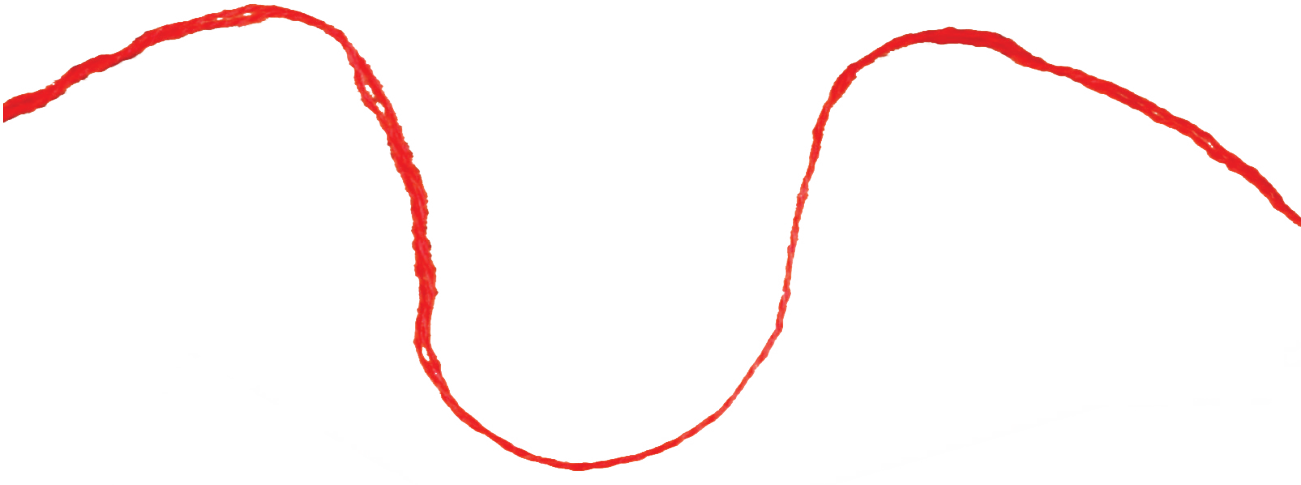
Part 1

CIS to MS



Chapter 1

General introduction



Preface

Multiple sclerosis (MS) is a chronic, disabling disease of the central nervous system (CNS), with an age of onset between 20-40 years. It was first identified as a distinct disease by Jean-Martin Charcot in the second half of the 19th century. Charcot named the disease *la sclerose en plaques* based on the sclerotic plaques that were seen on pathologic examination [1].

To date, over 150 years later, the exact etiology of MS is still not exactly known. The disease is thought to manifest itself in genetically susceptible individuals, after exposure to certain environmental factors, resulting in a pathology characterized by inflammation, axonal loss and demyelination. In recent years, evidence has been accumulating that not only myelinated brain tissue, but also gray matter is affected by the disease.

In 85% of MS patients, the disease starts with a subacute episode of symptoms resulting from inflammation of the optic nerve, spinal cord, cerebrum, cerebellum or brain stem, called clinically isolated syndrome or CIS. When a patient has experienced a CIS, there is a 30-70% chance that he or she will eventually be diagnosed with MS [2]. When this happens and these patients experience a second clinical attack, they enter the relapsing-remitting phase of the disease. In the majority of patients this is followed in later years by a secondary progressive phase, characterized by a slow progression of disability but lacking evident relapses (figure 1). Because of the presence of multiple brain lesions visible on the MRI scans of most CIS patients at the time of first symptoms, it is thought that an asymptomatic preclinical phase precedes the first clinical symptoms. In 10-15% of MS patients the disease is progressive from onset; this is called primary progressive MS (PPMS). This thesis will focus on the relapsing-remitting form of MS; the factors that are predictive of a next attack.

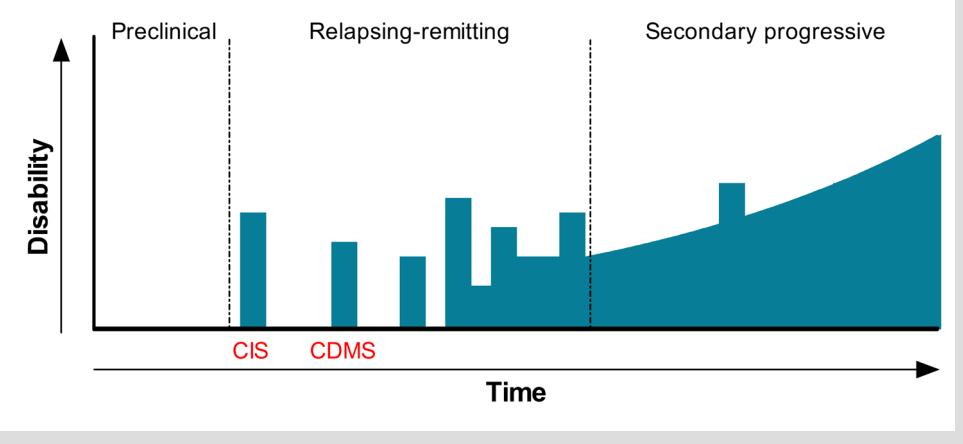


Figure 1. Clinical course of multiple sclerosis as it observed in the majority of patients: a first attack (clinically isolated syndrome or CIS) is followed by a second attack defining clinically definite MS (CDMS). The relapsing-remitting disease course is followed in later years by a secondary progressive phase.

Prognostic factors in MS

MS is a complex disease characterized by a large heterogeneity in radiological and pathological findings but also in clinical disease course and treatment response [3-5].

Prognostic factors are needed to 1. reliably counsel patients about their prognosis, 2. differentiate between CIS/MS and other causes of the symptoms, 3. be able to start the appropriate treatment at the right moment in the right patient, and 4. gain more insight into the pathogenesis of MS (and 5. to try to prevent MS in some cases, knowing that the incidence of MS is increasing [6], probably caused by a, so far unknown, environmental factor).

This thesis focuses on predictive factors in patients with CIS and relapsing-remitting MS (RRMS). In this introduction, an overview of the known prognostic factors is given, including clinical and bedside factors as well as genetics and body fluid biomarkers. First, an overview of risk factors for MS in the general population is given. Second, predictive factors for the next attack are described for patients with CIS and RRMS. For patients with CIS, the next attack is disease-defining, leading to the diagnosis of clinically definite MS (CDMS). In patients with RRMS, there is some controversy regarding the importance of relapses. Because the progressive phase of the disease (see figure 1) causes most of the long-term disability in MS patients, and this is independent of the location, severity and recovery of previous relapses [7], some people feel that relapses do not matter in relation to long-term disability. However, it is also known that relapses are associated with residual neurological deficit in 40-50% of cases [8], and that relapses early in the disease course (year 1 and 2) do seem to affect later disability [9]. Furthermore, relapses have physical, emotional and financial consequences, and may lead to hospitalization and time away from work and home [10]. Especially in early disease, relapses are the main cause of disability in MS patients. Because relapses are also the main target for all current MS therapies, it is an important topic in MS research. Because progressive MS was not investigated in the research described in this thesis, these MS subtypes are not discussed here.

The risk for MS

Around the world, over 2.5 million people suffer from MS [11]. The disease incidence and prevalence are distributed unevenly over the world: a high prevalence is found in Western Europe and North America, and the lowest prevalence in Asia, the Middle East and Africa [6]. The risk for MS is determined by both genetic and environmental factors.

Biological factors

Genetics and ethnicity

Arguments for the influence of genetic factors on the risk for MS include the increased risk for MS in family members of patients (figure 2), the regional differences in MS prevalence, and the difference in MS prevalence between ethnic groups; MS is very rare in Samis, Turkmen, Uzbeks, Kazakhs, Kirgizis, native Siberians, North and South Amerindians, Canadian Hutterites, Chinese, Japanese, African blacks and New Zealand Maoris, and there is a high

risk for MS among Sardinians, Parsis and Palestinians [12]. More than 150 susceptibility loci for MS have been described. These loci, all associated with relatively small risks, lie mostly in genes with functions in immunity. The greatest effect on individual MS risk is exerted by variation in the major histocompatibility complex (MHC), especially the HLA-DRB1*15:01 allele [13-15].

Gender

MS is more common in women than in men, and this sex ratio (female: male exceeding 3.2:1) [16] seems to be increasing. Worldwide, the incidence of MS has been increasing over the last decades, particularly in women [6]. The increasing female: male ratio makes it likely that there is in fact a true increase in incidence, explained by a changing environmental exposure, particularly in women. Although there are some obvious candidates, for example smoking or use of contraceptives, the real culprits have not been identified yet.

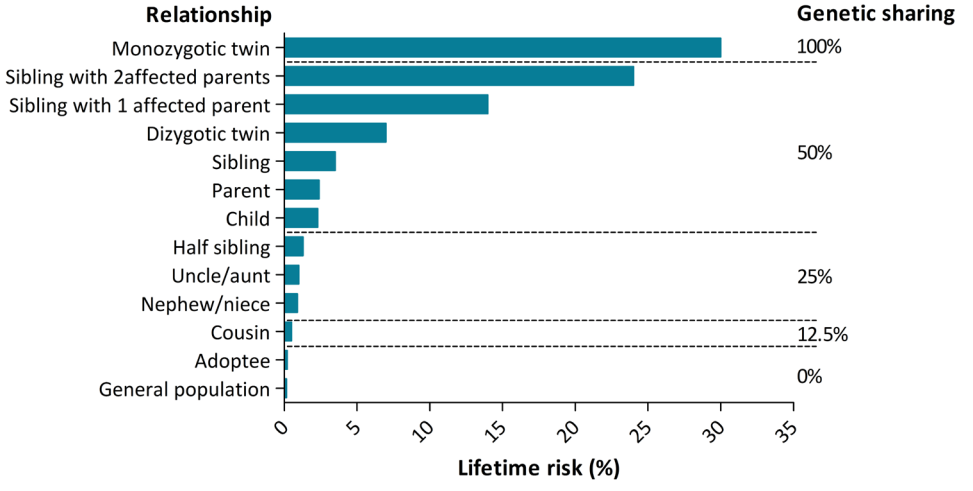


Figure 2. Recurrence risks for multiple sclerosis. Age-adjusted recurrence risks for different relatives of probands with multiple sclerosis, and degree of genetic sharing between relative and proband. Pooled data from population-based surveys. Based on: Compston and Coles, Multiple Sclerosis, Lancet 2008 [4].

Vitamin D and UV light

MS prevalence has repeatedly been reported to have a latitudinal gradient; being more prevalent in temperate areas further away from the equator [17-19]. This latitudinal gradient is one of the reasons for sunlight or vitamin D to be regarded as an environmental factor for MS risk; one of the strongest epidemiological arguments being the fact that migrants from the United Kingdom to the south of Australia have a significantly higher MS risk than those who migrated to the north of Australia [20]. However, recent evidence suggests that the latitudinal gradient might be decreasing[21] or might not have existed at all in some are-

as [6]. Nevertheless, there are also many studies that found an inverse association between MS prevalence and solar radiation [22, 23] and MS prevalence and serum vitamin D levels [24]. Furthermore, there is biological plausibility for a role of vitamin D in MS, as vitamin D has immunological properties, and vitamin D related genes have been associated with MS risk [14, 25]. UV-light could also act as an immune modulator separately from vitamin D [26, 27].

Taken together this suggests that there is indeed an association between latitude and MS risk, possibly attributable to UV-light and vitamin D, but that the latitudinal gradient has been leveled out on the northern hemisphere. This is possibly due to differences in sunbathing behavior between northern and southern Europeans, to higher dietary intake of vitamin D in northern countries, or to migration of many people from around the equator to more prosperous northern countries. Perhaps genetic factors also play a role here [6, 28]. A recent meta-analysis confirmed the association between latitude and MS prevalence [28].

Infections and Epstein-Barr virus (EBV)

The 'hygiene hypothesis' is based on the theory that exposure to infections in early childhood is protective against autoimmunity, possibly because this exposure is needed for the immune system to develop normally [29]. According to this hypothesis, when exposure to early childhood infections is reduced (due to increased standards of hygiene, vaccinations and widespread use of antibiotics), risk of MS or other autoimmune diseases increases with increasing age at infection. This hypothesis is attractive because it may partly explain several epidemiological features of MS, such as the latitudinal gradient [30]. However, one aspect that is not explained by the hygiene hypothesis is the association of MS with the Epstein-Barr virus (EBV). Nearly all MS patients are seropositive for anti-EBV antibodies, and people who are seronegative have practically no risk of developing MS. It appears that EBV infection is a prerequisite for the development of MS [31]. Especially after EBV-induced infectious mononucleosis, the risk of MS is increased, even more so if infectious mononucleosis occurs after the age of 15 [32]. It should be noted however, that in matched controls of MS patients, EBV seropositivity is also very common (90-95%), so most EBV infected people do not develop MS [31].

Hormonal factors (parity, use of contraceptives)

Because of the increasing female/male ratio in MS epidemiology, sex hormones are suspected to influence MS risk. Several studies have studied different aspects of this, but no consistent associations were found. An Australian study showed that the number of pregnancies was inversely associated with the risk of a first demyelinating event. The effect of the number of family pregnancies was only seen in the mothers, not in the fathers, arguing for a role of biological/hormonal factors in the mother and against postnatal environmental factors that could also affect the fathers [33]. However, a previous study found no association of parity with MS incidence, and also failed to show an association of oral contraceptives use and MS risk [34]. Other studies on the use of contraceptives and MS risk have also shown conflicting results [35, 36].

Biomarkers

In 2012, a group from Germany identified the ATP-sensitive inward rectifying potassium channel KIR4.1 as target of IgG antibodies in multiple sclerosis. Serum levels of antibodies to KIR4.1 were higher in MS patients than controls [37]. The same group also found higher levels of KIR4.1 titers in children with demyelinating disease [38]. However, these findings could not be replicated by others [39, 40]. These discrepancies are possibly at least in part due to technical issues, but further research on this topic is needed.

Clinical factors

Lifestyle factors (smoking, obesity, alcohol, stress)

One of the best-studied environmental factors associated with the development of MS is smoking, with a consistent increased risk for MS in smokers compared to never-smokers across the different studies [41-44]. A Swedish study comparing the risk for MS in never-smokers who had been exposed to passive smoking to never-smokers without exposure, found that passive smoking was also associated with an increased MS risk. The use of tobacco in the form of moist snuff is not associated with an increased risk, making it plausible that the negative effect of smoking results from some sort of irritation in the lungs, and not nicotine exposure [41, 45].

Recently, a case-control study from Sweden showed that alcohol consumption was associated with a decreased risk for MS, in a dose-dependent fashion. Furthermore, the use of alcohol appeared to reduce the negative effect of smoking [46].

A number of studies from different countries reported an association of obesity during childhood and adolescence with increased MS risk, particularly in girls [47-50]. Although some of these studies are based on self-reported data, the outcome is fairly consistent. The inverse association of obesity with vitamin D levels may play a role in the effect of obesity on MS risk. Possibly, the obesity epidemic partly explains the increasing incidence of MS.

It has been suggested that the occurrence of stressful life events increases MS risk [51]. However, a recent large cohort study from Denmark could not provide any evidence for the association between major life events and subsequent MS risk [52].

In conclusion, the epidemiology of MS is determined by multiple factors, both genetic and environmental. Furthermore, several of the known environmental risk factors (e.g. smoking, obesity, EBV) have been shown to interact with HLA genotype to influence MS risk [53-55]. A genetic predisposition in combination with EBV infection seems to be essential to get the disease. Furthermore, vitamin D and sunlight are important contributors to MS risk. The increasing incidence of MS particularly in women argues for other, thus far unknown, environmental factors to be important. Lifestyle factors such as obesity probably play a role here.

The risk for attacks

When a patient has experienced a first demyelinating event or CIS, there is a 30-70% chance that he or she will eventually be diagnosed with MS [2]. Although the second attack, defining clinically definite MS (CDMS), comes within the first 5 years for the majority of patients, there is a large variability in time to second attack, and in some patients, the disease will always remain monophasic.

Also after the diagnosis MS has been made, the course of the disease is highly heterogeneous. About 10% of patients will have a benign disease course, defined as 'disease in which the patient remains fully functional in all neurologic systems'[56]. In the long term, after 20 years, 50% of patients will be wheelchair dependent (DSS score of 7). Factors with a negative association with long-term prognosis in RRMS patients are male sex, older age at onset, a higher relapse rate early in the disease course, greater disability in the first 5 years, the involvement of more systems (especially ataxia, bowel and bladder symptoms, and cognitive impairment), and incomplete recovery from an attack[57].

Apart from factors associated with the long term prognosis and disability, some factors have been identified that are associated with the development of relapses in the short run. The below overview describes these factors, both for CIS patients and RRMS patients. Where possible, the data on CIS patients are based on outcome data from untreated CIS patients from natural history studies or the placebo arms of treatment studies (the North American Optic Neuritis Treatment Trial (ONTT)[58], the Controlled High Risk Subjects Avonex Multiple Sclerosis Prevention Study (CHAMPS)[59], the Rebif Flexible Dosing in early MS study (REFLEX)[60], the Early Treatment of Multiple Sclerosis Study (ETOMS)[61], the Betaferon/Betaseron in Newly Emerging Multiple Sclerosis for Initial Treatment study (BENEFIT)[61]. Where no prospective data from untreated patients are available, the best available evidence from the literature is used.

Biological factors

Genetics and ethnicity

The association of ethnicity with a diagnosis of CDMS in CIS patients was investigated in the ONTT and CHAMPS studies. No significant association was found, but there was a trend for a higher risk in white patients compared to black or non-white patients [58, 59], although both studies included only a small number of non-white patients. In contrast, in an older study [63], a significantly higher risk for an early second attack was found in non-white patients.

Similarly, there seems to be no association with conversion to CDMS of a positive family history for MS, although also here the number of included patients with positive family history was low [58, 59].

In a study performed with data from 2215 patients with CIS or MS and 2 validations sets,

weighted genetic risk scores of CIS and MS patients had similar distributions, suggesting that genetic susceptibility for MS and CIS is comparable. The weighted genetic risk score was not associated with time to conversion to CDMS [64].

Thus, the genetic susceptibility for CIS and MS appears to be the same. Genetics and ethnic background seem to co-determine an individual's risk to develop a CIS, but not the subsequent risk to have a second, disease-defining attack. Larger studies are needed to clarify this.

The association between known MS-risk-associated SNPs and relapses was investigated in a recent prospective study from Australia [65]. HLA-DRB1*15:01 was not associated with the occurrence of relapse, but five SNPs tagging other genes were. Although these results did not remain significant after correction for multiple testing, there was a clear allele dose-response effect for two of these SNPs tagging EVI5 and MAPK1, suggesting a true association but a lack of power. No association with disability progression was found. Other studies on the association of genetics with clinical disease course have not identified significant associations besides an earlier age of onset associated with HLA-DRB1*15:01 [66, 67]. This topic is currently being studied more extensively in international collaborations.

Age

The influence of age on the risk of conversion to CDMS is not completely clear. Some, but not all, studies found a higher risk for conversion in younger age groups [59, 62, 63]. In RRMS patients, age is an important risk factor for entering the secondary progressive phase of the disease, and older age is associated with a lower relapse risk [7, 68].

Gender

The risk of having a first demyelinating event is higher for women than for men. However, the effect of gender on a subsequent diagnosis of CDMS is less clear. In many studies, men and women have an equal risk for CDMS, and only few studies found a higher risk in women [58, 69]. A meta-analysis including studies with a total of 4732 patients indeed found a high risk for CIS in females compared to males (RR 2.12), but only a moderately increased risk for CDMS in female CIS patients, which was not significant (RR 1.20)[70]. Not only do women have a higher risk for MS, it was also recently shown in a large international study including over 80.000 patient years of relapse-onset MS, that female patients have a higher relapse rate than males throughout the course of the disease [68].

Vitamin D

So far, two studies have investigated the association between 25-OH-vitamin D levels and conversion to CDMS [71-73]. The first one [71] found increased risk of CDMS only in women with low 25-OH-D levels. The second one [72], found significantly increased risks for new lesions and increased lesion volumes on MRI for patients with low 25-OH-D levels, but only a borderline increased risk for CDMS. The association of vitamin D levels with disease activity in patients with relapsing-remitting MS will be described in this thesis.

Infections and viruses

As mentioned before, an infection with EBV is probably a prerequisite to develop a CIS, but the association of EBV antibody titers with conversion to CDMS in CIS patients is less clear. One study in 147 patients with a mean follow-up of 7 years found an association of higher Epstein-Barr Virus-Encoded Nuclear Antigen 1 (EBNA1) IgG titers with risk for McDonald MS according to the 2001 revised criteria, but not with risk for clinically definite MS [74]; the association with McDonald MS was only statistically significant in the univariate analysis. In this study, anti EBNA1 titers were also associated with increased disability. Others reported a higher conversion risk in patients with anti-CMV IgG positivity, but not anti-EBV positivity [75].

In RRMS, it has been hypothesized that EBV-reactivation is associated with relapse risk. However, studies evaluating serological and viral load markers of EBV reactivation and clinical disease course have shown not shown convincing evidence for such an association [76-78].

Apart from EBV infections previously in life, patients with CIS often report symptoms of a viral infection in the days or weeks before onset of CIS (29% in the ONTT study)[58]. The predictive value of these viral symptoms is not clear. In the ONTT study, patients with viral symptoms preceding CIS had a higher conversion risk, but this was only true for patients with monofocal optic neuritis without T2 lesions on brain MRI [58]. In contrast, in a cohort of Australian pediatric patients, preceding infection was protective against a subsequent diagnosis of MS [79]. In patients with RRMS, strong evidence suggests that relapses can be triggered by infections [80-82], possibly through induction of secretion of pro-inflammatory cytokines by infectious agents or immune activation by interaction of the host immune system with viral superantigens. However, the association of infections with the appearance of new MRI lesions has not been as consistent. Some of the clinical worsening associated with infection may also be due to pseudorelapses [83].

Although some case-reports suggest an increased risk of relapse following vaccination, evidence is scarce and the few larger studies do not support an association of vaccination and relapse risk [84, 85].

Hormonal factors (parity, contraception, assisted reproduction technology)

There is no information on parity, the use of contraceptives or assisted reproduction technology in CIS patients regarding the risk of a subsequent diagnosis of MS. However, there is much evidence that hormonal factors are associated with relapse risk. For example, it is now well-known that the relapse rate decreases during pregnancy, particularly during the third trimester, with a decrease in relapse rate of around 70% [86, 87]. After delivery, the relapse risk increases again. It is not fully known what causes this increased postpartum relapse risk. Assisted reproduction technology is also associated with increased disease activity in the following 3 months, especially when the procedure did not result in pregnancy and gonadotrophin-releasing hormone [88]. There is inconclusive evidence on the use of oral contraceptives and clinical disease course [89].

Biomarkers

Biomarkers are defined by the Biomarkers Definitions Working Group as 'a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention' [90]. Comabella and Montalban have recently reviewed biomarkers currently available for MS [3]. 'Validated' biomarkers (that is, replicated in relatively large cohorts of patients and the potential to become clinically useful) for CDMS in CIS patients are shown in Table 1, and biomarkers associated with relapses in RRMS patients are shown in Table 2.

Biomarker	Body fluid	Comment
Free kappa light chains	CSF and serum	Plasma cells produce excess kappa and lambda light chains, which are secreted as free light chains and can be detected in CSF and serum. KFLC are raised in CSF from patients with MS and their presence might support disease diagnosis [91-93] but they do not seem superior to OCB [94]
IgM oligoclonal bands	CSF	IgM antibodies play a part in the inflammatory response in patients with MS and are associated with an increased risk for conversion to CDMS [95, 96]
Measles, rubella and varicella zoster reaction	CSF	Corresponds with a polyspecific, intrathecal B-cell response characterised by antibody production to neurotropic viruses (measles, rubella, and varicella zoster). Measles, rubella, and varicella zoster reaction has been associated with an increased risk for conversion to MS [97, 98]
Chitinase3like1	CSF	Glycoside hydrolase that is secreted mainly by activated macrophages. Concentrations are increased in patients with clinically isolated syndromes who later convert to clinically definite MS compared with patients who remain with clinically isolated syndromes. The same group of investigators validated the findings in an independent cohort of patients with clinically isolated syndromes [99]

Table 1. Potential biomarkers associated with CDMS in CIS patients (based on: Comabella and Montalban, Body fluid biomarkers in multiple sclerosis, Lancet Neurology, 2014 [3])

Biomarker	Body fluid	Comment
IgM oligoclonal bands	CSF	IgM OCB are associated with an aggressive disease course, more relapses and higher T2 lesion load [100,101]
Nitric oxide metabolites	CSF, serum, plasma, urine	Oxidative stress plays a part in MS disease. Concentrations of NO and its metabolites (nitrates and nitrites) increase during acute exacerbations and are associated with radiological inflammatory activity and disability progression [102, 103]
Matrix metallo-proteinase 9 (MMP9)	CSF, serum and PBMC	Matrix metalloproteinases are involved in myelin breakdown, release of proinflammatory cytokines, and axonal damage. MMP9 concentrations increase in patients with MS during relapses and are linked to radiological disease activity[104-107]
Myelin basic protein (MBP)	CSF	During myelin injury, MBP and its fragments are released into the CSF. MBP is increased during clinical attacks and associated with gadolinium enhancement [108-110]
Osteopontin (OPN)	Serum, plasma, CSF	Integrin-binding protein with pleiotropic roles, including inflammation, cell-mediated immunity, and cell survival. Concentrations are increased in patients with RRMS during clinical relapses [111-113]
Chemokine ligand 13 (CXCL13)	CSF	Has a role in B-cell recruitment to the CNS during inflammation. Concentrations are increased in patients with active disease [114-116]
Brain-derived neurotrophic factor (BDNF)	Serum, plasma and PBMC	Neurotrophic factor secreted not only by neurons, but also by immune cells with roles in CNS neurogenesis, neuroprotection, and neuroregeneration. Concentrations are increased in patients during relapses compared with patients with stable clinical phases [117-119]
Complement factor H	Serum	Regulates the formation and function of complement factors C3 and C5. Concentrations are increased in patients with progressive forms of MS and patients with relapsing-remitting MS in relapse. The same group of researchers did an in-house clinical replication of the findings [120]
B-cell activating factor (BAFF)	CSF and PBMC	Member of the TNF superfamily with a role in B-cell homeostasis. Concentrations in CSF are increased in patients with MS compared with patients with other neurological disorders, and concentrations are higher in patients with secondary progressive MS and RRMS during relapses than during remittance [116, 121].

Table 2. Biomarkers associated with relapses (based on: Comabella and Montalban, Body fluid biomarkers in multiple sclerosis, Lancet Neurology, 2014 [3])

Clinical factors

Clinical symptoms at onset

No differences in MS risk are found between different clinical syndromes (e.g. optic neuritis, transverse myelitis, brainstem syndrome)[59, 62]. It was suggested that patients with optic neuritis had a more benign disease course[122], but this is only true for optic neuritis patients without lesions on brain MRI [58]. A more severe disease course in patients with multifocal onset has been mentioned by some [63] but is not confirmed by others [60, 122].

Lifestyle factors (smoking, obesity, alcohol, stress, diet)

Smoking is a risk factor for CIS and MS, and one study has shown that, in CIS patients, it is also a risk factor for conversion to CDMS, with a hazard ratio of 1.83 for smokers [123]. Of note, in this study, only patients with white matter lesions on MRI and positive oligoclonal bands in CSF were included. Nothing is known about alcohol use or obesity and CDMS risk in CIS patients. In RRMS patients, smoking has been associated with conversion to secondary progressive MS, but the association with relapses is not clear. An important clinical factor associated with relapses is stress. The occurrence of stressful life events has been shown to be a risk factor for an MS attack in several studies [124, 125].

Recently, a study has been published that showed that high salt intake was associated with an increased relapse rate [126]. Indeed, it has recently been shown that salt could stimulate the development of pro-inflammatory Th17 cells and worsen EAE [127], so there is also biological plausibility that increased dietary salt intake might represent an environmental risk factor for MS.

MRI

An abnormal brain MRI scan is found in about 60% CIS patients [128] and this is the most important predictive factor for CDMS after CIS: the risk for CDMS is 72-82% if the baseline scan shows abnormalities compared to 21-24% in patients with a normal MRI [58, 129]. Therefore, MRI abnormalities have been included in the diagnostic criteria for MS since 2001[130], and since the latest revisions in 2010, the diagnosis of MS can be made in some patients after only 1 attack if the MRI scan fulfills certain criteria [131]. Typical MS lesions are ovoid shaped lesions that are hyperintense on T2, and localized in the following regions of the CNS: periventricular, juxtacortical, infratentorial [132], in the spinal cord [133], or the corpus callosum [134]. Both the number of lesions and the localization of the lesions have predictive value. In addition to T2 lesions, gadolinium enhancement of lesions is associated with increased risk of conversion to CDMS [59, 62]. Gadolinium enhancing lesions are regarded as 'active' lesions, and the simultaneous presence of both enhancing and non-enhancing lesions can account for dissemination in time according to the most recent diagnostic criteria for MS [131]. Black holes, which are lesions with low signal on T1, are sometimes regarded as markers of more severe demyelination and axonal damage, but their predictive value has been shown to be low [135]. Emerging MRI techniques include diffusion tensor imaging (DTI), magnetization transfer imaging, MR spectroscopy and functional MR imag-

ing; these are less often used in clinical practice and their prognostic potential has not been convincingly demonstrated yet [136].

A special situation is the so-called radiologically isolated syndrome (RIS), which defines the incidental identification of white matter abnormalities suggestive for MS on MRI, in people without typical MS symptoms [137]. People with RIS are at risk to develop CIS or MS; in a large study including 451 RIS subjects, 34% had a clinical event within 5 years. Risk factors associated with clinical symptoms were younger age, male sex and spinal cord involvement [138].

Although the correlation between MRI lesion load and clinical manifestations is weak, conventional MRI techniques are frequently used as a surrogate marker for MS disease activity, both in clinical practice and in clinical trials. Gadolinium-enhancing lesions and the appearance of new T2 lesions are associated with relapses, whereas brain atrophy and T1 hypointense lesions (black holes) are associated with disease progression [139-141].

Evoked potentials

Evoked potentials were included in the revised diagnostic criteria for MS by Poser et al. in 1983 [142] as paraclinical evidence of an MS lesion. However, their prognostic value, alone or in combination with MRI, was found to be modest [143]

OCT

Optical Coherence Tomography (OCT) is an ophthalmologic instrument that measures the retinal nerve fiber layer (RNFL) thickness with a method analogous to ultrasound, but using near-infrared light instead of sound. The RNFL contains ganglion cell axons that are continuous with the optic nerve and are unmyelinated, which makes them suitable for measuring neurodegeneration. It is known that after optic neuritis, RNFL thickness rapidly decreases, but also in MS patients that did not suffer from optic neuritis, RNFL thickness has also been shown to decrease over time [144, 145]. However, it is not yet known if OCT measures can be used to predict conversion to CDMS in CIS patients.

Cerebrospinal fluid oligoclonal bands

IgG oligoclonal bands (OCBs) in the cerebrospinal fluid are a sign of intrathecal IgG synthesis, without corresponding IgG in serum. They likely represent the humoral immune activation in the CNS of CIS patients. OCBs in the CSF of CIS patients are a known predictor for CDMS. However, they can also be present in other inflammatory diseases such as sarcoidosis or SLE [146]. A recent meta-analysis including 2685 CIS patients showed that OCBs were present in 69.5% of patients. Of OCB-positive patients, 64.1% later reached CDMS, compared with 22.6% of OCB-negative patients (OR 9.88, $p<0.00001$)[147]. In the latest version of the diagnostic criteria for MS, CSF OCBs can no longer be used to reduce the MRI requirements for dissemination in space [131]. Although several studies have shown that OCBs are predictive for CDMS independent of MRI measures [148, 149], the additional diagnostic value of OCBs with the latest imaging criteria remains to be confirmed.

Scope of this thesis

This thesis focuses on the prediction of the next attack in MS. In patients with a first demyelinating event, CIS, the next attack is disease-defining, diagnosing the patients with clinically definite relapsing-remitting MS. In patients with RRMS, we investigated factors that influence the relapse risk. This is important, because 50% of relapses cause increased sustained disability [8].

In the first part of this thesis, prognostic factors in CIS patients are described. First, in **chapter 2**, the accuracy of the most recent revisions to the McDonald diagnostic criteria for MS was assessed by applying these criteria to our cohort of CIS patients. In **chapter 3 and 4**, we aimed to find new prognostic markers, both clinical and biomarkers. In **chapter 3**, we investigated the prevalence and prognostic value of fatigue in CIS patients, because this is a well-known feature of MS but little was known about fatigue in CIS patients. In this chapter, we also evaluated vitamin D-levels in CIS patients. In **chapter 4**, proteomics analysis of cerebrospinal fluid of CIS patients and controls was performed, to look for biomarkers that could differentiate CIS patients from controls and differentiate monophasic CIS patients from future MS patients. In **chapter 5**, various predictors are integrated into a predictive model for the diagnosis of clinically definite MS in CIS patients.

The second part of this thesis investigates prognostic factors in patients with relapsing-remitting MS. There was accumulating evidence for a role of vitamin D in MS, but little was known about its association with clinical disease course in MS patients. Therfore, in **chapter 6**, serum vitamin D levels were measured at regular time intervals in patients with RRMS to investigate their association with relapse risk. In **chapter 7**, vitamin D levels are measured during pregnancy in MS patients and healthy women, and the association of pregnancy vitamin D levels and the risk for a postpartum relapse is investigated. In **chapter 8**, a possible association of vitamin A levels and relapse rate is investigated. **Chapter 9** discusses osteopontin plasma levels and their association with relapse risk.

The main findings of this thesis and an interpretation of the results are discussed in **chapter 10**.

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Chapter 2

Application of the 2010 revised criteria for the diagnosis of multiple sclerosis to patients with clinically isolated syndromes.

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Eur J Neurol, 2013

Abstract

Background and purpose

Recently, the McDonald criteria for the diagnosis of multiple sclerosis (MS) have been revised, with the aims to diagnose earlier and to simplify the use of brain MRI. To validate the 2010 revised criteria they were applied to a cohort of patients with clinically isolated syndromes (CIS).

Methods

In all, 178 CIS patients were followed from onset. Test characteristics were calculated after 1, 3 and 5 years and compared between the 2005 and 2010 revised criteria. The time to diagnosis of the 2005 and 2010 criteria was compared using survival analysis and the log-rank test. Clinical evidence for dissemination in space and time was the gold standard for clinically definite MS (CDMS).

Results

During follow-up, 76 patients converted to CDMS (mean time to conversion 23.9 months). At 1 year, the specificity and accuracy of the 2005 criteria were a little higher than those of the 2010 criteria (98.0% and 98.4% vs. 86.3% and 88.5%). However, at 5 years, differences completely disappeared (specificity 85.7% and accuracy 93.3% for both criteria). MS diagnosis could be made significantly faster with the 2010 criteria (P = 0.007). Using the 2010 criteria, in 19% of patients the diagnosis could already be made at baseline.

Conclusions

By applying the 2010 revised criteria a diagnosis of MS can be made earlier, whilst prediction of disease progression is maintained. This validation brings along great advantages, for treatment possibilities as well as patient counselling.

Introduction

Since the publication of the Poser criteria in 1983 [1], the diagnosis of multiple sclerosis (MS) has been based on the demonstration of dissemination in space (DIS) and time (DIT): evidence that the disease has affected more than one part of the central nervous system on more than one occasion. Patients with MS often present with a first episode of symptoms suggestive for demyelination, such as optic neuritis or transverse myelitis. This first episode is called clinically isolated syndrome (CIS). MS is a disabling disease that affects especially young people in the prime of their lives. Patients with CIS face a very insecure future, not knowing whether or not they will go on to develop MS and, if they do, how the disease course will be [2]. To be able to advise CIS patients as well as possible, it is very important that a diagnosis of MS can be made quickly and accurately. Also, the possibility of starting appropriate treatment early may be beneficial for disease outcome in MS [3].

For these reasons, several attempts have been made over the years to adjust the criteria in such a way that the diagnosis of MS can be made earlier and more easily. Several revisions to the diagnostic criteria have been published by the International Panel on the Diagnosis of MS. In 2001, the MRI scan was added as an important diagnostic tool that could be used for the criterion of DIS [4]. In 2005 [5] and 2010 [6], the criteria were revised again. The revised diagnostic criteria of 2005 and 2010 for relapsing-remitting MS are shown in Table 1.

In the latest revisions of 2010, aims were to simplify the use of brain MRI for the diagnosis of MS and to allow for earlier diagnosis in different populations. As can be seen from the table, with these criteria the diagnosis of MS can sometimes already be made in patients with only a single attack (CIS), after a single baseline brain MRI scan. If these criteria prove to work well, this would be a huge progress in the diagnostics of MS. However, the risk of false positive diagnoses is not imaginary. For example, in a study by Chard et al. [7] it has been shown that 10%-15% of patients that were diagnosed with MS based on the 2005 MRI criteria never had a clinical second attack in up to two decades.

To investigate what the 2010 criteria really add to the existing diagnostics in MS, their accuracy was investigated and compared with the 2005 criteria by applying them to our cohort of CIS patients.

Patients and methods

Patients

Patients were included in the neurology outpatient clinic of the Erasmus MC University Hospital, a tertiary referral center for MS patients. In our center, all patients aged 18–50 years with a first episode suggestive for demyelination are followed prospectively if they give informed consent (approved by the Erasmus MC ethics committee). For the present study, all patients were included who had experienced a first episode suggestive of demyelination, had a baseline MRI scan performed within 3 months of symptom onset and had at least 1 year of follow-up. All patients were clinically assessed at baseline and thereafter seen regularly for reassessment. Exacerbation was defined as a worsening of existing symptoms or the appearance of new symptoms lasting for more than 24 h, after a period of more than 30 days of improvement or stability, confirmed by neurological examination [8]. A temporary neurological deterioration associated with fever was not considered as an exacerbation. Clinically definite MS (CDMS) was diagnosed when there was clinical evidence for DIS and DIT as described by Poser and colleagues [1]. This was used as the gold standard for the diagnosis of multiple sclerosis. At baseline, MRI and laboratory tests were performed to rule out alternative diagnoses. This was repeated during follow-up if necessary. Patients with alternative diagnoses were not included in the analyses.

Procedures

All brain MRI scans were performed on 1.5 T scanners with a standard head coil (Philips, Best, The Netherlands, or General Electric, Milwaukee, WI, USA). Scans typically consisted of an axial T1-weighted sequence, an axial spin echo proton-density weighted (PDW) and a T2-weighted sequence, and an axial fluid-attenuated inversion recovery (FLAIR) sequence, with 2–5 mm images. Post-gadolinium T1- weighted sequences were added by the radiologist on indication in patients with T2 lesions suggestive of demyelination. Since spinal cord scans were not systematically performed, these were not included in the analysis.

Baseline scans were scored for Barkhof-Tintoré criteria and Swanton criteria for DIS, and for criteria for DIT according to the 2010 revisions to the McDonald criteria. These criteria are described in Table 1.

Statistical methods

The following test characteristics were calculated:

Sensitivity:
The proportion of patients with the disease who have a positive test result. This was calculated as true positives/(true positives + false negatives).

Specificity:
The proportion of patients without the disease who have a negative test result. This was calculated as true negatives/(true negatives + false positives).

Positive predictive value (PPV):
The proportion of patients who have the disease amongst the patients with positive test results. This was calculated as true positives/(true positives + false positives).

Accuracy:
The proportion of true results of a test. This was calculated as (true positives + true negatives)/(true positives + false positives + true negatives + false negatives).

Test characteristics for the criteria for DIS of the 2005 and 2010 criteria and for DIT of the 2010 criteria based on the baseline scan were calculated after 1, 3 and 5 years of follow-up. For the calculations regarding MRI criteria for DIT at baseline according to the 2010 criteria, only patients for whom post-gadolinium images were available or scans that showed no abnormalities were taken into account (n = 114).

	McDonald 2005	**McDonald 2010**
DIS	a) 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) ≥3 of the 4 Barkhof-Tintoré criteria fulfilled: - ≥9 T2 hyperintense lesions or 1 gadolinium-enhancing lesion - ≥3 periventricular lesions - ≥1 juxtacortical lesion - ≥1 infratentorial lesion (1 spinal cord lesion can substitute for 1 brain lesion and spinal cord lesions can be included in the total T2 lesion count)) c) ≥2 T2 lesions plus positive CSF (isoelectric focusing evidence of oligoclonal bands and/or elevated IgG index)	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 (Swanton criteria): - Periventricular - Juxtacortical - Infratentorial - Spinal cord (symptomatic lesions in patients with brainstem or spinal cord syndrome are excluded)
DIT	a) ≥2 attacks separated by a period of at least one month b) 1 gadolinium-enhancing lesion ≥3 months after CIS if not at the site corresponding to CIS c) A new T2 lesion compared with a previous scan obtained ≥30 days after CIS	a) ≥2 attacks separated by a period of at least one 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

Table 1. Overview of revisions of the diagnostic criteria for MS and. DIS= dissemination in space; DIT= dissemination in time; CNS= central nervous system; CSF= cerebrospinal fluid; CIS= clinically isolated syndrome; MRI= magnetic resonance imaging. Based on 2005 revisions to the McDonald criteria [5] and 2010 revisions to the McDonald criteria [6].

Test characteristics of the 2010 diagnostic criteria (DIS + DIT) were calculated after 1, 3 and 5 years of follow-up and compared with the 2005 criteria. For these calculations, only patients who had at least one follow-up scan were included in the analyses (n = 61).
Time to diagnosis with the 2005 and 2010 criteria was analysed using Kaplan–Meier survival analyses and compared with a log-rank test. Survival analysis included all patients with a follow-up scan and/or a diagnosis of MS according to 2010 criteria. Statistical analyses were performed using SPSS version 17.0 for Windows.

Results

Patients

In all, 187 patients from our CIS cohort met the inclusion criteria. Nine patients (4.8%) were diagnosed with diseases other than MS (four neuromyelitis optica, two Leber's hereditary optic neuropathy, one chronic relapsing inflammatory optic neuropathy, one vascular, one psychogenic); these patients were not included in the analysis, which left 178 patients eligible for analysis (figure 1). Median follow-up time of the patients was 44.5 months (range 12–174).

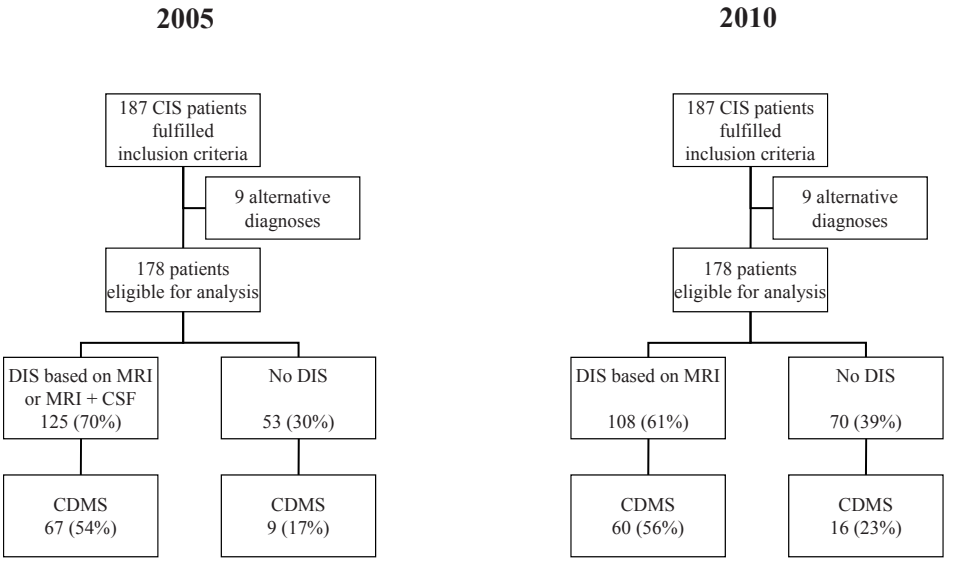

Figure 1. Flowcharts of patients in analysis and percentages of patients in the different groups that had clinically definite MS during follow up; left for 2005 revised criteria and right for 2010 revised criteria. DIS = dissemination in space, DIT = dissemination in time, CDMS = clinically definite MS.

Baseline characteristics of the included patients are shown in Table 2. Seventy-six patients (42.7%) had at least one relapse during follow-up leading to the diagnosis of CDMS. Mean time to conversion was 23.9 months (median 16.5, range 1–86). Twenty-four patients

(13.5%) received immune-modulating therapy before they had a second clinical attack. Sixty-one patients (34.3%) had at least one follow-up MRI scan.

Characteristic	N (%)		
Gender			
Male	52 (29.2%)		
Female	126 (70.8%)		
Ethnicity			
Caucasian	168 (94.4%)		
Asian	1 (0.6%)		
Black	3 (1.7%)		
Mediterranean	6 (3.4%)		
Clinical syndrome			
Optic neuritis	74 (41.6%)		
Brainstem	27 (15.2%)		
Spinal cord	39 (21.9%)		
Cerebellum	9 (5.1%)		
Cerebral hemispheres	20 (11.2%)		
Other	9 (5.1%)		
CDMS	76 (42.7%)		
Age at onset (years)	mean 32.7	median 33.0	(range 16-54)
Time to baseline MRI (weeks)	mean 4.8	median 4.0	(range 0-13)
Follow-up time (months)	mean 51.7	median 44.5	(range 12-174)
Time to CDMS (months)	mean 23.9	median 16.5	(range 1-86)

Table 2. Characteristics of patients (n=178). CDMS = clinically definite MS

DIS and DIT criteria of baseline scans

When comparing the 2005 and 2010 criteria for DIS, the following could be seen in our cohort (see also Fig. 1 and Table 3A). Seventy-two of 178 patients (40.4%) fulfilled the Barkhof-Tintoré criteria; 125 patients (70.2%) fulfilled DIS 2005 criteria including cerebrospinal fluid (CSF). Sixty-seven (53.6%) of the DIS 2005 positive patients converted to CDMS during follow-up. Nine (17.0%) of 53 patients who did not fulfil DIS 2005 criteria converted to CDMS during follow-up. The sensitivity of the DIS 2005 criteria was 88.2% (95% CI 80.9–95.4) and the specificity was 43.1% (95% CI 33.5–52.8). The PPV for DIS 2005 was 53.6% (95% CI 44.9–62.3) and the accuracy 62.4% (95% CI 55.2– 69.5).

The Swanton criteria for DIS as used in the 2010 revisions to the McDonald criteria were fulfilled in 108 (60.7%) of our patients. Sixty (55.6%) of them developed CDMS during follow-up. Sixteen (22.9%) of 70 patients who did not fulfil the Swanton DIS 2010 criteria converted to CDMS. The sensitivity of the Swanton criteria was 79.0% (95% CI 69.8–88.1) and the specificity was 52.9% (95% CI 43.3–62.6). The PPV and accuracy were 55.6% (95% CI 46.2–64.9) and 64.0% (95% CI 57.0–71.1), respectively.

When examining the 2010 criteria for DIT, it was found that 36 (31.6%) of 114 patients fulfilled the criteria at baseline. Sixteen (44.4%) of them converted to CDMS during follow-up. Twenty-seven (34.6%) of 78 patients who did not fulfil the new DIT criteria converted to CDMS. The sensitivity of the DIT criteria was 37.2% (95% CI 22.8–51.7) and the specificity was 71.8% (95% CI 61.4–82.3). Thirty-three (18.5%) patients fulfilled criteria for both DIS and DIT 2010 at baseline.

2010 vs. 2005 revised criteria

Of the 61 patients for whom a follow-up scan was available, 43 (70.5%) had the diagnosis MS according to the 2005 criteria, with a mean time to diagnosis of 26.1 months (SD 22.4). Forty-four (72.1%) patients received the diagnosis MS according to the 2010 criteria, with a mean time to diagnosis of 23.6 months (SD 24.0). Thirty-eight of those patients had a second clinical attack during follow-up leading to CDMS. Mean time to CDMS was 27.9 months (SD 23.9).

At 1 year of follow-up, 58.8% of patients who were diagnosed with MS according to the 2010 criteria had had a second clinical attack leading to CDMS. At 3 years, this was 86.4%. At 5 years of follow-up, 18 of 30 patients (60.0%) were diagnosed with MS according to the 2010 criteria. At this time, 16 (53.3%) patients had CDMS; so at 5 years two patients (6.7%) who had the MS diagnosis based on 2010 criteria would still never have had a second clinical attack. However, one of these two patients had a second clinical attack at 5 years and 4 months after CIS. The PPV of the 2010 criteria increases from 58.8% at 1 year to 86.4% at 3 years and 88.9% at 5 years. The specificity at 1, 3 and 5 years is 86.3%, 87.5% and 85.7%, respectively. The specificity (and thus also the number of false positives) of the 2010 criteria at 5 years is the same as for the 2005 criteria. The test characteristics are shown in Table 3B.

The survival curves of time to diagnosis with the 2005 and 2010 criteria are depicted in figure 2; the main difference is the much larger number of diagnoses made at baseline with the 2010 criteria, with a steep drop at this point. Time to diagnosis between the two methods differed significantly (P = 0.007).

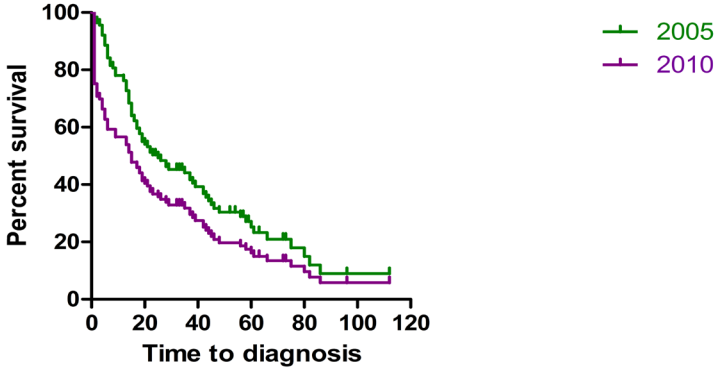

Figure 2. Survival curve of time to diagnosis with both criteria: 2010 criteria allow for earlier diagnosis.

A				B	
	DIS 2005 (Barkhof- Tintoré + CSF)	DIS 2010 (Swanton)	DIT 2010 Baseline MRI	2005 DIS + DIT (n=61)	2010 DIS + DIT (n=61)
Sensitivity					
1 yr (n=178)	92.3% (82.1-100)	80.8% (65.6-95.9)	62.5% (38.8-86.2)		
3 yr (n=110)	92.3% (83.9-100)	84.6% (73.3-95.9)	52.4% (31.0-73.7)		
5 yr (n=54)	84.4% (71.8-97.0)	84.4% (71.8-97.0)	61.5% (35.1-88.0)		
Total f-up (n=178)	88.2% (80.9-95.4)	79.0% (69.8-88.1)	37.2% (22.8-51.7)		
Specificity					
1 yr (n=178)	33.6% (26.1-41.1)	42.8% (34.9-50.6)	73.5% (64.7-82.2)	98.0% (94.2-100)	86.3% (76.8-95.7)
3 yr (n=110)	31.0% (20.2-41.7)	47.9% (36.3-59.5)	76.7% (64.1-89.4)	91.7% (80.6-100)	87.5% (74.3-100)
5 yr (n=54)	31.8% (12.4-51.3)	54.6% (33.7-75.4)	90.9% (73.9-100)	85.7% (67.4-100)	85.7% (67.4-100)
Total f-up (n=178)	43.1% (33.5-52.8)	52.9% (43.3-62.6)	71.8% (61.4-82.3)	78.3% (61.4-95.1)	73.9% (56.0-91.9)
PPV					
1 yr (n=178)	19.2% (12.3-26.1)	19.4% (12.0-26.9)	27.8% (13.2-42.4)	90.9% (73.9-100)	58.8% (35.4-82.2)
3 yr (n=110)	42.4% (31.9-52.9)	47.1% (35.5-58.8)	52.4% (31.0-73.7)	90.5% (77.9-100)	86.4% (72.0-100)
5 yr (n=54)	64.3% (49.8-78.8)	73.0% (58.7-87.3)	88.9% (68.4-100)	88.9% (74.4-100)	88.9% (74.4-100)
Total f-up (n=178)	53.6% (44.9-62.3)	55.6% (46.2-64.9)	50.0% (30.8-69.2)	88.4% (78.8-98.0)	86.4% (76.2-96.5)
Accuracy					
1 yr (n=178)	42.1% (34.9-49.4)	48.3% (41.0-55.7)	71.9% (63.7-80.2)	98.4% (95.2-100)	88.5% (80.5-96.5)
3 yr (n=110)	52.7% (43.4-62.1)	60.9% (51.8-70.0)	68.8% (57.4-80.1)	95.4% (89.1-100)	93.0% (85.4-100)
5 yr (n=54)	63.0% (50.1-75.8)	72.2% (60.3-84.2)	75.0% (57.7-92.3)	93.3% (84.4-100)	93.3% (84.4-100)
Total f-up (n=178)	62.4% (55.2-69.5)	64.0% (57.0-71.1)	58.8% (49.7-67.8)	91.8% (84.9-98.7)	90.2% (82.7-97.6)

Table 3A and B. Test characteristics of criteria for DIS and DIT of baseline scans (A) and for 2005 and 2010 criteria as a whole (B). For the latter only patients for whom a second scan was available are taken into account. Test characteristics are shown at 1, 3 and 5 years of follow-up and total follow-up. PPV = positive predictive value. DIS = dissemination in space. DIT = dissemination in time.

Discussion

In this study the performance of the new diagnostic criteria for MS was investigated. The criteria for DIS and DIT used in the 2005 and 2010 criteria in our cohort of 178 CIS patients were also tested with an average follow-up time of over 4 years.

Many studies have calculated test characteristics for the Barkhof-Tintoré criteria [9–18]. To be able to better compare DIS criteria in the 2005 and 2010 revisions to the diagnostic criteria for MS, the test characteristics for DIS 2005 were calculated including both Barkhof-Tintoré criteria and CSF analysis. In doing so, a relatively high sensitivity (88.2%) for DIS 2005 was found compared with other studies, but a lower specificity (43.1%) [9–18]. The DIS 2010/Swanton criteria showed a somewhat lower sensitivity (79.0%) and a higher specificity (52.9%) compared with DIS 2005. Overall, the DIS criteria of 2005 and 2010 performed

similarly in the prediction of CDMS (accuracy 62.4% and 64.0%). The new criteria for DIT had a reasonable specificity (71.8%) but a low sensitivity (37.2%), in our study even somewhat lower than in the study by Gomez-Moreno et al. [18].

When comparing the 2005 and 2010 criteria taking both DIS and DIT into account, a somewhat lower specificity was found for the 2010 criteria (73.9%) compared with the 2005 criteria (78.3%), and a similar PPV (86.4% vs. 88.4%). However, it should be noted that the PPV for the 2010 criteria strongly increases with follow-up time, whereas it remains stable for the 2005 criteria (as shown in Table 3): at 1 year, specificity and PPV of the 2005 criteria were a little higher than those of the 2010 criteria, but at 5 years differences were completely gone. This reflects the fact that in many patients a diagnosis can be made much earlier with the 2010 criteria, whilst some of them will have their second clinical attack only many years later. So, the longer the follow-up time is, the better the test characteristics for the 2010 criteria get. In our cohort, after 5 years only two patients got a diagnosis of MS with the new criteria but did not experience a second clinical attack ('false positives'). One of them did have a second clinical attack but after 5 years and 4 months of follow-up, so the number of false positives would decrease even further with increasing follow-up time.

At 5 years of follow-up, the number of false positives for both diagnostic methods is the same, but diagnosis was made significantly faster with the 2010 criteria. For a serious disease with such a considerable impact on one's life as MS, it is of great importance to have as few false positives as possible. However, it is also very valuable to be able to give a patient some certainty early in a disease process that brings already many uncertainties with it. Also, an early diagnosis allows for earlier treatment, which may be beneficial for disease outcome [3], although it might be debatable if this is really an advantage for patients who turn out to have a mild disease course.

Since the introduction of the 2010 revisions, the CSF examination is no longer included in the diagnostic criteria for relapsing-remitting MS. However, as is acknowledged, CSF can still be important to evaluate alternative diagnoses [6,19]. This was not tested in this study because of the low number of patients with alternative diagnoses in our cohort. Those patients were excluded from the analyses.
There are some shortcomings to our study. Not all patients underwent spinal cord MRI and not all patients had a follow-up MRI scan performed. For this reason, test characteristics for the 2005 and 2010 criteria as a whole were calculated in the subgroup of patients for whom a second scan was available. A small number of patients (13.5%) received immune-modulating therapy before a second attack. These 'high risk' patients were not excluded from this study because they would probably have provided more bias if they were excluded than now being included. In this cohort, the frequency of diagnoses other than MS was low (4.8%). In this situation, specificity (reflecting the proportion of patients without the disease that have a negative test result) functions to differentiate between monophasic and progressive disease, more than between MS and other diagnoses. To be noted, other studies testing diagnostic criteria for MS have also excluded alternative diagnoses [14,15,20]. It remains questionable whether the criteria that work well in such cohorts retain their specific-

ity when applied to populations in general hospitals. At least for the Swanton criteria for DIS, this seems to be the case [21]. Still, it should be emphasized, especially for use in more general patient populations, that it is always necessary to rule out alternative diagnoses first.

In a cohort of 178 patients it was shown that the diagnosis of MS could be made easier and faster with the 2010 revised criteria compared with the 2005 criteria. One other study applied the 2010 criteria to a cohort of CIS patients [18] and, although in this study no follow-up scans were included, it also confirmed the value of the new criteria. As recent posters at the ECTRIMS congress showed (e.g. [22]), the new criteria are starting to be validated globally. In our cohort, in a substantial number of CIS patients (33; 18.5%) the diagnosis could already be made at baseline. Test characteristics of the 2010 and 2005 criteria are similar, but because test characteristics of 2010 criteria increase with follow-up time, those criteria might perform better when tested in a cohort with an even longer follow-up time. The fact that the diagnosis of MS can be made earlier with the 2010 criteria is a great advantage, giving CIS patients at least a glimpse of certainty after a life-changing event.

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Chapter 3

Fatigue at time of CIS is an independent predictor of a subsequent diagnosis of multiple sclerosis

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Abstract

Objective

Fatigue is a common, disabling symptom of multiple sclerosis (MS), but little is known about fatigue in patients with clinically isolated syndrome (CIS), often the presenting symptom of MS. We aimed to investigate the prevalence and severity of fatigue in patients with CIS, and its association with a diagnosis of clinically definite MS (CDMS).

Methods

127 patients were consecutively included in our ongoing prospective CIS study. At baseline, clinical, demographic, laboratory and MRI data were collected, and fatigue severity was assessed using Krupp's Fatigue Severity Scale (FSS); fatigue was defined as FSS≥5.0. Fatigue scores were compared with scores of 113 healthy controls and with scores from the literature. The association of fatigue with CDMS was calculated using Cox regression models.

Results

The mean FSS of patients with CIS was 4.3, similar to MS patients, and significantly higher than that of healthy individuals ($p<0.001$). Fatigue prevalence in patients with CIS (46.5%) was significantly higher than in controls ($p<0.001$). Fifty-two patients (40.9%) reached CDMS during follow-up. Fatigue was associated with a diagnosis of CDMS in univariate analysis (HR 2.6, 95% CI 1.5 to 4.6) and in multivariate analysis correcting for sex, age, neuroanatomical localisation of CIS, 25-OH-vitamin D, anxiety, depression, MRI dissemination in space and gadolinium enhancement (HR 4.5, 95% CI 1.9 to 10.6).

Conclusions

Already at the stage of CIS, fatigue is a very common symptom, with a severity similar to fatigue in MS patients. This fatigue seems unrelated to the type or severity of the attack. Importantly, we found that fatigue was an independent predictor of a subsequent diagnosis of MS.

Introduction

Fatigue is a very common and disabling symptom in patients with multiple sclerosis (MS), reported by over 75% of MS patients at some point in the course of the disease [1–3]. Nevertheless, the mechanism of fatigue in MS patients remains poorly understood. The proposed causes range from altered cerebral activation or influences and effects of pro-inflammatory cytokines to sleep disorders or depression[4]. Even less is known about fatigue among patients with a clinically isolated syndrome (CIS), often the presenting symptom of MS.

In this study, we aimed to investigate the prevalence and severity of fatigue in patients with CIS, and its association with a diagnosis of clinically definite MS (CDMS).

Low vitamin D has been associated with fatigue in several conditions, such as systemic lupus erythematosus (SLE)[5,6] and traumatic brain injury[7], and is considered to be involved in the development and disease course of MS[8–10]. Therefore, a second aim of this study was to investigate if fatigue was associated with the vitamin D status of patients with CIS. To do this, fatigue, 25-OH-D concentrations (the metabolite best reflecting vitamin D status) [11] and other clinical parameters were measured in our ongoing prospective multicenter CIS study.

Methods

Participants

Patients were consecutively included in our ongoing prospective CIS study, the PROUD study (Predicting the Outcome of a Demyelinating event). This observational study is a multicenter study of our tertiary referral center for MS in collaboration with several regional hospitals. Patients with CIS suggestive of MS are included if they give informed consent and fulfil the following inclusion criteria: age between 18 and 50 years, inclusion within 6 months after symptom onset and no serious comorbidity. At baseline, clinical and demographic data are collected, an MRI is performed and fatigue is assessed. For the present investigation, we applied the following supplementary inclusion criterion: time between fatigue assessment and blood sampling less than 2 months. Patients with alternative diagnoses were excluded from the analyses, as well as patients with comorbidity likely to cause fatigue, other than depression. All patients were followed prospectively and were seen regularly for clinical re-assessment. The Ethics Committee of the Erasmus MC University Medical Center Rotterdam approved the study protocol and informed consent was obtained from all participants.

To compare the prevalence and severity of fatigue between patients with CIS and controls, we used data from a group of 113 healthy controls included previously in our center[12]. These controls were volunteers recruited from hospital personnel, relatives or friends of patients visiting the outpatient clinic, and volunteers unfamiliar with the study. They had declared themselves to be healthy and not taking any medication that could contribute to fatigue. We also compared the fatigue of patients with CIS with healthy controls and MS patients from the literature[13,14].

Definitions

Exacerbation was defined as a worsening of existing symptoms or the appearance of new symptoms lasting for more than 24 h, after a period of more than 30 days of improvement or stability, confirmed by neurologic examination[15]. A temporary neurological deterioration associated with fever was not considered as an exacerbation. All patients in this study were termed CIS, and also included patients who fulfilled the 2010 McDonald criteria. CDMS was diagnosed when there was clinical evidence for dissemination in space and time as described by Poser et al.[16].

Instruments and clinical data

Fatigue was assessed using Krupp's Fatigue Severity Scale (FSS)[17]. This is a self-administered questionnaire that is widely used and has been validated for use in patients with MS[13,14,17]. It has nine items and seven possible responses per item, ranging from 1 (strong disagreement) to 7 (strong agreement). The mean value of the nine items is the final score. Fatigue is defined as an FSS score of ≥5.0 [18–20].

Since fatigue is known to be associated with depression, we also measured depression, using the Hospital Anxiety and Depression Scale (HADS). The HADS is a self-administered questionnaire consisting of 14 items measuring symptoms of anxiety (7 items) and depression (7 items)[21,22].

Neuroanatomical localization of CIS was determined at baseline and divided into the following groups: optic nerve, spinal cord, brain stem, other and multiregional.

Measurement of 25-OH-D

Concentrations of 25-OH-D were determined by a radioimmunoassay method (DiaSorin, USA) using an extraction method. The inter-assay variation coefficient at a concentration of 62 and 109 nmol/L is 11.6% and 10.3%, respectively. The intra-assay variation coefficient at the levels is 5.7% and 6.6%, respectively.

MRI

All brain MRIs were performed on 1.5 T scanners with a standard head coil (Philips, Best, the Netherlands, or General Electric, Milwaukee, Wisconsin, USA). Scans typically consisted of an axial T1-weighted sequence, an axial spin echo proton density-weighted and T2-weighted sequence, and an axial fluid-attenuated inversion recovery sequence, with 2–5 mm images. Post-gadolinium T1-weighted sequences were added by the radiologist on indication in patients with T2 lesions suggestive of demyelination. Scans were scored for dissemination in space according to the 2010 McDonald criteria (≥1 lesion in at least two of the following areas: periventricular, juxtacortical, infratentorial or spinal cord)[23] and for gadolinium enhancement. All brain MRIs were performed within 3 months of symptom onset.

Statistical analysis

Fatigue was analyzed both as a continuous variable (FSS) and as a dichotomous variable (yes/no with FSS 5.0 as a cut-off value). Comparison of continuous variables between groups was done using Student t test or ANOVA. Student t test was also used to compare the mean FSS of patients with CIS with values from the literature. Dichotomized variables were compared using the $\chi 2$ test. To calculate the association of two continuous variables, correlation analysis was used. The association of fatigue with the diagnosis of CDMS was analyzed using survival analysis with univariate and multivariate Cox regression models. In these analyses, 25-OH-D levels were divided into two groups (low: <50 nmol/L, and high: >50 nmol/L). Anxiety and depression were included in the model as dichotomous variables. All calculations were done using SPSS V.21 for Windows.

Results

Participants

Of the 137 patients in our ongoing CIS study who fulfilled the inclusion criteria, 7 were excluded from the analyses because of alternative diagnoses and 3 were excluded from the analyses because of comorbidity likely to cause fatigue (1 Crohn's disease, 1 untreated hypothyroidism and 1 panhypopituitarism). This left 127 patients for the analyses: baseline characteristics of patients and 113 healthy controls are depicted in table 1. During follow-up, 52 patients (40.9%) met the criteria for CDMS, with a mean time to diagnosis of 21.1 months (SD 17.0). Blood samples for vitamin D measurement were available for 104 of 127 patients.

	CIS-patients n=127	Controls n=113
Sex (nr of females), n(%)	98 (77.2%)	54 (47.8%)
Age (years) mean (SD)	34.0 (8.8)	54.2 (14.8)
Follow-up (months) mean (SD)	35.4 (19.3)	
FSS mean (SD)	4.3 (1.9)	2.9 (1.1)

Table 1. Baseline characteristics and FSS outcome for CIS patients and healthy controls.

Fatigue

Fifty-nine patients (46.5%) suffered from fatigue, and the mean FSS for patients with CIS was 4.3 (SD 1.9). In the control group, fatigue was significantly less prevalent (5.3%, $p<0.001$) and less severe (mean FSS 2.9 (SD 1.1), $p<0.001$). Similarly, in healthy individuals from the literature, the mean FSS was 3.00 (SD 2.24)[13] and 3.31 (SD 1.38),[14] which was significantly lower than in patients with CIS (both $p<0.001$). The FSS of our patients with CIS was similar to that of the MS patients in the cited studies[13, 14] (mean 4.66, SD 1.64 and mean 4.81, SD 1.46, both $p>0.05$). FSS scores did not depend on gender or age.

The mean FSS did not differ significantly between patients with different neuroanatomical localizations of CIS. FSS scores did not depend on the time between symptom onset and filling out of the questionnaires.

Fatigue was not associated with 25-OH-D levels: neither with FSS as a continuous variable, nor with fatigue as a dichotomous variable. FSS was not associated with age, nor with MRI measures (number of T2 lesions, gadolinium enhancement, and normal versus abnormal MRI). FSS correlated with both anxiety (r=0.44, p<0.001) and depression (r=0.51, p<0.001).

In univariate analysis, fatigue was associated with a diagnosis of CDMS both as a continuous variable (HR 1.3, 95% CI 1.1 to 1.6) and as a dichotomous variable (HR 2.6, 95% CI 1.5 to 4.6). Thirty-two (61.5%) of 52 patients who reached CDMS suffered from fatigue, versus 27 (36.0%) of 75 patients who did not reach CDMS. Patients suffering from fatigue had a shorter time to diagnosis than non-fatigued patients: 32 months in fatigued patients versus 54.8 months in non-fatigued patients (Log Rank test: p=0.001). The Kaplan-Meier curve of time to CDMS for patients with and without fatigue is shown in figure 1.

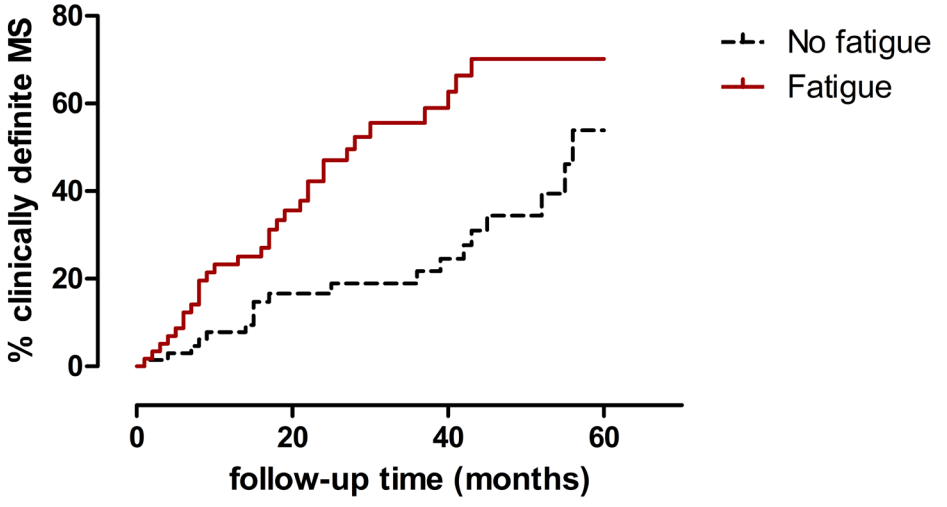

Figure 1. Kaplan-Meier curves for time to CDMS for patients with and without fatigue (FSS ≥5.0) . Log Rank test: p=0.001.

25-OH-D

The mean 25-OH-D concentration of patients with CIS was 67.6 nmol/L (SD 34.4). 25-OH-D levels showed a seasonal fluctuation with the peak level in July and nadir in January. Thirty-one patients (24.4%) had low vitamin D levels(<50 nmol/L). We found no association between 25-OH-D levels and a diagnosis of CDMS (HR for the low-level group 1.5, 95% CI 0.8 to 2.8). Since in a recent study[10] an increased risk for CDMS was found for only the lowest 10% of 25-OH-D concentrations, we also analyzed the risk of CDMS for 25-OH-D divided into 10 equal groups, but we did not find an increased risk in the lowest 25-OH-D level group, not even when men and women were analyzed separately. 25-OH-D levels were also not associated with time to CDMS, MRI measures and presence of anxiety or depression.

Multivariate analysis

In a multivariable model correcting for sex, age, localization of symptoms, vitamin D, anxiety, depression, number of T2 lesions and gadolinium enhancement, the association of fatigue with CDMS was significant, with an HR for CDMS in fatigued patients of 4.5 (95% CI 1.9 to 10.6). Also in the multivariable model, low 25-OH-D levels were not associated with CDMS (HR 0.9, 95% CI 0.3 to 2.1). In fact, fatigue turned out to be the most significant predictor for CMDS in the model. HRs are shown in table 2.

Variable		n	Events	Hazard ratio (95% CI)	P-value
Age	<30	52	21	1 (ref)	-*
	30-40	40	16	0.8 (0.3-1.9)	0.63
	>40	35	15	0.4 (0.1-1.2)	0.09
Sex	Male	29	8	1 (ref)	-
	Female	98	44	1.3 (0.6-3.1)	0.52
DIS criteria McDonald	Not fulfilled	45	12	1 (ref)	-
2010	Fulfilled	70	34	1.7 (0.7-4.4)	0.27
Gadolinium	No	45	16	1 (ref)	-
enhancement	Yes	31	19	1.8 (0.7-5.0)	0.22
Vitamin D	<50	31	16	0.9 (0.3-2.1)	0.75
	>50	73	26	1 (ref)	-
Fatigue	No	68	20	1 (ref)	-
	Yes	59	32	4.5 (1.9-10.6)	0.001
Anxiety	No	79	31	1 (ref)	-
	Yes	45	20	0.9 (0.4-2.2)	0.89
Depression	No	97	39	1 (ref)	-
	Yes	27	12	1.7 (0.6-5.0)	0.34
Symptom localization	Optic nerve	42	14	1 (ref)	-
	Spinal cord	35	13	1.0 (0.3-3.1)	0.98
	Brain stem	12	6	0.4 (0.1-1.1)	0.25
	Multiregional	22	11	3.2 (1.1-9.4)	0.03
	Other	15	8	1.3 (0.4-5.0)	0.67

*p for trend 0.22

Table 2. Hazard ratios for clinically definite MS according to the multivariate analysis. Events = a diagnosis of CDMS

Discussion

In this study, we show that fatigue is a common symptom in patients with CIS, with almost half of patients (46.5%) suffering from it. The prevalence and the severity of fatigue in patients with CIS are significantly higher than in healthy persons, and similar to the fatigue in MS patients. Importantly, we found that fatigue is associated with a greater risk of CDMS,

even independent of MRI measures. The mean time to diagnosis is significantly shorter in patients suffering from fatigue than in non-fatigued patients.

There are several possible explanations for fatigue in patients with CIS. First, it is known that fatigue correlates with neurological disability[24], so the fatigue in patients with CIS could be related to the attack itself. However, one would expect this fatigue to differ among patients with different neuroanatomical localizations of CIS (e.g., lower FSS in patients with optic neuritis) or lesion loads, and to improve along with amelioration of symptoms; we did not find any association with neuroanatomical localization, nor with lesion load, nor with time from symptom onset. Second, fatigue could be due to the psychological impact of getting a possible diagnosis of MS. Indeed, we found an association with anxiety and depression, a phenomenon that has been shown previously[25]. Nevertheless, correction for anxiety and depression in the multiple regression analysis did not influence the association between fatigue and CDMS. Third, fatigue could be related to the second attack, which would explain the association with CDMS. However, as the mean time to the second attack in patients who reach a diagnosis of CDMS is 21.1 months, this does not seem very likely. We favor a fourth explanation: that the fatigue of patients with CIS is the MS-related fatigue presenting itself already at the moment of CIS, independent of the type or severity of CIS. One previous study[26] has shown that fatigue sometimes precedes other symptoms in MS and another study[27] described fatigue in early MS; however, in both studies, fatigue was not studied separately in patients with CIS. Although fatigue is very frequently seen in all subtypes of MS[28], its pathophysiology is still not well understood. It is likely to be multifactorial, with major roles for inflammatory cytokines associated with the disease combined with CNS dysfunction and secondary mechanisms such as sleep disorders or depression[4,28]. We did not find associations of fatigue with MRI measures. In this study, we also tested the hypothesis that vitamin D is involved in fatigue, but we did not find any evidence for this in patients with CIS. Also, we could not confirm the earlier described association between low vitamin D concentrations in patients with CIS and risk of CDMS[10,29]. Here, a lack of power cannot be ruled out, but the association, if it exists, will be small as the HR is only 1.2.

Fatigue is a serious disabling symptom of MS, with a negative impact on quality of life. Our study is the first to show that already at the stage of CIS fatigue is very common, with a severity similar to fatigue in MS patients. This fatigue is unrelated to the type or severity of the attack. In addition, we found that fatigue was predictive for definite MS, independent of vitamin D and known predictors of a subsequent diagnosis of MS such as the McDonald 2010 criteria.

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Chapter 4

Decreased neuro-axonal proteins in CSF at first demyelinating event

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In preparation

Abstract

Background

There is a substantial need for biomarkers in patients with clinically isolated syndrome (CIS), the first presenting symptom of multiple sclerosis (MS), for several reasons. First, biomarkers could provide more insight into the pathophysiology of the early events leading to MS, which is still largely unknown. Second, the course of the disease after CIS is very heterogeneous and not well predictable, so biomarkers could improve patient counselling and enable early treatment in the right patients.
In recent studies we have shown the usefulness of cerebrospinal fluid (CSF) proteomics studies for the detection of pathologically relevant proteins for multiple sclerosis (MS).

Objectives

To compare the proteome of CIS patients and controls to find disease-related proteins.
To investigate the proteins associated with a first demyelinating attack and with progression to clinically definite MS.

Methods

Proteomics analysis was performed in CSF samples of 47 CIS patients with clinical and MRI data collected within 2 months after symptom onset , and 45 controls. CSF samples were enzymatically digested and subsequently measured by LC-MS/MS on a LTQ-Orbitrap mass spectrometer. The mass spectra were analyzed and differential abundance of the identified proteins between groups were investigated.

Results

A total of 3159 peptides were identified, relating to 485 proteins. Only 1 protein was significantly more abundant in CSF of CIS patients than in controls: Ig kappa chain C region. Thirty-five proteins were lower in CIS patients than controls, most of them with functions in nervous system development and function (such as contactin 1, contactin 2 and neuronal cell adhesion molecule). These 35 proteins were even lower in patients with the highest MRI lesion loads. We observed no significant differences in specific peptide abundance levels between patients who did and did not reach a diagnosis of clinically definite MS.

Conclusions

In conclusion, we found no difference in protein abundance relating to disease progression, but we did observe a remarkably lower abundance of neuro-axonal proteins in patients with a first demyelinating event. It remains to be determined whether this is a reflection of an MS predisposing gray matter disturbance, or rather a result of disease pathology itself.

Introduction

Multiple sclerosis (MS) is a common chronic, disabling disease of the central nervous system (CNS). Although its pathophysiology is still not completely clear, it seems to result from an interplay of genetic susceptibility and environmental exposure[1]. In most MS patients, the disease starts with a subacute episode of symptoms (such as optic neuritis, transverse myelitis or brainstem syndrome) called clinically isolated syndrome (CIS). However, only 30-70% of CIS patients will eventually develop MS[2]. In some patients the diagnosis of MS can already be made after the first attack, based on the most recent diagnostic criteria[3], but in the majority of CIS patients those criteria are not met, and it is hard to predict who will develop MS and who will not. The discovery of biomarkers could lead to better predictors of disease course in CIS patients, and could also shed new light on the pathophysiologic mechanisms of MS.

In recent studies, we and others have shown the usefulness of cerebrospinal fluid (CSF) proteomics studies for the detection of pathologically relevant proteins for MS[4-8].

In the present study we aimed to identify novel markers for a first demyelinating event and further MS disease progression, using advanced mass spectrometry techniques.

Methods

Participants

Patients were included at the neurology outpatient clinic of the Erasmus MC University Hospital, a tertiary referral center for MS patients. In our center, all patients aged 18–50 years with a first episode suggestive for demyelination are followed prospectively if they give informed consent (approved by the Erasmus MC ethics committee). For the present study, we included patients with CSF samples, clinical and MRI data collected within 2 months after symptom onset.

Clinically definite MS (CDMS) was diagnosed if there was clinical evidence for dissemination in space and time as described by Poser and colleagues.[9]

Controls were ‘symptomatic controls’ as defined in [10]; persons who underwent lumbar puncture at the outpatient clinic of our hospital for reasons other than neuroinflammatory diseases; these were negative neurosyphilis (40.0%), acute headache without neurological disease after follow up (e.g. no subarachnoid hemorrhage, no inflammation or idiopathic intracranial hypertension, 20.0%) and other (e.g. back pain, muscle pain without neurological disease, 40.0%).

Sample preparation, mass spectrometer measurements and analysis

CSF samples were taken from patients and controls and immediately centrifuged for 10 minutes at 3000 rpm to discard cells and cellular elements. The samples were subsequently used for routine CSF diagnostics, which included quantification of total protein concentra-

tion. The remaining volume of the samples was aliquoted and stored at -80°C, where they remained until sample preparation for this study.

From each CSF sample, 20 µL was added to 20 µL of 0.2% Rapigest (Waters, Milford, MA) in 50 mM ammonium bicarbonate buffer. After 30 minutes incubation periods with 1,4-dithiothreitol (60 °C) and, subsequently, iodoacetamide (37 °C), 4 µL of 0.1 µg/µL gold-grade trypsin (Promega, Madison, WI)/3 mM Tris-HCl (pH 8.0) was added to each sample. The samples were incubated overnight at 37 °C. To adjust the pH of the digest to pH < 2, trifluoroacetic acid (TFA) was added to the mixture prior to the final incubation step at 37°C for a duration of 45 minutes to stop the enzymatic digestion reaction.

Mass spectrometry measurements were carried out on a Ultimate 3000 nano LC system (Dionex, Germering, Germany) online coupled to a hybrid linear ion trap/Orbitrap mass spectrometer (LTQ Orbitrap XL; Thermo Fisher Scientific, Germany). Five µL digest (i.e. 2 µL CSF) were loaded on to a C18 trap column (C18 PepMap, 300 µm ID ×5 mm, 5 µm particle size, 100 Å pore size; Dionex, The Netherlands) and desalted for 10 minutes using a flow rate of 20 µL/min 0.1% TFA. Then the trap column was switched online with the analytical column (PepMap C18, 75 µm ID ×150 mm, 3 µm particle and 100 Å pore size; Dionex, The Netherlands) and peptides were eluted with following binary gradient: 0%–25% solvent B in 120 minutes and 25%–50% solvent B in further 60 minutes, where solvent A consist of 2% acetonitrile and 0.1% formic in water and solvent B consists of 80% acetonitrile and 0.08% formic acid in water. Column flow rate was set to 300 nL/min. For mass spectrometry detection a data dependent acquisition method was used: high resolution survey scan from 400–1800 Th. was performed in the Orbitrap (value of target of automatic gain control AGC 106, resolution 30,000 at 400 m/z; lock mass was set to 445.120025 u (protonated (Si(CH3)2O)6). Based on this survey scan the 5 most intensive ions were consecutively isolated (AGC target set to 104 ions) and fragmented by collision-activated dissociation (CAD) applying 35% normalized collision energy in the linear ion trap. After precursors were selected for MS/MS, they were excluded for further MS/MS spectra for 3 minutes.

The raw data was pre-processed using the Progenesis LC-mass spectrometry software package (version 4.0, Nonlinear Dynamics, Newcastle-upon-Tyne, United Kingdom). Peptides were identified and assigned to proteins by exporting features, for which MS/MS spectra were recorded, using the Bioworks software package (version 3.2; Thermo Fisher Scientific, Germany; peak picking by Extract_msn, default settings). The resulting .mgf file was submitted to Mascot (version 2, Matrix Science, London, United Kingdom) for identification to interrogate the UniProt-database (release 2013_07; taxonomy: Homo sapiens, containing 20265 sequences). Only ions with charge states between +2 and +8 were considered and only proteins with at least two unique peptides (Mascot ions sore > 25, (i.e. a peptide probability cut off value of 0.01)) assigned to them were accepted as true identifications. Modifications: carbamidomethylation of cysteine was set as fixed and oxidation of methionine as variable modification, allowing a maximum of 2 missed cleavages. Mass tolerance for precursor ions was set to 10 ppm and for fragment ions at 0.5 Da. The Mascot search results were imported back into the Progenesis software to link the identified peptides to

the detected abundances of these peptides. The peptide abundances were normalized to the total ion current to compensate for experimental variations using an algorithm available in the analysis software. Subsequently the data were exported in Excel format.

All CSF samples were tested for blood contamination by checking for the presence of the blood-specific proteins hemoglobin and apolipoprotein B-100; none were found to be contaminated.

The reproducibility of the measurement procedure was tested by measuring a single CSF digest multiple times over the course of the experiment. This sample was measured after every six CSF samples and analysis of these runs showed no significant changes in machine function over the course of the study.

Statistical analysis

The raw abundances of all identified peptides were compared between the groups of samples by performing a non-paired 2-tailed t-test on all individual peptides. Proteins of which 50% or more of the peptides had a low p-value in this t-test ($p<0.05$), and 25% of the peptides of the protein had a very low p-value ($p<0.01$) were deemed to be significantly differentially abundant between the two groups. An additional condition for proteins to be deemed significant was that 75% or more of the peptides must be altered in the same direction between the groups.

The following clinical variables were used in the analyses: conversion to CDMS (dichotomous), sex (dichotomous), age (dichotomous; older or younger than median, i.e. 33 years), optic neuritis vs. other symptom localization, and positive CSF defined as the presence of oligoclonal bands and/or IgG-index ≥ 0.68 (dichotomous). The following MRI parameters were used: normal MRI vs. abnormal MRI (defined as ≥ 1 T2 hyperintense lesion), gadolinium enhancement (dichotomous), fulfilling of criteria for dissemination in space according to 2010 McDonald criteria (dichotomous), and the number of T2 hyperintense lesions (in three groups: 0 lesions, 1-9 lesions, >9 lesions).

Protein function and network analysis

We searched for the functions of the identified proteins in online databases (www.uniprot.org) and previous reports using Pubmed. Identified proteins were uploaded in the Ingenuity Pathways Analysis service (Ingenuity Systems, Redwood City, CA, USA) for network analysis of their biological context.

Variable	Patients n=47	Controls n=45	P
Sex, nr of females (%)	34 (72.3%)	20 (44.4%)	0.006
Age (years)	34.8 (8.9)	32.6 (9.4)	0.26
Protein concentration (g/l)	0.40 (0.16)	0.34 (0.15)	0.09
Albumin concentration (g/l)	0.22 (0.11)	0.20 (0.10)	0.39
CIS type			
- Optic neuritis	17 (36.2%)		
- Transverse myelitis	13 (27.7%)		
- Brainstem	13 (27.7%)		
- Cerebral	2 (4.3%)		
- Cerebellar	2 (4.3%)		
CDMS	21 (44.7%)		
Time between CIS and LP (weeks)	3.7 (2.7)		
Time to CDMS (months)	31.4 (26.3) range 1-103		
Follow-up (months)	49.1 (36.6) Range 0-141		

Table 1. Baseline characteristics of patients and controls as mean (SD) unless otherwise specified.

Results

Participants

Fifty-four patients and 45 controls with other neurological diseases fulfilled the inclusion criteria. Seven CIS samples were not measurable and therefore excluded from the analysis (2: very low signal and 5: bad retention time alignment in the analysis software). This left 47 patients and 45 controls for the analysis. During a mean follow-up time of more than 4 years, twenty-one CIS patients reached a diagnosis of clinically definite MS, with a mean time to diagnosis of 31.4 months (SD 26.3). No significant differences were observed between patients and controls in terms of age, total protein and albumin concentrations. Among the patients were more females than among controls. Characteristics of patients and controls are shown in Table 1.

Protein identification

The Mascot database search against the human subset of the Uniprot database resulted in the identification of 3159 peptides that related to 485 proteins.

CIS versus controls

Using stringent statistical criteria, we found 36 proteins significantly different between patients and controls. Only 1 protein was significantly more abundant in the CSF of CIS patients than in controls: Ig kappa chain C region. Thirty-five proteins were significantly less abundant in CIS. Most identified proteins that were lower in CIS than controls have functions in the development and maintenance of the nervous system. Uploading of the differentially

abundant proteins in the Ingenuity Pathway analysis software showed 21 of the differentially abundant proteins in a network relating to nervous system development and function, 8 proteins in a network relating to cell-to-cell signaling and interaction, and 6 proteins in a network relating to cell morphology, cell death and survival. The identified proteins that were differentially abundant in CSF of CIS patients and controls are shown in table 2. Comparing these identified proteins between patients with the highest MRI T2 lesion load (>9 lesions) and patients without any MRI abnormalities, we found the majority lower in the group with the highest lesion load, although this did not reach significance. This is also shown in table 2.

CIS patients: MS or no MS

There were no significant differences in peptide abundance between patients who did and did not reach a diagnosis of clinically definite MS during follow up. Next we questioned whether the CSF peptide abundances from 'fast converters', i.e. patients who reached CDMS within two years, were different from the monophasic CIS group, but this was not the case. We observed no differentially abundant proteins related to gender, nor between patients with normal versus abnormal MRI, nor between patients with and without gadolinium enhancement.

When comparing patients with and without positive CSF (defined as the presence of oligoclonal bands or IgG index ≥ 0.68), we found, unsurprisingly, peptides relating to multiple immunoglobulin proteins: Ig kappa chain V-I region, Ig kappa chain C region, Ig gamma-1 chain C region, Ig kappa chain V-III region, and Ig kappa chain V-II region.

When looking at type of CIS, we found that only 2 proteins were significantly more abundant in patients with brainstem symptoms compared to patients with optic neuritis: these were pleiotrophin (PTN) and extracellular superoxide dismutase [Cu-Zn] (SOD3); the latter was also on the list of differentially abundant proteins in patients and controls (table 2).

Discussion

We performed Orbitrap-based high-resolution proteomics analysis of CSF in, to our knowledge, the largest set of CIS patients so far. We found several proteins that were clearly differentially abundant in CIS patients and controls. Interestingly, these were for the main part markers related to gray matter development and function that we found to be less abundant in CIS.

Consisted with our findings, some of the identified proteins have been implicated in MS before. For example, contactin 2, which is a neuronal membrane protein expressed at the juxtaparanodal region, and contactin 1, which is thought to be involved in the formation of axon connections, have been found to be differentially abundant in CIS patients and controls by others[11, 12]. Contactin-2 directed autoimmunity is identified in multiple sclerosis patients and immunity directed against its rat homologue TAG-1 mediates gray matter pathology in animal models[13].

The finding that many gray matter related proteins and no myelin products were differentially abundant at this early stage of demyelinating disease feeds the hypothesis that gray matter pathology is not secondary to demyelination but might be the primary characteristic of the disease pathology. This is in line with recent work by Dhaunchak et al. in pediatric patients [14] and Schutzer et al.[12] who showed in a smaller cohort of 9 CIS patients, 12 relapsing-remitting MS patients and 6 controls that several gray matter related proteins were quantitatively different at the time of CIS. It is also congruent with natural history studies of MS showing that disease progression, which is probably caused by neurodegeneration, starts early and progresses at similar pace among all disease subtypes of MS, independent

Protein	Genetic name	Fold change	Function	MRI 0 lesions vs. >9 lesions
Pathway: Nervous system development and function				
Receptor-type tyrosine-protein phosphatase N2	PTPRN2	0.579	Implicated in development of nervous system. Involved in lipid signaling, cell signaling and membrane trafficking	Lower in >9 lesions
Neurotrimin	NTM	0.723	Neural cell adhesion molecule. Mediates effects on neurite outgrowth	No difference
Neuroendocrine protein 7B2	SCG5	0.655	Chaperone for PCSK2/PC2, which is involved in maturation of neuroendocrine peptides	Lower in >9 lesions
Superoxide dismutase [Cu-Zn]	SOD1	0.905	Destroys radicals	Lower in >9 lesions
Neurosecretory protein VGF	VGF	0.710	Involved in regulation of cell-cell interactions and synaptogenesis during nervous system maturation. Antimicrobial.	Lower in >9 lesions
Major prion protein	PRNP	0.692	Involved in neuronal development and synaptic plasticity	Lower in >9 lesions
Metalloproteinase inhibitor 2	TIMP2	0.715	Metastasis suppressor, inhibits proliferation of endothelial cells. Important in tissue homeostasis	Lower in >9 lesions
Extracellular superoxide dismutase [Cu-Zn]	SOD3	0.737	Scavenges radicals	Higher in >9 lesions
Neurocan core protein	NCAN	0.796	May modulate neuronal adhesion and neurite growth during development	No difference
Neuronal growth regulator 1	NEGR1	0.832	Involved in cell adhesion. May function as trans-neural growth-promoting factor in regenerative axon sprouting.	
Amyloid-like protein 1	APLP1	0.665	Involved in post-synaptic function, regulation of neurite outgrowth and synaptic maturation	Lower in >9 lesions

Table 2. Differentially abundant proteins in CIS-patients and controls

Cartilage acidic protein 1	CRTAC1	0.764	Unknown	Lower in >9 lesions
Contactin-2	CNTN2	0.825	Cell adhesion molecule. Contributes to the organization of axonal domains at nodes of ranvier by maintaining VGKC at the juxtaparanodal region.	
Contactin-1	CNTN1	0.756	Cell adhesion molecule. May be involved in formation of axon connections in the developing nervous system	No difference
Cadherin-2	CDH2	0.745	Calcium-dependent cell-adhesion molecule. Neuronal recognition, regulation of dendritic spine density.	No difference
Neuronal cell adhesion molecule	NRCAM	0.829	Involved in neuron-neuron adhesion. Promotes directional signaling during axonal cone growth	Lower in >9 lesions
Cell adhesion molecule 3	CADM3	0.749	Involved in cell-cell adhesion	Lower in >9 lesions
Cadherin-13	CDH13	0.910	May act as negative regulator of neural cell growth. Cell migration, phenotypic changes.	Lower in >9 lesions
Calsyntenin-1	CLSTN1	0.794	Regulates calcium concentration, intracellular transport	Lower in >9 lesions
Collagen alpha-1(I) chain	COL1A1	0.790	Component of collagen	Higher in >9 lesions
Pathway: Cell-to-cell signaling and interaction				
Ig kappa chain C region	**IGKC**	**2.050**	**Antigen binding, immune response**	**Higher in >9 lesions**
Trans-Golgi network integral membrane protein 2	TGOLN2	0.616	Involved in regulating membrane traffic to and from trans-Golgi network	Lower in >9 lesions
Calsyntenin-3	CLSTN3	0.631	May modulate calcium-mediated postsynaptic signals	Lower in >9 lesions
CD59 glycoprotein	CD59	0.664	Inhibitor of complement action	No difference

Table 2. Differentially abundant proteins in CIS-patients and controls - continued

Transmembrane protein 132A	TMEM132A	0.581	Involved in embryonic and postnatal development of the brain, resistance to cell death	No difference
Seizure 6-like protein	SEZ6L	0.759	May contribute to specialized endoplasmic reticulum functions in neurons	Lower in >9 lesions
Tyrosine-protein phosphatase non-receptor type substrate 1	SIRPA	0.739	Supports adhesion of cerebellar neurons, neurite outgrowth and glial cell attachment. Mediates phagocytosis, mast cell activation and dendritic cell activation.	Lower in >9 lesions
Proline-rich transmembrane protein 3	PRRT3	0.780	Unknown	No difference
Pathway: Cell morphology, cell death and survival				
Ribonuclease pancreatic	RNASE1	0.670	Catalyzes RNA cleavage	Lower in >9 lesions
Extracellular matrix protein 1	ECM1	0.739	Stimulates proliferation of endothelial cells, promotes angiogenesis. May be involved in CNS repair	Lower in >9 lesions
Seizure 6-like protein 2	SEZ6L2	0.756	May contribute to specialized endoplasmic reticulum functions in neurons	Lower in >9 lesions
Testican-1	SPOCK1	0.708	Involved in cell-cell and cell-matrix interactions	No difference
Seizure protein 6 homolog	SEZ6	0.824	Involved in cell-cell recognition, neuronal membrane signaling, development of appropriate excitatory synaptic connectivity, dendrite outgrowth.	Lower in >9 lesions
Glucosidase 2 subunit beta	PRKCSH	0.700	Subunit of enzyme in endoplasmic reticulum	No difference
Pathway: Other				
VPS10 domain-containing receptor SorCS3	SORCS3	0.580	Neuropeptide receptor activity	Lower in >9 lesions
Protocadherin Fat 2	FAT2	0.868	Involved in migration of epidermal cells, cerebellar development	Higher in >9 lesions

Table 2. Differentially abundant proteins in CIS-patients and controls - continued

of the duration, amount and severity of previous relapses[15]. Furthermore, MRI studies show that gray matter lesions and atrophy can already be observed in CIS patients[16, 17].

A remarkable finding of our study is, that all differentially abundant proteins between CIS patients and controls except immunoglobulin, were less abundant in CIS patients. This is of note, because if these gray matter proteins in CSF would simply result from leakage or release as a consequence of tissue injury caused by the disease process, one would expect them to be more abundant in CIS patients than in controls. The relatively stringent criteria used here for the level of significance, the amount of molecules involved in the same physiological system, all different in the same direction (decreased) leaves little room for a false positive finding. Theoretically, the control group might have had disproportionally high levels of gray matter proteins for some reason. However, as the control group consisted of patients who did not have overt CNS disease, this seems unlikely. It should be acknowledged that the controls were not fully sex-matched. However, as no differences were observed between males and females it is highly unlikely that this has affected our results.

So, why are these molecules lower in patients already early after the first manifestation of the disease? Overall there are two possibilities: the lower abundance of proteins involved in the integrity of the CNS may either precede or even predispose for a first demyelinating attack, or alternatively be the consequence of the attack and subsequent smoldering chronic disease process.

It will be impossible to study whether lower abundance of these proteins really precedes the first attack, but in this light it is tempting to hypothesize that a disturbed neuro-axonal physiology, reflected by a lower abundance of functionally relevant proteins, predisposes for a first demyelinating event. Such a phenomenon would fit at least partially an "inside-out model" for MS pathogenesis. This would be consistent with reports from others who have described a lower abundance of proteins in patients compared to controls, not only in demyelinating disease [11, 12, 14], but also in other more clearly neurodegenerative diseases such as Alzheimer's, Huntington's and Parkinson's disease[18, 19]. In a recent study by Mapstone et al.[20] in Alzheimer patients, a depletion of several proteins was found in the serum of patients in the very early stage of the disease. The authors hypothesized that this revealed the breakdown of neural cell membranes in those individuals destined to phenoconvert from cognitive intactness to Alzheimer's disease. In a similar manner, in early MS it can be hypothesized that a dysfunction in CNS gray matter physiology can be observed as a depletion of proteins essential for normal development and function in the CSF in those destined to undergo a first demyelinating event. The fact that recently discovered new MS-related genes also included several gray-matter related genes might add to the credibility of this hypothesis[21].

The alternative option for lower abundance of proteins at first attack may be that the disease process, which has already started, directly leads to lower levels. The inflammatory neuropathological process may have a dampening effect on the production of certain proteins that are important in the physiologic maintenance of the CNS. This would rather fit

an “outside-in” model for the axonal and neuronal pathology of the disease. The active inflammatory process may also consume these proteins, for example by a higher rate of elimination of proteins from the CSF by macrophages. An argument in favor of this alternative explanation is the fact that most identified proteins were even lower in patients with the highest MRI T2 lesion load. Considering MRI T2 lesions as a measure for inflammation, the protein abundances seem to decrease with increasing inflammation.

We did not find any markers that could discriminate between monophasic CIS and patients who convert to relapsing-remitting MS, not even when only the fast converters were taken into account. Some other studies did show differences between monophasic CIS and RRMS-CIS patients [12, 22, 23], although differences between CIS and healthy controls seem to be more pronounced than between RRMS and CIS patients [12]. Which factors determine if a patient remains monophasic or goes on to develop MS remains to be elucidated.

In conclusion, we found no difference in protein abundance relating to disease progression, but we did observe a remarkably lower abundance of neuro-axonal proteins in patients with a first demyelinating event. It remains to be determined whether this is a reflection of an MS predisposing gray matter disturbance, or rather a result of disease pathology itself.

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Chapter 5

A clinical prediction model for definite multiple sclerosis in patients with clinically isolated syndrome

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In preparation

Abstract

Background

Clinically isolated syndrome (CIS) is often the first manifestation of multiple sclerosis (MS). However, not all patients with CIS will go on to develop multiple sclerosis (MS).

Objective

To develop a simple and reliable prediction model for MS in patients with CIS.

Methods

CIS patients were included with age 18-50 years, inclusion within 6 months after symptom onset and no serious comorbidity. Clinical, demographic, MRI, serum and CSF parameters were collected at baseline. The outcome measure was clinically definite MS (CDMS) as defined by Poser and colleagues. A multivariable Cox regression model was created after univariate screening of candidate predictors. A simple scoring system was then constructed giving equal weight to all predictors. Model performance was quantified according to the c statistic indicating discriminative ability, with internal validation using bootstrapping techniques.

Results

Of 431 patients in the analysis, 109 fulfilled the criteria for CDMS within 2 years. The following 5 variables were found to be the main predictors of CDMS: DIS+DIT2010 (the baseline scan fulfills criteria for dissemination in time and place according to the 2010 revised McDonald criteria), corpus callosum lesion, cerebrospinal fluid oligoclonal bands, fatigue and abnormal MRI. The final model had a reasonable discriminative ability with a c statistic of 0.71. Three risk groups were created: low risk (0-1 risk factor present), intermediate risk (2-3 risk factors) and high risk (4-5 risk factors). The 5-year risk for CDMS in the low-risk group was 19% versus 56% in the intermediate-risk group and 93% in the high-risk group.

Discussion

With the proposed clinical prediction model, 3 risk groups can be distinguished, with 5-year risks for clinically definite MS ranging from less than 20% in the low-risk group to more than 90% in the high-risk group. If further validated, the proposed simple tool can assist to inform CIS patients and to support decision making regarding the early start of immunomodulatory treatment.

Introduction

Clinically isolated syndrome (CIS) is a subacute manifestation of symptoms that is often the first manifestation of multiple sclerosis (MS). However, not all patients with CIS will go on to develop MS: after 20 years, about 63% will have experienced a second attack, defining clinically definite MS (CDMS)[1]. The uncertainty about whether or not a CIS patient will develop MS is problematic both for the wellbeing of the patient[2, 3] and for the possibility to start immunomodulatory medication early in the course of the disease[4]. Therefore, worldwide efforts are being made to find predictors for the course of the disease after CIS.

One major achievement is the publication of the 2010 revisions to the McDonald criteria for the diagnosis of MS.[5] With these revised criteria, the diagnosis of MS can already be made after one attack (CIS) in a subgroup of patients with specific abnormalities on the MRI scan. However, this subgroup includes only a minority of CIS patients[6], leaving most patients still in the dark.
For this reason, we set out to make a reliable prediction model for MS in patients with CIS, aiming to make it simple and clinically useful by using parameters that are readily available.

Methods

Patients

In the Rotterdam MS Center, patients with CIS suggestive of multiple sclerosis were included who fulfilled the following inclusion criteria: age between 18 and 50 years, inclusion within 6 months after symptom onset and no serious comorbidity. At baseline, clinical and demographic data were collected, an MRI scan was performed, a blood sample was taken and fatigue was assessed. All patients were clinically assessed at baseline and thereafter seen regularly for reassessment. Patients were instructed to contact the clinic in case of a suspected exacerbation. Patients with alternative diagnoses were excluded from the analyses. The Ethics Committee of the Erasmus MC University Hospital approved the 'PROUD' (Predicting the OUtcome of a Demyelinating event) study protocol.

Candidate predictors

Candidate predictors were chosen a priori based on existing literature and clinical expertise. Age, sex, localization of first symptoms, fatigue and presence of first or second degree relatives with MS were the clinical parameters selected. For simplicity and because optic neuritis appeared to be the localization with the highest discriminating ability, this localization was chosen in the model (optic neuritis yes/no) instead of localization (optic nerve/spinal cord/ brainstem/ other). Fatigue was assessed using the Krupp's Fatigue Severity Scale (FSS) [7]. This is a self-administered questionnaire that is widely used and has been validated for use in patients with MS [7-9]. It has nine items and seven possible responses per item, ranging from 1 (strong disagreement) to 7 (strong agreement). The mean value of the nine items is the final score. Fatigue was defined as an FSS score of ≥ 5.0[10]; this variable was used in the model after examination of the continuous FSS variable showed that such dichotomization did not cause much loss of the discriminative ability.

The following MRI parameters were considered: abnormal MRI (defined as 1 or more lesions), number of T2 lesions (with the following groups: 0 lesions, 1-9 lesions, >9 lesions), gadolinium enhancement, presence of a lesion in the corpus callosum, modified Barkhof criteria (defined as at least 3 of 4 criteria fulfilled[11, 12]), Swanton criteria[13], and DIS+DIT2010 (defined as: the baseline scan fulfills criteria for dissemination in time and place according to the 2010 revised McDonald criteria[5]). In the cerebrospinal fluid (CSF), the IgG index and the presence of oligoclonal bands were assessed. A serum sample was taken at baseline for the measurement of 25-OH-vitamin D.

Outcome measure

The outcome measure in the analyses was clinically definite MS (CDMS). This was diagnosed in case of clinical evidence for dissemination in space and time as described by Poser and colleagues[14].

Statistical analyses

Time to CDMS was calculated from start of first symptoms. Patients without CDMS were considered as censored observations. The 2- and 5-year cumulative incidence of CDMS were estimated using Kaplan-Meier curves. Missing values were imputed using a multiple imputation method, which is a sophisticated method to preserve data and reduce bias in case of missing data[15]. Cox proportional hazard regression analysis was used to calculate univariate and multivariable hazard ratios. A multivariable model was created including all predictors with univariate p-values <0.2. This multivariable model was then further simplified using stepwise backward selection. Non-linearity of continuous variables was checked using restricted cubic splines. A scoring system was constructed based on the regression coefficients of the multivariable model. To further facilitate clinical application, a scoring system was developed in which predictors with similar regression coefficients were given equal weight. The discriminative ability of the model was quantified with the c statistic. The c statistic is similar to the area under the curve of a receiver-operating characteristic curve for logistic regression models and can range from 0.5 to 1.0 for sensible models. The internal validity of the model was assessed by bootstrapping techniques. To determine how over-optimistic the model was, the discriminative ability of the model was determined on the bootstrap samples and on the full model. Statistical analyses were performed using SPSS 21.0 for Windows and R statistical software.

Results

Among 497 CIS patients consecutively included, 2 patients were found to have had previous symptoms (e.g., no CIS), 52 were lost to follow up and 12 patients were excluded because of alternative diagnoses (3 neuromyelitis optica, 2 sarcoidosis, 2 chronic relapsing inflammatory optic neuropathy, 5 other). This left 431 patients for the analysis. Of these patients, 109 patients met the criteria for CDMS within 2 years of follow up. Univariate hazard ratios and 2- and 5-year risk of CDMS for the variables are shown in table 1.

Variable		n	Events	2-year risk of MS (95% CI)	5-year risk of MS (95% CI)	Hazard ratio (95% CI)
Age	<30	164	69	30.7% (23.1-38.3)	51.4% (40.8-62.0)	1 (ref)
	30-40	156	63	27.4% (20.0-34.8)	51.1% (40.3-61.9)	1.0 (0.7-1.4)
	>40	111	44	26.9% (17.9-35.9)	63.1% (49.4-76.8)	0.9 (0.7-1.3
Sex	Male	117	42	25.5% (16.9-34.1)	50.0% (36.7-63.3)	1 (ref)
	Female	314	134	29.6% (24.1-35.1)	55.7% (48.1-63.3)	1.1 (0.8-1.6)
Optic neuritis	Yes	178	62	21.2% (14.7-27.7)	43.3% (33.5-53.1)	1 (ref)
	No	252	113	33.1% (26.8-39.4)	63.0% (54.0-72.0)	1.6 (1.2-2.2)
First or second degree relatives with MS	No	187	75	29.2% (22.1-36.3)	61.9% (50.7-73.1)	1 (ref)
	Yes	26	6	22.6 (5.2-40.0)	31.2 (8.9-53.5)	0.5 (0.2-1.2)
Nr of T2 lesions	0	54	6	6.1% (0.0-12.8)	13.1% (1.7-24.5)	1 (ref)
	1-9	186	63	23.4% (16.7-30.1)	49.5% (38.7-60.3)	4.2 (1.8-9.6)
	>9	154	92	39.4% (31.4-47.4)	70.1% (60.7-79.5)	7.4 (3.2-16.9)
Abnormal MRI	No	54	5	6.1% (0.0-12.8)	13.1% (1.7-24.5)	1 (ref)
	Yes	340	155	31.1% (25.8-36.4)	60.1% (52.7-67.5)	5.6 (2.5-12.7)
Gadolinium enhancement	No	186	54	20.2% (13.9-26.5)	41.9% (31.1-52.7)	1 (ref)
	Yes	65	42	50.4% (37.5-63.3)	84.3% (70.6-98.0)	3.5 (2.3-5.2)
DIS+DIT2010	No	352	128	23.5% (18.8-28.2)	48.6% (41.2-56.0)	1 (ref)
	Yes	51	36	54.4% (39.7-69.1)	94.9% (85.5-104.3)	3.6 (2.4-5.2)
Corpus callosum lesion	No	239	68	16.7% (11.6-21.8)	40.4% (31.4-49.4)	1 (ref)
	Yes	167	98	42.3% (34.5-50.1)	73.4% (63.8-83.0)	2.8 (2.0-3.8)
3 of 4 Barkhof criteria	No	253	73	18.9% (13.8-24.0)	39.7% (31.1-48.3)	1 (ref)
	Yes	151	92	41.6% (33.2-50.0)	75.5% (66.3-84.7)	2.5 (1.9-3.5)
Swanton criteria	No	167	39	15.0% (9.1-20.9)	31.6% (21.6-41.6)	1 (ref)
	Yes	219	119	36.9% (30.2-43.6)	68.9% (60.3-77.5)	3.0 (2.1-4.3)
CSF oligoclonal bands	No	107	33	18.2% 10.4-26.0)	38.9% (26.7-51.1)	1 (ref)
	Yes	167	86	33.9% (26.3-41.5)	67.4% (57.4-77.4)	2.4 (1.6-3.6)
IgG index	<0.7	115	36	18.1% (10.7-25.5)	45.1% (32.0-58.2)	1 (ref)
	>0.7	146	80	34.2% (26.0-42.4)	63.8% (54.0-73.6)	2.0 (1.4-3.0)
25-OH-vitamin D	<50	37	19	28.1% (13.2-43.0)	58.5% (39.9-77.1)	1.3 (0.8-2.3)
	>50	90	35	24.0% (15.0-33.0)	54.1% (38.8-69.4)	1 (ref)
Fatigue	No	85	28	17.3% (9.1-25.5)	47.4% (32.3-62.5)	1 (ref)
	Yes	62	36	40.6% (28.1-53.1)	79.8% (60.6-99.0)	2.5 (1.5-4.1)

Table 1. Potential predictors for CDMS risk among 431 CIS patients.

The following variables had strong univariable effects: age, optic neuritis, number of T2 lesions, abnormal MRI, gadolinium enhancement, DIS+DIT2010, corpus callosum lesion, Barkhof criteria, Swanton criteria, oligoclonal bands, IgG index and fatigue. We found no evidence for non-linearity of the continuous variable (IgG index) in this model. After further simplification, the multivariable model contained 5 predictors: DIS+DIT2010, corpus callosum lesion, oligoclonal bands, abnormal MRI and fatigue (table 2). Using a simple scoring system with equal weight for all predictors in the multivariable model resulted in similar predictive performance compared to a more detailed scoring system. The observed c statis-

tic for the final model was 0.72, indicating reasonable discriminative ability. After correction for over-optimism this was reduced to 0.71, indicating limited over-optimism.

With this simple scoring system, 3 groups were created: a low, intermediate and high risk group (table 3). The 5-year risk for CDMS in the low-risk group was 19% versus 56% in the intermediate-risk group and 93% in the high-risk group (figure 1). The c-statistic for the simple model with 3 groups was 0.66.

Risk variables	Hazard ratios (95% CI)	Score
DIS+DIT2010	2.2 (1.4-3.3)	1
Corpus callosum lesion	1.9 (1.2-2.9)	1
Oligoclonal bands	1.7 (1.1-2.6	1
Fatigue	2.3 (1.4-3.9)	1
Abnormal MRI	2.3 (0.9-6.0)	1
CDMS risk score		0-5

Table 2. Hazard ratios and scores for the final prediction model. DIS+DIT2010= the baseline scan fulfills criteria for dissemination in time and place according to the 2010 revised McDonald criteria[5]

Number of risk factors present DIS+DIT2010, corpus callosum lesion, oligoclonal bands, fatigue or abnormal MRI	**Risk category**	**2-year risk of MS**	**5-year risk of MS**
0 or 1	Low	6.1% (1.0-11.2)	19.4% (8.0-30.8)
2 or 3	Intermediate	27.8% (21.7-33.9)	56.0% (47.2-64.8)
4 or 5	High	56.5% (45.7-68.5)	92.5 (82.7-100)

Table 3. Risk of clinically definite MS for three risk groups at 2 and 5 years after CIS.

Discussion

The uncertainty of whether or not a patient will develop MS after a first demyelinating event is problematic for patient wellbeing and hinders early treatment. We propose a simple clinical prediction model for clinically definite MS to be used in patients with CIS, based on 5 readily available clinical parameters: 1. abnormal MRI, 2. DIS+DIT2010 (the baseline scan fulfills the criteria for dissemination in space and time according to the 2010 revised McDonald criteria), 3. corpus callosum lesion, 4. CSF oligoclonal bands, and 5. fatigue. With the model, 3 risk groups can be distinguished, with 5-year risks for clinically definite MS ranging from less than 20% in the low-risk group to more than 90% in the high-risk group.

The identified variables were combined by means of a simple scoring system, giving equal weight to all parameters. In comparison with a scoring system based on the regression coefficients, this simpler scoring performed equally well. To facilitate clinical use, we advocate the use of this simpler scoring system, possibly further simplified in 3 prognostic groups.

Of the MRI parameters, a lesion in the corpus callosum is less frequently mentioned in the MS literature than number of T2 lesions, Barkhof criteria, Swanton criteria, gadolinium enhancement and DIS+DIT2010 , and it is interesting that among all other parameters, this less-frequently used parameter was most relevant. However, as the Barkhof criteria, Swanton criteria and DIS+DIT2010 criteria all consider the same lesion locations (i.e. periventricular, juxtacortical, infratentorial and spinal cord) they might be expected to cancel each other out, and the predictive value of the callosal lesion independent of Barkhof criteria has been described before[11, 16].

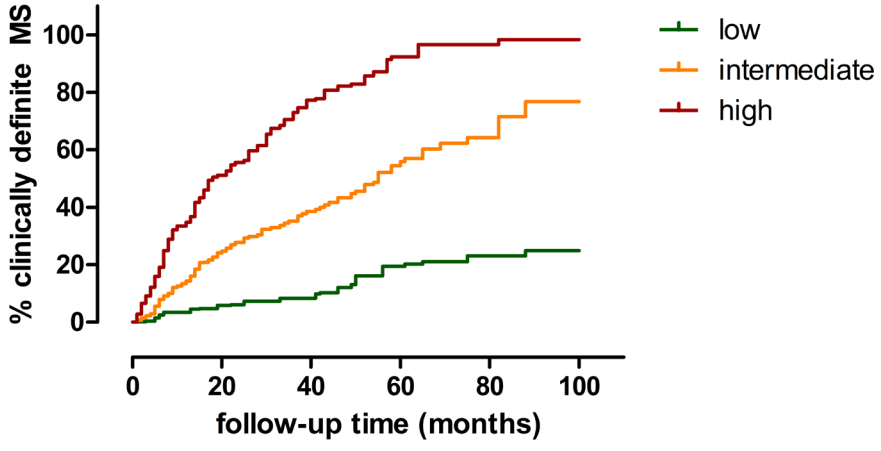

Figure 1. Cumulative incidence of CDMS for the three risk groups.

Another notable predictor is the abnormal MRI. Of course, the fact that an abnormal MRI was predictive for CDMS in CIS patients has been known for long [1, 17]. However, it is remarkable that an abnormal MRI adds to the risk of MS, even in addition to a lesion in the corpus callosum or the fulfilling of DIS+DIT2010 criteria, where the patient has an abnormal MRI per definition. Apparently, this parameter contributes to separate the high and low risk groups even further.

A relatively new predictor is fatigue, as defined by an FSS score of ≥5. Fatigue is a well-known feature of MS, but has not been extensively studied in patients with CIS. However, we recently investigated fatigue in CIS patients and found that fatigue was quite common and relatively severe, and that it was predictive of CDMS[18]. It was therefore decided to include fatigue as a parameter in this prognostic model. The fact that fatigue is a relatively strong and independent predictor even in a model including all well-known MS parameters suggests that it is related to intrinsic disease activity rather than a result of damage[18]. It also asks for more attention for this problem already at the very early stage of the disease. The FSS is a validated questionnaire that is available in many languages and can easily be filled out at the outpatient clinic.

In our model, oligoclonal bands were a significant predictor in addition to DIS+DIT2010 criteria. In the 2010 revised McDonald criteria, oligoclonal bands can no longer be used to reduce the MRI requirements for the diagnosis of MS, because the contribution of CSF status to the newest MRI criteria for DIS and DIT was never evaluated. Our results suggest that oligoclonal bands do in fact add predictive value when used in addition to these criteria, and may provide a reason to perform a lumbar puncture in CIS patients, not only to rule out other disease from the differential diagnosis, but also to predict the risk for CDMS[19].

A variable that had little predictive value was 25-OH-vitamin D. Two studies have investigated the predictive value of 25-OH-D in CIS patients before[20, 21]. The first one, in 465 patients[20], found significantly increased risks for new lesions and increased lesion volumes on MRI for patients with low 25-OH-vitamin D levels, but only a borderline increased risk for CDMS. The second study found increased risk only in women with very low 25-OH-vitamin D levels. In our study, the contribution of 25-OH-vitamin D to the model was very modest[21]. This does not exclude the possibility of a predictive value for vitamin D in CIS patients, but its effects are moderate compared to other more powerful predictors.

We chose not to include other known predictors for MS such as HLA and EBV antibody titers, because these are not easily and routinely available in all patients presenting with CIS, and would therefore make the prediction model less useful in clinical practice.

There are several limitations to our study. First, data were not fully complete in all individuals, especially on the 25-OH-D measurements and family history. Furthermore, we did not have the possibility to validate our results in an external cohort of patients yet, which is a necessary step before introducing the model in clinical practice. Internal validation revealed a relatively good discriminative ability. We started building this model with well-known predictors for MS in CIS patients, obtained after years of international investigations. Therefore it seems unlikely to find additional predictors amongst the currently available parameters that would add substantially to the discriminative ability of the currently proposed model.

This is the first clinical prediction model for CDMS in patients with CIS, distinguishing a low, intermediate and high-risk group based on widely available clinical parameters. MS and its disease course are very heterogeneous, and this model is far from perfect in separating those who will and will not develop MS. Nevertheless, if further validated, this model can be a practical tool to inform and provide support to CIS patients and support decision-making regarding the early start of immunomodulatory treatment.

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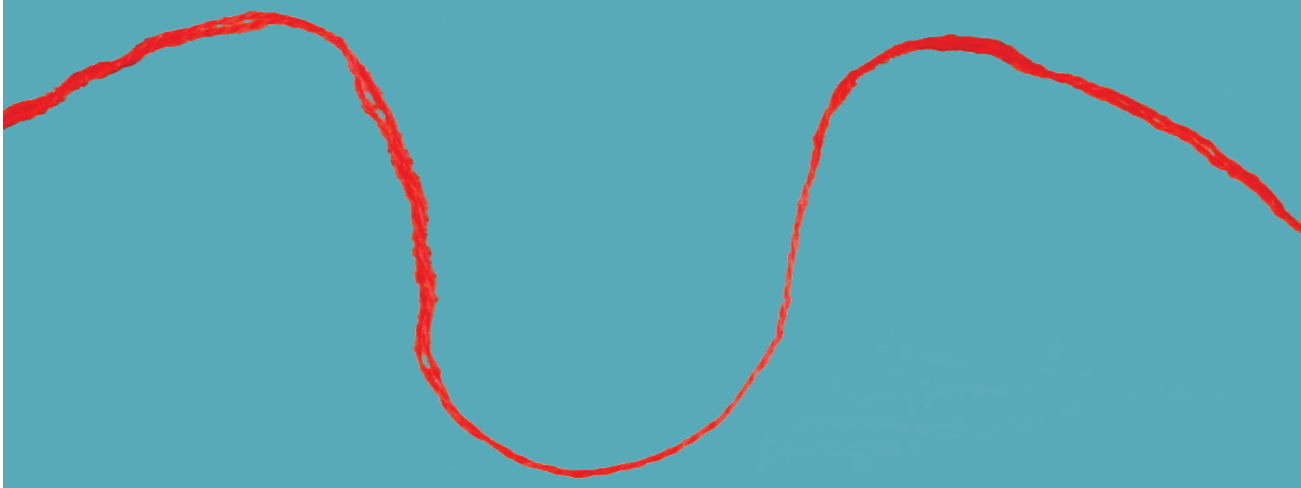
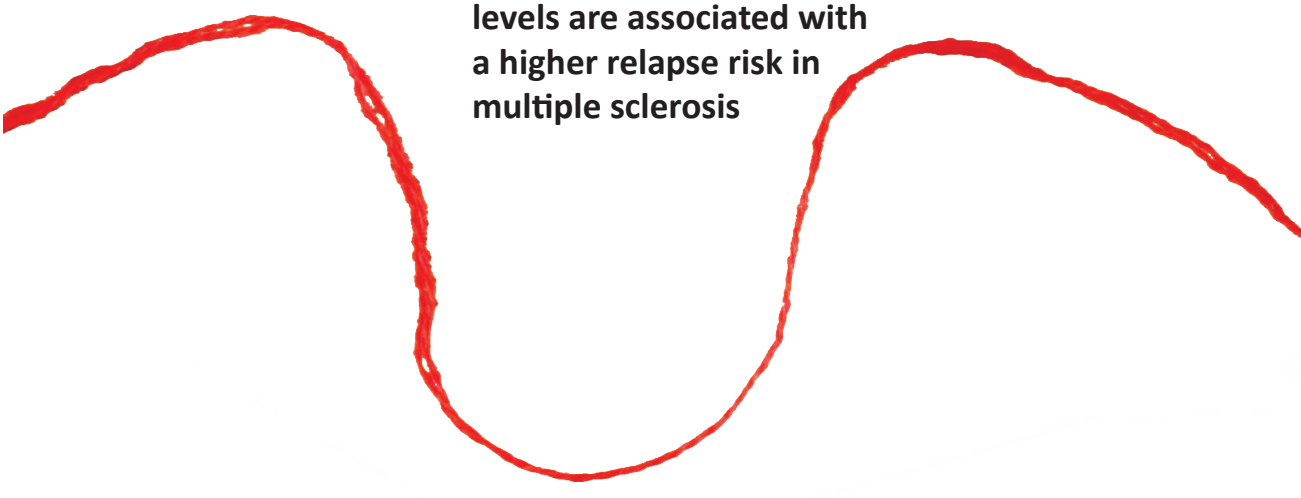
Part 2

RRMS to next attack



Chapter 6

Lower serum vitamin D levels are associated with a higher relapse risk in multiple sclerosis



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Neurology, 2012

Abstract

Objective

There is increasing evidence that vitamin D can be protective against the development of multiple sclerosis (MS), but it may also be beneficial for the clinical course of the disease. Our objective was to prospectively investigate if 25-hydroxy-vitamin D (25-OH-D) levels are associated with exacerbation risk in MS in a study with frequent serum measurements.

Methods

This was a prospective longitudinal study in 73 patients with relapsing-remitting MS. Blood samples for 25-OH-D measurements were taken every 8 weeks. Associations between 25-OH-D levels and exacerbation rates were assessed using Poisson regression (generalized estimating equations) with the individual serum levels as time-dependent variable.

Results

During follow-up (mean 1.7 years), 58 patients experienced a total of 139 exacerbations. Monthly moving averages of 25-OH-D levels were categorized into low (<50 nmol/L), medium (50–100 nmol/L), and high (>100 nmol/L) levels. Exacerbation risk decreased significantly with higher serum vitamin D levels: respective relative exacerbation rates for the medium and high level category as compared to the low-level category were 0.7 and 0.5 (p value for trend: p=0.007). The association between 25-OH-D concentrations and exacerbation rate was log linear without a threshold. With each doubling of the serum 25-OH-D concentration the exacerbation rate decreased by 27% (95% confidence interval 8%–42%, p=0.008).

Conclusions

Our finding that higher vitamin D levels are associated with decreased exacerbation risk in relapsing-remitting MS suggests a beneficial effect of vitamin D on disease course in MS. However, the possibility of reverse causality cannot be ruled out completely. Randomized intervention studies are therefore needed to investigate the effect of vitamin D supplementation in MS.

Introduction

Multiple sclerosis (MS) is a chronic disease of the CNS that starts in most patients with a relapsing-remitting disease course. The etiology of MS is multifactorial. Both genetic susceptibility and environmental exposure contribute to the pathogenesis[1]. One of the environmental factors associated with the development of MS is vitamin D[2].

Vitamin D is a group of fat-soluble prehormones, related to steroid hormones. It can be absorbed from food, but in the human body the main source for vitamin D is the production in the skin under influence of UVB light. Although the active metabolite of vitamin D is 1,25-dihydroxy-vitamin D (1,25-diOH-D), or calcitriol [3], the metabolite best reflecting the vitamin D status of the patient is 25-hydroxy-vitamin D (25-OH-D), or calcidiol[4].

A protective effect of vitamin D on the onset of MS is supported by many studies in epidemiology as well as in basic and clinical science[5–10] but recent evidence suggests that vitamin D might also influence the clinical course of the disease. Several studies report lower serum 25-OH-D concentrations during exacerbations then during remission[11–13]. So far, only one prospective study was conducted to investigate the association between serum 25-OH-D concentrations and exacerbation rate[14]. In that study, higher serum 25-OH-D concentrations were found to be associated with a lower hazard for exacerbation. However, calculations were based on only 2 measurements of serum 25-OH-D concentrations per person per year. Because serum 25-OH-D concentrations are known to be fluctuating with season, more frequent sampling would allow for a more accurate estimation of vitamin D levels. The aim of this study is therefore to investigate the association between serum 25-OH-D concentrations and disease course in relapsing-remitting MS prospectively with serum measurements every 8 weeks.

Methods

Standard protocol approvals, registrations, and patient consents

Data and samples were collected in the Rotterdam Study on Exacerbations in Multiple Sclerosis, a longitudinal prospective study in patients with relapsing-remitting MS[15]. Patients were included sequentially during an inclusion period of 1.7 years in 1997–1999. Serum 25-OH-D measurements were performed in 2010. At the time of recruitment and data collection, written informed consent was obtained from all patients. The Medical Ethical Committee of the Erasmus MC approved the study protocol.

Patients

Patients aged 18–55 years could be included in the study if they had clinically definite MS with a relapsing remitting disease course. Patients were excluded from participation if they suffered from other serious diseases.

Definitions

All patients fulfilled the McDonald criteria for the diagnosis of MS[16]. Exacerbation was defined as a worsening of existing symptoms or the appearance of new symptoms lasting for more than 24 hours, after a period of more than 30 days of improvement or stability, confirmed by neurologic examination[17]. A temporary neurologic deterioration associated with fever was not considered as an exacerbation.

Because infection is a known risk factor for exacerbations in MS, the at-risk period around infection was used as a covariate in this study[15]. Infection was defined as the appearance of coryza, sore throat, flu-like feeling, myalgia, fever, diarrhea, or a urinary infection lasting >24 hours. The at-risk period for infection was defined as the period of 2 weeks before until 5 weeks after the onset of a clinical infection, as described previously[18].

Visits, samples, and measurement of exacerbations

All patients visited the outpatient clinic of the Erasmus Medical Centre University Hospital regularly every 8 weeks. On every visit, samples for 25-OH-D measurements were taken and disability was measured using the Kurtzke Expanded Disability Status Scale (EDSS)[19]. Furthermore, patients were instructed to contact the study center when they experienced symptoms of infection or neurologic impairment. In case of a suspected infection or exacerbation, an additional visit to the outpatient clinic was arranged within 3 days, to confirm the infection or exacerbation. Serum samples were stored at -80°C until serum 25-OH-D measurement, performed in 2010.

Measurement of 25-OH-vitamin D

Concentrations of 25-OH-D have been determined by a RIA method (DiaSorin, USA) using an extraction method. The interassay variation coefficient at a concentration of 62 and 109 nmol/L is 11.6% and 10.3%, respectively. The intra-assay variation coefficient at the levels is 5.7% and 6.6%, respectively. Only serum samples taken at the regular 8-weekly visits were used for 25-OH-D measurements; samples taken during exacerbation visits were not evaluated.

Statistical analysis

Graphical display of individual serum 25-OH-D concentrations vs calendar time indicated a sinusoidal pattern. Therefore a sinusoidal model was used to describe the 25-OH-D concentrations along calendar time:

$$\text{Log10(concentration)} = a + b \sin(2\pi[t + c].$$

In this formula the parameter a represents the mean logarithmically transformed concentration, b represents the amplitude, c denotes the phase of the sinusoidal curve, and t represents the day of the year the blood sample was taken. Concentrations were transformed logarithmically in order to get approximately normal distributions.

Nonlinear regression was used to estimate the parameters in the regression model and the parameters a and b were allowed to differ from patient to patient, i.e., individual's levels and amplitudes were taken as random effects in the regression model. SAS software (PROC NLMIXED; SAS Institute Inc., Cary, NC) was used in the calculations.

To assess the association between individual serum 25-OH-D concentrations and the incidence rate of exacerbations, the follow-up time for each patient, which covered a maximum period of 2.3 years, was split into intervals of 1 week each. For each of these intervals the number of exacerbations was determined. The individual exacerbation rate was assumed to depend on the geometric mean serum 25-OH-D concentration during the previous 4 weeks. To obtain the latter mean level, for each individual the 4-weekly levels between measurements were determined using interpolated values. In case a planned sample was missing for a patient, and his last as well as the next observation occurred more than 4 weeks earlier, respectively later, than the date at which patients were estimated to have their maximal or minimal value in view of the sinusoidal pattern, this interpolation was not done and interpolated values were set missing. This was done to avoid weekly estimates of the concentration that were likely to be either too low or too high in view of the sinusoidal pattern. During the period of 4 weeks following an exacerbation, the individual was not considered at risk for another exacerbation. A priori it was decided to categorize the geometric mean serum levels of the 4 preceding weeks into a low level (<50 nmol/L), a medium level (50–100 nmol/L), and a high level (>100 nmol/L). The relationship between serum 25-OH-D concentrations and the incidence rate of exacerbations was assessed using Poisson regression models with the geometric mean individual serum levels as a time-dependent variable. Generalized estimating equations with an exchangeable covariance matrix for the subsequent study weeks were used in the calculations (SAS PROC GENMOD). The effect of other factors including gender, age, EDSS, number of exacerbations before study entry, and use of interferon-during the study was also estimated using a multivariable generalized linear model with a log-link function. To evaluate whether the occurrence of exacerbations after entry into study affected the dropout rate, Cox regression was used with the cumulative number of exacerbations as a time dependent variable. P=0.05 (2-Sided) was considered the limit of significance in all analyses.

Results

Patient characteristics

A total of 73 patients were included in this study. Mean follow-up time of all patients was 1.7 years (range 0.4 –2.3). Nine patients had dropped out of the study before intended completion date (1 patient because of participation in another study; for the 8 other patients no reason was known). All patients were Dutch Caucasians; baseline characteristics of included patients are shown in table 1. In addition to the 13 patients who used interferon- β at study entry, 15 patients started to use interferon-β during follow-up; 28 patients used interferon-β at some point during on average 56 weeks. Vitamin supplements were not widely used among the patients: 5 patients used vitamin B complex and 2 used multivitamin pills not containing vitamin D. One patient took calcium supplements; it was unknown if those contained vitamin D.

Variable (n=73)	Mean (range)	Std. Deviation
Age, years	39.4 (19-55)	9.1
Disease duration, years	5.2 (0-25)	4.1
Disability (EDSS) *	2.5 (0-6.0)	1.6
Exacerbations in previous 2 years	2.2 (1-8)	1.3
Variable (n=73)	**Proportion (%)**	
Gender, F/M	77% / 23%	
IFN use, N/Y	82% / 18%	

* EDSS is a method for quantifying disability in MS, ranging from 0.0 (normal neurological exam) to 10.0 (death due to MS)

Table 1. Characteristics of patients at baseline.

A total of 58 patients experienced a total of 139 exacerbations during this study. Median time from inclusion to first exacerbation was 20 weeks. Thirty-three patients had more than 1 exacerbation; the average exacerbation rate was 1.2 per year (range 0–6.2 per year). Three patients experienced a sixth exacerbation during follow-up.

Serum 25-OH-D concentrations

Serum 25-OH-D concentrations showed a seasonal sinusoidal fluctuation (figure 1). Serum concentrations were high in summer and low in winter, with peak levels in mid-August and nadirs in mid-February. The fitted sinusoidal curve resulted in a geometric mean 25-OH-D concentration of 69 nmol/L. There was a considerable variation in mean levels between patients (coefficient of variation 41%).

Association between serum 25-OH-D concentrations and exacerbation risk

Exacerbation rates were found to decrease with increasing levels of serum 25-OH-D con-

centrations (figure 2A). For the low (<50 nmol/L), medium (50 –100 nmol/L), and high (>100 nmol/L) category the monthly exacerbation rates were 0.15 (95% confidence interval [CI] 0.12– 0.20), 0.10 (95% CI 0.08–0.14), and 0.07 (95% CI 0.05– 0.12), respectively. The risk of an exacerbation was significantly increased in the group with low serum 25-OH-D concentrations (<50 nmol/L) compared to the group with high serum concentrations (>100 nmol/L). Rate ratios for the low and medium group were 2.0 and 1.4, respectively (p for trend = 0.007).

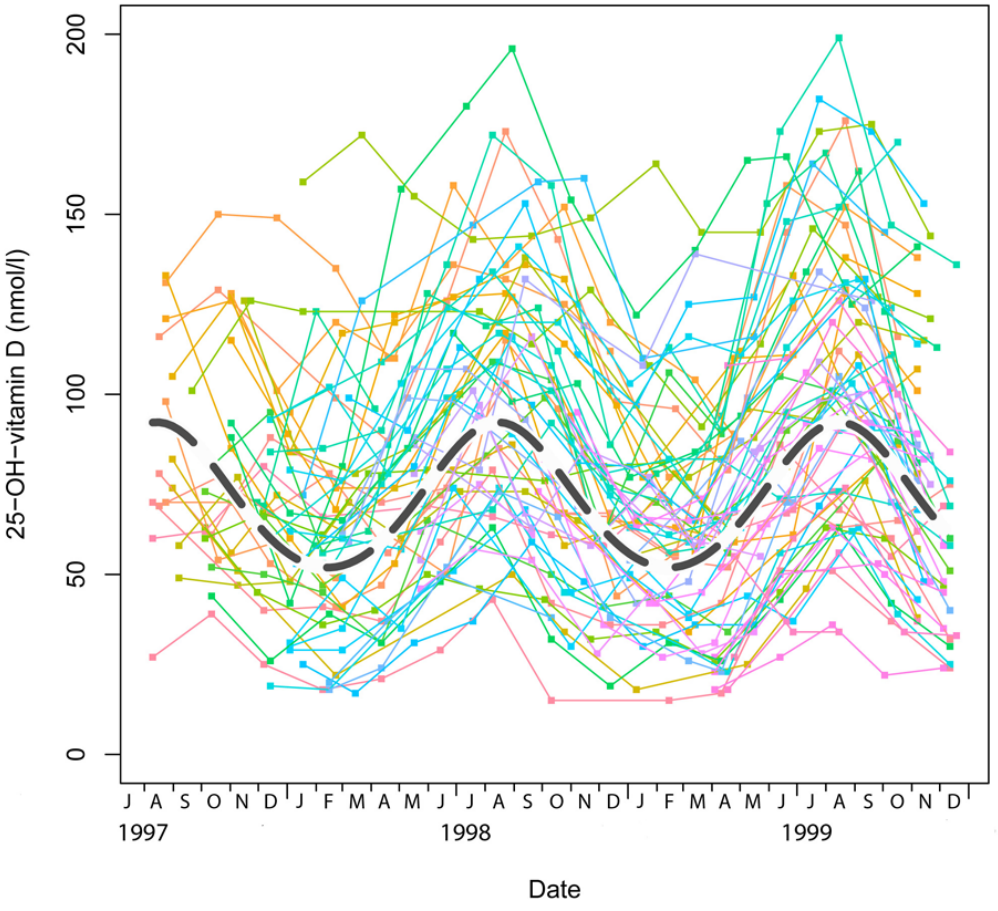

Figure 1. 25-OH-D concentrations per patient versus date of blood sampling. Data of individual patients are connected by straight lines. The dotted curve represents the overall fitted sinusoidal curve. Estimates (+/- standard error) of the parameters of the model Log10(concentration) = a + b sin(2π(t+c)) are a=1.837 +/- 0.022, b=0.125 +/- 0.009 and c=-0.367 +/- 0.006.

In univariate analysis it was also found that infections were associated with the risk of an exacerbation. The exacerbation rate within an at-risk period was 2.1-fold increased (95% CI 1.6

–2.8, p < 0.001, figure 2B). Simultaneous evaluation of categories of levels of serum 25-OH-vitamin and infections showed that both factors were related to the exacerbation rate (table 2). Also the effect of one factor did not depend on the other (interaction: p = 0.18).

Other characteristics (gender, age, EDSS, use of interferon- β, and number of exacerbations in the 2-year period before entry into the study) were not significantly associated with the exacerbation rates. This applied in univariate (all p > 0.18) as well as multivariable analysis (all p > 0.17). In particular, the effect of vitamin D on exacerbations was not modified by interferon use (p = 0.78 for the interaction effect).

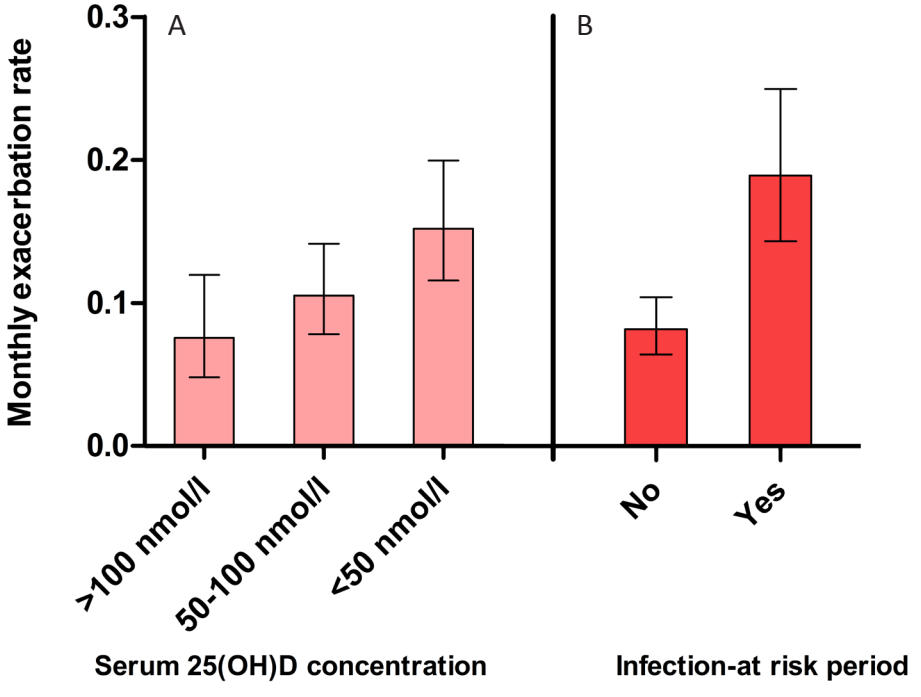

Figure 2. (A) Monthly exacerbation rates for the different groups of serum 25-OH-D concentrations, and (B) for at risk period for infections. Error bars denote 95% confidence intervals. Serum 25-OH-D concentrations: p (trend) = 0.007; infections p<0.001.

No significant differences among the 4 seasons were found regarding exacerbation rates, in univariate, or in multivariate analysis. Analyzing logarithmically transformed serum 25-OH-D concentrations on a continuous scale showed that a doubling of serum 25-OH-D concentrations caused a decrease of the exacerbation rate by 27% (95% CI 8%–42%, p = 0.008) (adjusted for the effect of infections). Adding quadratic and cubic terms of the linear predictor, i.e., log(serum concentration), did not significantly improve the fit of the model, indicating

the linearity of the association and the absence of a threshold.
Analyzing the 9 dropouts it was found that the dropout rate did not significantly correlate with the cumulative number of exacerbations during the study (Cox regression: p =0.29), nor with any baseline characteristic.

		Relative exacerbation rate	95% Confidence Interval	P-value
ARP infection	Yes	2.3	1.7 – 3.1	<0.001
	No	1 (reference)	-	
Vit D level	Low <50nmol/l	1.9	1.1 – 3.2	0.013 #
	Medium 50-100 nmol/l	1.4	0.8 – 2.2	0.235 #
	High >100 nmol/l	1 (reference)	-	

p-value for trend: 0.012

Table 2. Association between exacerbation rate and serum 25-OH-D concentrations and infection according to multivariable analysis.

Discussion

In the present study we show that lower 25-OH-D levels are significantly associated with a higher exacerbation risk in patients with relapsing-remitting MS. In the category of low 25-OH-D levels, the risk for an exacerbation was 2 times higher than in the category of high levels. This association was log linear without a threshold effect; a doubling of serum 25-OH-D concentrations lowered the exacerbation risk by 27%. Adjustment for potential confounders, including infection, gender, disability (EDSS), and use of immunomodulatory therapy, did not alter this association. In particular, although in experimental autoimmune encephalomyelitis the effect of vitamin D is found to be stronger in female mice[10], we did not find an influence of gender on the association between 25-OH-D and exacerbations in this study.

The key strength of this study is the frequent measurement of serum 25-OH-D concentrations. So far, only one prospective study has been performed to investigate the association between serum 25-OH-D concentrations and disease course in patients with relapsing-remitting MS with more than 1 serum measurement[14]. In that study, a significant association between 25-OH-D levels and exacerbation risk was found, but this was based on only 2 serum measurements per patient per year. It is well known that vitamin D levels fluctuate with season. Also, there is great interindividual variation of the mean vitamin D level and the amplitude of the seasonal fluctuation (also shown in our data). This makes extrapolating data from only 1 or 2 measurements per year using sinusoidal models very imprecise. With the present study, the suggested association between 25-OH-D levels and exacerbation risk can now be confirmed with much more accurate data on vitamin D levels. Furthermore, we included infection as a confounder and provide more frequently measured information on disability.

An additional strength of this study is the infrequent use of interferon-β among the patients. This allowed us to study the sole effect of vitamin D. Although some studies suggest an additional beneficial effect of vitamin D in interferon-β users[20,21], we did not find an additional effect of interferon-β treatment in this study. The fact that the association between vitamin D and exacerbation risk held in patients who used interferon-β might suggest that the effect of vitamin D is additive to the effect of interferon-β. However, this study was not set up to measure such an additive effect.

Our study was limited by the lack of information on the amount of sunshine the patients had during this study. We do not expect sun exposure and subsequent cutaneous vitamin D production to differ much among patients, as all patients were Caucasians from the Rotterdam region, but sun exposure is a potential confounder that we could not adjust for.

Although we found a strong seasonal fluctuation of vitamin D levels, the association between clinical disease activity and season was not significant in this study. Several other studies describe seasonality of MS disease activity, mostly with higher activity in spring. What we show here is an association between vitamin D and exacerbation risk that persists through all seasons. In this respect it should be noted that vitamin D and some other UV-mediated immune processes can act separately on the immune system, as recently described[22,23].

In studies on vitamin D and disease course in MS, there is often the issue of reverse causality: the possibility that the higher relapse rate is not caused by lower vitamin D levels, but that the low vitamin D levels are caused by increased disability that prevents patients from spending time outdoors. In this study, 2 facts argue against such a phenomenon. First, for every week the influence of the vitamin D levels during the preceding 4 weeks on exacerbations was calculated. Secondly, adjustment for disability (EDSS) did not alter the inverse association between 25-OH-D levels and exacerbation risk. Furthermore, only the serum samples taken at the regular 8-weekly visits were used in the sinusoidal model, and samples taken during exacerbation visits were not evaluated.

The biological plausibility for a protective role of vitamin D on the disease course of MS has been given in many experimental settings. Different cells of the immune system, including macrophages and activated T lymphocytes[24] and B lymphocytes[25], contain vitamin D receptors. 1,25-(OH)2 D has been shown to inhibit the production of inflammatory cytokines in vitro[26] and to promote the development of regulatory T lymphocytes[27,28]. Furthermore, a correlation between serum 25-OH-D concentrations and a more anti-inflammatory Th1/Th2 ratio has been found[20]. Also in other autoimmune diseases, such as rheumatoid arthritis, type 1 diabetes, and systemic lupus erythematosus, evidence for a protective role of vitamin D is growing[29]. However, in these ailments prospective studies are scarce.

To evaluate if the association between vitamin D levels and exacerbations in MS could be causal, Hill's criteria of causation can be used[30]. Hill's criteria are as follows: 1) strength of the association, 2) consistency of the association, 3) temporality, i.e., does the exposure

precede the disease (this is the issue of reverse causality), 4) biological gradient, or dose-response curve, 5) plausibility of the causation, and 6) experiment: have there been experiments to investigate if a change in exposure alters disease frequency. In the present study, we found a moderately strong association (criterion 1), which was linear (criterion 4). Our findings are consistent with previous research and are biologically plausible (criteria 2 and 5). Despite arguments provided above, the theoretic possibility of reverse causality (criterion 3) cannot be ruled out completely. Therefore clinical intervention trials are needed to further investigate the relationship between vitamin D supplementation and disease course in MS.

In this prospective cohort study we have demonstrated that lower serum vitamin D levels are associated with increased exacerbation risk in patients with relapsing-remitting MS; for each doubling of the serum 25-OH-D concentration the relapse risk in MS decreases by 27%.

Vitamin D has the advantages of being cheap, safe, and easy to administer, and could therefore be a valuable addition to the existing treatment opportunities in MS.

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Chapter 7

The influence of vitamin D on postpartum relapse and quality of life in pregnant multiple sclerosis patients

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Abstract

Background

In relapsing-remitting MS patients, lower serum vitamin-D concentrations are associated with higher relapse risk. In a number of conditions, low vitamin D has been associated with fatigue. Pregnant women are at particular risk for vitamin-D insufficiency.

Objective

To investigate whether vitamin-D status is associated with postpartum relapse and quality of life during pregnancy.

Methods

43 pregnant RRMS patients and 21 pregnant controls were seen at regular times before, during and after pregnancy. At every visit clinical assessment, samples for 25-OH-D measurements, and QOL questionnaires were taken.

Results

Lower 25-OH-D concentrations were not associated with postpartum relapse risk. Pregnancy-25-OH-D levels of patients and controls were not significantly different. In controls, but not patients, higher 25-OH-D concentrations were correlated with better general health, social functioning and mental health, but not with vitality.

Conclusion

Low vitamin-D levels are not associated with postpartum relapse. In pregnant MS patients, vitamin-D levels are similar to levels in healthy women and are not associated with quality of life. Therefore, with regard to QOL and postpartum relapse, we found no arguments for advising pregnant MS patients to take more vitamin-D supplements than healthy women.

Introduction

Vitamin D insufficiency is not only thought to be involved in the development of multiple sclerosis (MS)[1], but also in its disease course: in patients with relapsing-remitting MS (RRMS), lower serum vitamin D concentrations are associated with a higher relapse risk[2].

In a number of conditions, low serum vitamin D concentrations have also been associated with worse scores on quality-of- life items[3, 4] such as fatigue[5, 6].

It is known that vitamin D deficiency is very common in pregnant and lactating women throughout the world [7, 8]. In MS, although the relapse rate generally decreases during pregnancy, there is an increased risk of relapse in the first 3 months after delivery[9]. It is not fully known what causes this increased postpartum relapse risk, but it could be hypothesized that low serum vitamin D levels during or after pregnancy play a role. Vitamin D might also be associated with quality of life during and after pregnancy.

In this study we aimed to investigate whether vitamin D is associated with the postpartum relapse risk, and whether it is associated with quality of life. The Rotterdam Study on Pregnancy in MS is a longitudinal study in pregnant MS patients, with serum sampling before conception, in the first and third trimester of pregnancy and after delivery. Because it is not known if vitamin D status of MS patients during pregnancy and lactation differs from the vitamin D status of healthy pregnant women, we also included a control group.

Patients and Methods

Participants and study design

Study design and recruitment of patients and healthy controls in the Rotterdam Study on Pregnancy in MS was described previously[10-12]. In brief, ambulant relapsing-remitting MS patients were recruited before conception and healthy controls were included in the first trimester of pregnancy. Visits were planned at the end of the first trimester (at 10-12 weeks of pregnancy), at the beginning of the third trimester (at 28-30 weeks of pregnancy) and at 4-8 weeks after delivery. MS patients also had a visit at least 9 months after delivery, at a time point where there was no infection or recent disease activity. At every visits blood samples were taken and disability was measured using Kurtzke Expanded Disability Status Scale (EDSS)[13]. Patients were included between October 2002 and February 2009.

The study was approved by the ethics committee of the Erasmus MC and all participants provided written informed consent.

Definitions

All patients fulfilled the McDonald criteria for the diagnosis of multiple sclerosis[14]. Relapse was defined as a worsening of existing symptoms or the appearance of new symptoms lasting for more than 24 hours, after a period of more than 30 days of improvement or stability, confirmed by neurologic examination[15]. A temporary neurological deterioration

associated with fever was not considered as a relapse. Postpartum relapse was defined as a relapse occurring in the first 3 months after delivery. Duration of disease was measured from time of diagnosis.

Measurement of 25-OH-vitamine D

Concentrations of 25-OH-vitamin D were determined by a RIA method (DiaSorin, USA) using an extraction method. The inter-assay variation coefficient at a concentration of 62 nmol/L was 11.6% and at 109 nmol/L it was 10.3%. The respective intra-assay variation coefficients at these levels were 5.7% and 6.6%, respectively. All samples were analyzed at the same time, and samples of MS patients and controls were run in the same batch.

Measurement of quality of life

To measure quality of life, the MOS 36 item short form health survey questionnaire (SF-36) [16] was used. The SF-36 consists of four physical health domains and four mental health domains and has been used before in MS patients[17, 18]. The physical health domains are physical functioning, role physical functioning, bodily pain, and general health. The mental health domains are social functioning, vitality, role emotional functioning, and mental health. For each domain a score is generated ranging from 0 (poor health) to 100 (optimal health). The vitality domain has implications for a patients' fatigue.

Statistical analysis

Comparisons of patient characteristics were performed using Student's t test for continuous variables or chi-square test for categorical variables. Relapse rate and serum 25-OH-D levels at different time points of pregnancy were compared using mixed linear model analysis, with season as covariable; seasons were defined as spring (March-May), summer (June-August), autumn (September-November) and winter (December-February). For the analysis of the quality-of-life scores, correlation analysis was used (Pearson or Spearman depending on the distribution). The differences of 25-OH-D levels of patients and healthy women and patients with and without postpartum relapses were calculated with Student's t test and with regression analysis to correct for season.

All calculations were performed using SPSS 20 for Windows.

Results

Participants

43 patients and 21 healthy controls were included in the study. 26 patients were included before pregnancy, 16 during the first trimester and 1 during the third trimester. All controls were included in the study during the first trimester of pregnancy. As described previously[11], healthy controls were recruited from the outpatient clinic of the obstetrics department in our hospital, reasons for referral being: previous cesarean section (six), history of cardiac problems (three, of which one also had twin pregnancy), medically assisted preg-

nancy (two), prematurity in previous pregnancy (two), previous post-natal depression (one), uterus duplex (one), epilepsy (one), twin pregnancy (one), metabolic disorder (one). Two women had no specific medical indication. Data on pregnancy and birth outcome are shown in table 1.

	Patients (n=43)	Controls (n=21)
Maternal age (±SD)(years)	31.5 (±3.8)	31.2 (±4.5)
Nulliparous	28 (65%)	7 (33%)
Ceasarian section	6 (14%)	5 (24%)
Assisted vaginal delivery (forceps/vacuum)	5 (12%)	1 (5%)
Twin pregnancy	0 (0%)	2 (10%)
Birth weight (±SD)(grams)	3,347 (±385)	3,223 (±860)
Gestational age at delivery (±SD)(weeks)	39.3 (±1.5)	38.2 (±2.7)
Prematurity[1]	1 (2%)	3 (14%)
Small for gestational age[2]	1 (2%)	3 (14%)
Breastfeeding >2 months	25 (58%)	9 (43%)
(Pre)eclampsia	0 (0%)	0 (0%)
Use of vitamin supplements containing vitamin D	20 (46.5%)	7 (33.3%)
Duration of disease (±SD)(years)	5.5 (±6.0)	
Median EDSS first trimester (IQR)	1.5 (1.0-2.0)	

1 Defined as birth <37 weeks of gestation, not including twin pregnancies
2 Defined as birth weight under -2SD, using standardized intrauterine growth charts

Table 1. Data on pregnancy and birth outcome of patients and controls. SD = standard deviation, IQR = interquartile range.

In the MS patients, disease activity decreased during pregnancy: mean annualized relapse rate (ARR) was 0.47 in the year before pregnancy and lowered to 0.09 in the third trimester. After delivery, there was a significant increase in relapse rate (the ARR in the first 3 months after delivery was 1.02; 11 women experienced a postpartum relapse). During the study (mean follow-up time 19.8 months), a total of 31 relapses were observed. None of the patients received medication during the study except for three patients who received intravenous immunoglobulin directly after delivery, with the aim to protect for possible exacerbations[19]. Eighteen MS patients did not use any vitamin supplements during the study. Twenty patients and 7 controls used vitamin supplements containing vitamin D at some point during follow-up. Of 5 patients and 3 controls, use of vitamin supplements was not known. There was no association between supplements use and disease activity before pregnancy (nor with EDSS nor with relapse in the year before pregnancy).

Serum 25-OH-D concentrations

Serum 25-OH-D concentrations of patients and controls showed a strong seasonal fluctuation, with the peak level in August and nadir for patients in February. Mean 25-OH-D concentrations per month are shown in supplemental figure 1.

We found a rise in 25-OH-D levels in the third trimester of pregnancy, and a decrease after delivery. This fluctuation was stronger in patients than in controls, but in both groups it was significant ($p<0.001$ for patients and $p<0.05$ for controls, corrected for seasonal influences). The differences in serum 25-OH-D concentrations between patients and healthy controls were not statistically significant (all $p>0.7$). In figure 1, serum 25-OH-D concentrations during pregnancy are depicted for patients and controls. Because parity has been associated with vitamin D status during pregnancy[20] we investigated this but we found no difference in 25-OH-D concentrations between nulliparous and multiparous women.

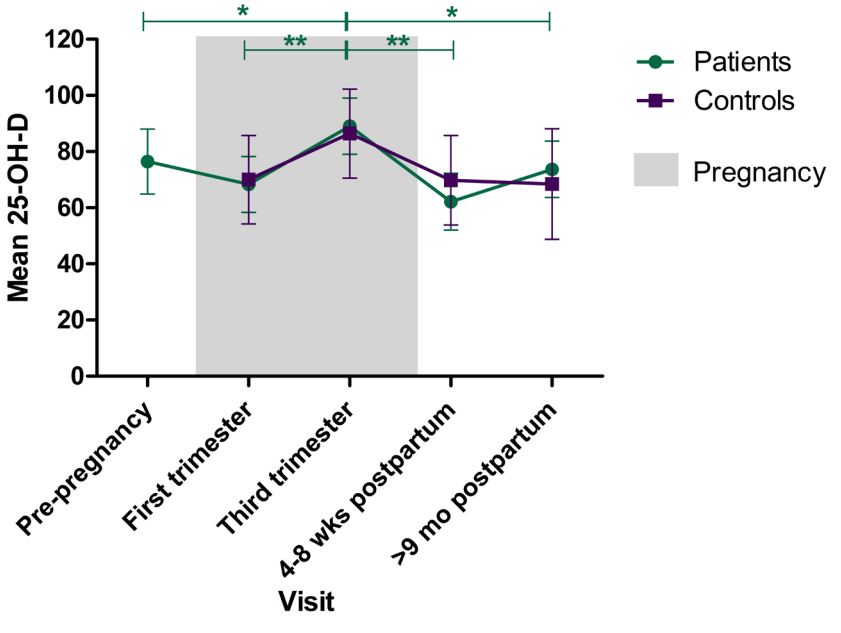

Figure 1. Mean serum 25-OH-D levels (nmol/l) of patients and controls before, during and after pregnancy. Depicted serum 25-OH-D values are corrected for season.

* $p<0.05$ only for patients

**$p<0.001$ for patients and $p<0.05$ for controls

Association between serum 25-OH-D concentrations and postpartum relapse and breast-feeding

The serum 25-OH-D concentrations during pregnancy were not associated with the occurrence of a postpartum relapse, also not when corrected for season. 25-OH-D concentrations of patients with and without a postpartum relapse are shown in figure 2. The proportion of patients that used vitamin supplements was the same among patients with and without a postpartum relapse. Also, the percentage of women who breastfed was not different among the women who did and did not experience a postpartum relapse (63.6% vs 65.6%). When looking at the relation of breastfeeding and vitamin D levels, we found that 25-OH-D levels were not significantly different between breastfeeding and non-breastfeeding women. We found that 25-OH-D levels were not associated with annualized relapse rate.

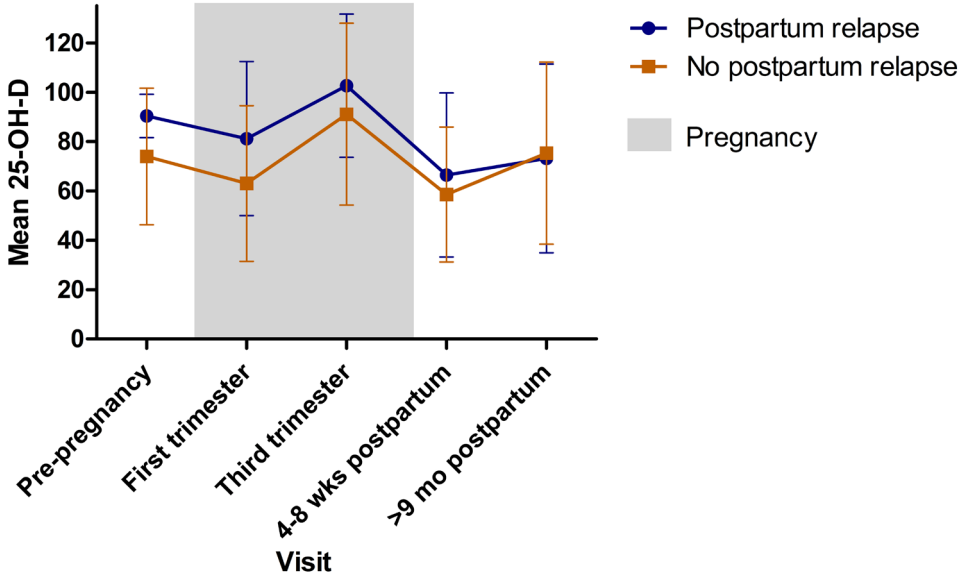

Figure 2. Mean serum 25-OH-D levels (nmol/l) before, during and after pregnancy for patients who did and did not experience a postpartum relapse.

Association between serum 25-OH-D concentrations and quality-of-life scores

In controls, but not in MS patients, a higher vitamin D concentration was positively correlated with better scores on several quality-of-life measures: these were general health (r=0.442), social functioning (r=0.365) and mental health (r=0.386). Vitamin D concentrations were not associated with vitality (r=-0. 04), not in healthy women nor in MS patients.

Discussion

The main finding of this study is that low vitamin D levels are not associated with postpartum relapse. It is hypothesized that in MS, but also in other autoimmune diseases like rheumatoid arthritis[21-23], during pregnancy there is a shift from a predominantly Th1 cell pro-inflammatory immunologic state to a more anti-inflammatory Th2 profile, reducing disease activity. After delivery, the immunologic state shifts back into the more pro-inflammatory profile resulting in the higher postpartum relapse risk. The changes in immune tolerance and reactivity during pregnancy are highly complex and not yet fully known. A major factor is thought to be the change in hormone levels during pregnancy, especially a strong increase in the levels of estrogens and cortisol. Other possible contributing factors include the increased secretion of anti-inflammatory cytokines, peptides, proteins and hormones by the fetal-placental unit [24], the downregulation during pregnancy of inflammation-related genes that are abnormally upregulated in MS [25], or microchimerism [26]. Because higher vitamin D is also associated with a more anti-inflammatory immunological profile[27], and has been associated with lower relapse risk in MS[2], we hypothesized that low vitamin D levels during pregnancy might also play a role in the postpartum relapse risk. However, we did not find any evidence for this.

One previous study, by Langer-Gould et al.[28], found no evidence for an association of low vitamin D levels and postpartum relapses; this study even found that postpartum levels of 25-OH-D were significantly higher in patients with a postpartum relapse than in patients without a relapse. The authors explained this by the fact that more women who relapsed did not breastfeed: in their study, breastfeeding was associated with lower vitamin D levels, although this was not significant when corrected for season. However, in our study, breastfeeding did not seem to influence 25-OH-D levels, nor did women with and without postpartum relapse differ significantly in their breastfeeding behavior. Because it is known that severity of the disease before pregnancy is a predictor of postpartum relapse[29], women with more severe disease might be expected to take more vitamin supplements; however, we found no evidence for difference in supplements use in our cohort. Vitamin D has immunological properties, and very likely plays a role in MS[1]. A possible explanation for the fact that we did not find any association with postpartum relapse is that vitamin D has a relatively small effect that is overridden by the much stronger hormonal effects on immunity.

A second finding of our study is that serum 25-OH-D concentrations changed under the influence of pregnancy, and did so equally in patients and controls; we did not find any significant difference between 25-OH-D levels of patients and controls. Higher 25-OH-D levels in the third trimester have been described before[30]. It is known that vitamin D metabolism changes during pregnancy. This might be related to altered calcium needs in the pregnant woman, but this does not seem completely true: during pregnancy, there is an uncoupling of vitamin D metabolism from calcium so that 1,25-diOH-D levels can increase without a concurrent rise in calcium concentrations, driven solely by 25-OH-D availability[7, 30, 31]. This uncoupling is only observed during pregnancy and at no other point in normal human physiology. Reasons for this possibility of vitamin D levels to rise independently of calcium

might include the altered immune function with increased innate immunity and decreased adaptive immunity, but also the possibility to provide the baby with sufficient levels of vitamin D; at term, for cord blood to attain a 25-OH-D level of 50 nmol/l, the maternal level would need to be at least 80 nmol/l[31, 32].

Finally, we found that vitamin D was associated with better general health, social functioning and mental health in healthy pregnant women. To our knowledge, this has not been studied before. No association was found with vitality. The fact that we did not find an association of vitamin D and improved quality of life in MS patients could be explained by the fact that pregnancy generally ameliorates MS. Therefore, MS patients already feel better during pregnancy than before[11], and may not notice a relatively small beneficial effect of vitamin D.

This study has some limitations, including small sample sizes at some time points and lack of dose information on vitamin D supplementation in some patients. Furthermore, we defined breastfeeding as a yes/no question and did not distinguish between exclusive and non-exclusive breastfeeding.

In conclusion, we found that although low vitamin D has been associated with MS relapse risk, and vitamin D levels are generally lower after delivery, there was no association with postpartum relapse. The exact mechanism behind the postpartum relapse in MS and the role of vitamin D in immunity during pregnancy remain to be further elucidated. In pregnant MS patients, vitamin D levels are similar to levels in healthy women and are not associated with quality of life. Therefore, with regard to QOL and postpartum relapse, we conclude that there is no evidence for a need to advise MS patients to take more vitamin D supplements during pregnancy than healthy pregnant women.

However, the following should be kept in mind when advising pregnant MS patients: the possibility that maternal vitamin D may influence the risk of the fetus to develop MS, and the impact of low vitamin D levels on bone health and other systems in any pregnant woman. For these reasons, it is important to make sure that 25-OH-D levels during pregnancy are sufficient, for all women, with or without MS.

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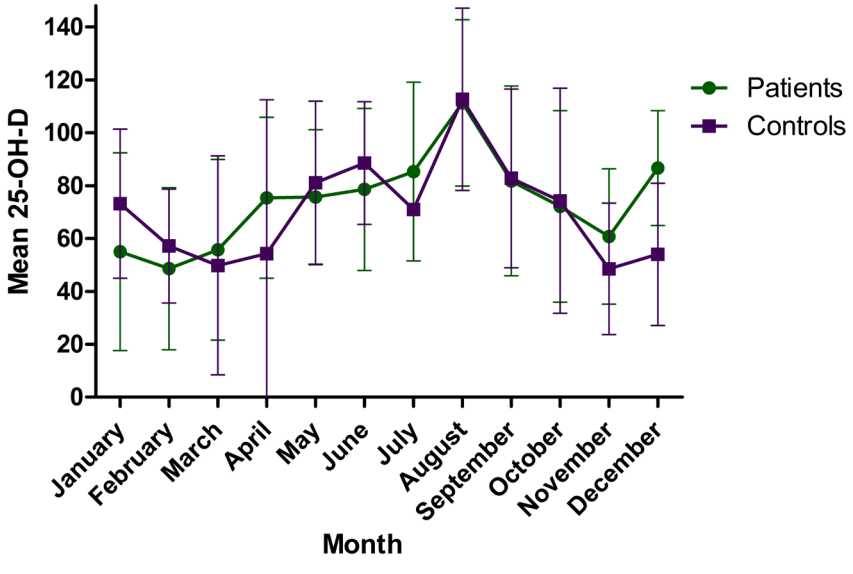

Supplemental figure 1. Mean serum 25-OH-D levels (nmol/l) of patients and controls per calendar month.

Chapter 8

Vitamin A is not associated with exacerbations in multiple sclerosis

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Mult Scler Relat Disord, 2014

Abstract

Background

Vitamin A is a multifunctional vitamin that can inhibit the formation of Th17 cells, which are probably involved in the development of relapses in MS. Furthermore, it promotes Treg formation. Therefore, vitamin A can be hypothesized to be lower in patients than in healthy controls, and to decrease relapse risk in relapsing–remitting MS(RRMS) patients.

Objectives

To compare vitamin A levels in MS patients and controls, and to investigate whether vitamin A levels are associated with relapse risk.

Methods

In a case-control study all-trans-retinol levels were compared between 31 RRMS patients and 29 matched controls.

In a prospective longitudinal study in 73 RRMS patients, serum samples for all-trans-retinol measurements were taken every eight weeks. Associations between all-trans-retinol concentrations and relapse rates were calculated using Poisson regression with the individual serum levels as time-dependent variable. Associations between vitamin A and vitamin D were calculated.

Results

Mean vitamin A levels were lower in patients (2.16 µmol/l) than in controls (2.44µmol/l) but with borderline significance (p=0.05). In the longitudinal study, during follow-up (mean 1.7 years), 58 patients experienced a total of 139 relapses. Monthly moving averages of all-trans retinol levels were categorized into tertiles: a low (<2.9 µmol/l), medium (2.9-3.7 µmol/l) and high level (>3.7 µmol/l). Relapse rates were not associated with serum all-trans retinol levels (p>0.2), in univariate nor in multivariate analysis.

Serum concentrations of all-trans-retinol and 25-OH-vitamin D were positively correlated, although this correlation was weak (r=0.15).

Conclusions

We did not find evidence for a role for vitamin A in the disease course of RRMS. We did find an association between vitamin A and D levels in the RRMS patients, possibly explained by dietary products that contain both fat-soluble vitamins.

Introduction

Vitamin A or retinol is a fat-soluble vitamin with multiple functions, such as those in vision, growth and the normal differentiation of epithelia[1,2]. Over the last 2 decades, it has become clear that vitamin A also has important roles in immune functioning, both in immunological tolerance and in adaptive immune responses [2].

After absorption from food, most vitamin A is stored in the liver, from where it is added to the circulation bound to retinol-binding protein (RBP)[3,4]. Inside the target cells, retinol is oxidized by alcohol dehydrogenase (ADH) into retinal, which can then be oxidized into the active form retinoic acid (RA) by the more selectively expressed retinaldehyde dehydrogenase (RALDH)[2].

RA is a signaling molecule that can control gene expression, mainly through the activation of nuclear retinoid receptors. There are three subgroups of retinoid receptors: retinoic acid receptors (RARα-γ), retinoid X receptors (RXRα-γ), and retinoic acid orphan receptors (RORα-γ)[5]. RXR can form heterodimers with other nuclear receptors, such as vitamin D receptor (VDR)[4].

RA has been shown to inhibit the formation of Th17 cells, which are probably involved in the development of relapses in MS[6], in vitro[7] via binding to RARα[8,9]. It can do so synergistically with 1,25-diOH-vitamin D[10]. In EAE, a synthetic retinoid could also inhibit Th17 cell differentiation and ameliorate EAE[11]. Furthermore, RA can promote the formation of anti-inflammatory Treg cells expressing Foxp3[7,8,12]. Recently, it was also found that vitamin A is possibly associated with MS risk[13] and MRI outcomes in MS[14].

Because of the roles described for vitamin A on Th17 and Treg cells, we hypothesized that 1) vitamin A would be lower in MS patients than in healthy controls, and 2) MS patients with higher vitamin A levels would have a lower relapse risk. To investigate this, we performed two studies: a case-control study of vitamin A levels, and a prospective longitudinal study of vitamin A levels in relapsing-remitting MS (RRMS) patients to investigate the association between vitamin A and relapse risk. Because of possible synergistic effects, we also looked at the associations of vitamin A and vitamin D in the longitudinal study.

Patients and methods

1. Case control study on vitamin A and MS

Patients and controls

We randomly selected 31 patients with RRMS from our pool of MS patients, subsequently selecting 29 controls matched for age and sex. All controls had signed for informed consent, and all patients were aware that serum would be stored for later use. The Medical Ethical Committee of the Erasmus Medical Center University Hospital approved the use of these materials.

Measurement of vitamin A

All-trans retinol was measured because this is the main form of retinol in the circulation[4,15,16], and also the best measure of vitamin A status. For analysis of retinol levels, the samples were extracted with hexane and, after evaporation, dissolved in methanol. Retinol levels were measured through reverse-phase high-performance liquid chromatography (HPLC) (column: 150 × 4.8 mm, Polaris C18A, Waters Alliance HT2795; detector: Waters 2475 [Waters, Milford, MA]), with excitation at 328 nm and detection of emission at 468 nm. The intra-assay variability of retinol measurements was 3.9% and the inter-assay variability was 5.1%.

Statistical analysis

Student's T-test was used to compare mean all-trans retinol levels between RRMS patients and healthy controls. ANOVA was used to adjust for age. P=0.05 (two-sided) was considered the limit of significance in all analyses. All calculations were done using SPSS 20.0 for Windows.

2. Longitudinal study on vitamin A and exacerbations

Patients

For the longitudinal study, data and samples were collected in the Rotterdam Study on Exacerbations in MS, a prospective study in patients with relapsing-remitting MS[17]. Patients aged 18-55 years could be included in the study if they had clinically definite MS with a relapsing-remitting disease course and at least two exacerbations in the previous 2 years. Patients were excluded from participation if they suffered from another serious disease. All patients signed for informed consent. The study protocol was approved by the Medical Ethical Committee of the Erasmus Medical Center University Hospital.

Definitions

Exacerbation was defined as a worsening of existing symptoms or the appearance of new symptoms lasting for more than 24 hours after a period of more than 30 days of improvement or stability, if confirmed by neurologic examination[18]. A temporary neurological deterioration associated with fever was not considered to be an exacerbation.
Because infection is a known risk factor for exacerbations in multiple sclerosis, the 'at risk period' around infection was used as a covariate in this study, as described previously[17].
Visits, samples and measurement of exacerbations

All patients visited the outpatient clinic of the Erasmus Medical Centre University Hospital regularly every 8 weeks. At every visit, blood samples were taken and disability was measured using the Kurtzke Expanded Disability Status Scale (EDSS)[19]. In the event of a suspected exacerbation or infection, additional visits were arranged within 3 days. Serum samples were stored at -80ºC until serum vitamin A measurement.

Measurement of vitamin A and vitamin D

Measurements of all-trans retinol were as described above. To investigate the association between vitamin A and D, we used a RIA method (DiaSorin, USA) using an extraction method, to measure 25-OH-vitamin D levels. The inter-assay variation coefficient at a concentration of 62 nmol/L was 11.6%; at 109 nmol/L it was 10.3%. The respective intra-assay variation coefficients at these levels were 5.7 and 6.6%. Only serum samples taken at the regular eight-weekly visits were used for the measurement of all-trans retinol and 25-OH-vitamin D; samples taken during exacerbation visits were not evaluated.

Statistical analysis

To assess the association between individual serum all-trans retinol concentrations and the incidence rate of exacerbations, we split the follow-up time for each patient, which covered a maximum period of 2.3 years, into intervals of one week each. The number of exacerbations was determined for each of these intervals. The individual exacerbation rate was assumed to depend on the mean serum all-trans retinol concentration over the previous 4 weeks. To obtain this mean level, the weekly levels between measurements were determined per individual using interpolated values. These interpolated values were subsequently averaged. During the period of 4 weeks that followed an exacerbation, an individual was not considered to be at risk for another exacerbation. It was decided a priori to categorize the mean serum levels of the 4 preceding weeks into tertiles: low (<2.9 µmol/l), medium (2.9-3.7 µmol/l) and high (>3.7 µmol/l). The relationship between serum all-trans retinol concentrations and the incidence rate of exacerbations was assessed using Poisson regression models with the mean individual serum levels as a time-dependent variable. In the calculations we used generalized estimating equations with an exchangeable covariance matrix for the subsequent study weeks. The effect of other factors, including gender, age, EDSS, number of exacerbations before study entry and use of interferon-β during the study, was also estimated using a multivariable generalized linear model with a log-link function. Associations between measured vitamin A and vitamin D concentrations, the latter log-transformed to get an approximate normal distribution, were calculated using mixed model regression analysis for repeated measurements. P=0.05 (two-sided) was considered the limit of significance in all analyses. All calculations were done using SPSS 20.0 for Windows.

Results

1. Case control study on vitamin A concentrations and MS

Patient characteristics

The baseline characteristics of the 31 patients and 29 controls included are shown in table 1. We had no information on the use of vitamin supplements of patients and controls. None of the patients were using interferon β at the time of blood sampling, because sampling was done during a clinical workup for therapy advice within our MS center.

Characteristic	Patients	SD	Controls	SD
Sex (f/m)	22/7		24/7	
Age mean (range)	35.4 (19-56)	10.3	35.9 (19-56)	10.4
Ethnicity (%)				
White caucasion	77.4		89.7	
Meditterranean	16.1		10.3	
Black	6.5			
Disease duration (years) mean (range)	4.5 (0-29)	5.4		

Table 1. Characteristics of patients and controls of the case-control study

Serum vitamin A concentrations

For most patients and controls, serum all-trans retinol concentrations fell within the normal range. None of the patients and none of the controls were vitamin-A deficient. As figure 1 shows, all-trans retinol concentrations where somewhat lower in patients than in controls (mean 2.16 ± 0.55 µmol/l vs. 2.44 ± 0.52 µmol/l), but this difference was only borderline significant (p= 0.050). Vitamin A depended on age in the case-control study, but not in the longitudinal study. It did not depend on sex. When adjusting for age, there was no significant difference between patients and controls (p=0.070).

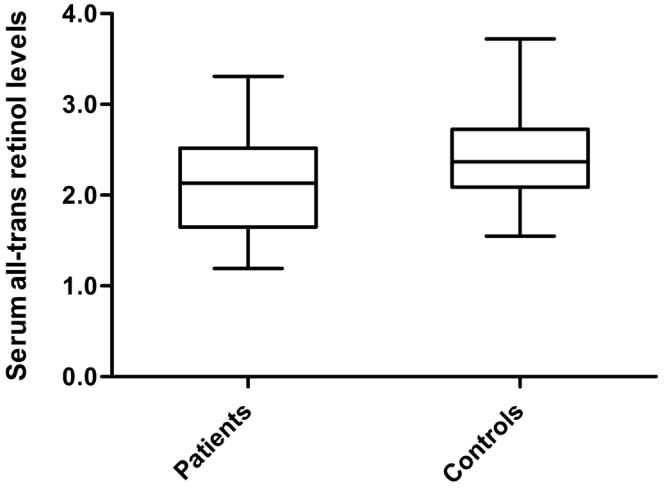

Figure 1. Serum all-trans retinol concentrations of relapsing-remitting MS patients and healthy controls.

2. Longitudinal study on vitamin A and exacerbations

Patient characteristics

73 patients were included in this study. The mean follow-up time of all patients was 1.7 years (range 0.4-2.3). Nine patients had dropped out of the study before the intended completion date, one due to participation in another study, the other eight for unknown reasons. All patients were Dutch Caucasians; their baseline characteristics are shown in table 2. In addition to the 13 patients who used interferon-β at study entry, 15 started to use interferon-β during follow-up; these 28 patients used interferon-β at some point during an average of 56 weeks. Vitamin supplements were not widely used among the patients: five used vitamin B complex (without vitamin A) and two used multivitamin pills, one of which contained 6 mg of beta-carotene.

Variable (n=73)	Mean (range)	Std. Deviation
Age, years	39.4 (19-55)	9.1
Disease duration, years	5.2 (0-25)	4.1
Disability (EDSS) *	2.5 (0-6.0)	1.6
Exacerbations in previous 2 years	2.2 (1-8)	1.3
Variable (n=73)	Proportion (%)	
Gender, F/M	77% / 23%	
IFN use, N/Y	82% / 18%	

* EDSS is a method for quantifying disability in MS, ranging from 0.0 (normal neurological exam) to 10.0 (death due to MS)

Table 2. Characteristics of patients of the longitudinal study at baseline.

58 patients experienced a total of 139 exacerbations during this study. Median time from inclusion to first exacerbation was 20 weeks. Thirty-three patients had more than one exacerbation; the average exacerbation rate was 1.2 per year (range 0-6.2 per year). Three patients experienced a sixth exacerbation during follow-up.

Serum vitamin A concentrations

Serum all-trans retinol concentrations fluctuated considerably, without a seasonal pattern or other clear pattern. Mean serum all-trans retinol concentration was 3.31 ± 1.14 µmol/l. Mean levels varied substantially between patients (Anova: $P<0.001$). Within patients there was also a considerable variation between measurement occasions. Of the total variation in levels 40% was due to differences between patients while 60% was due to differences within patients.

Association between serum vitamin A concentrations and exacerbation risk

Univariate analysis did not show exacerbation rates to be associated with serum all-trans retinol levels (p>0.2).
As shown previously[20], infections were associated with the risk of an exacerbation, the exacerbation rate within an "at risk period" being 2.1-fold higher (95% CI 1.6 to 2.8, p<0.001). Also, vitamin D (25-OH-D) serum levels were found to be associated with exacerbation risk. In the multivariate model including 25-OH-D and infections, infections and 25-OH-D were both still related to the exacerbation rate, whereas all-trans retinol was not (table 3). Adding gender or the use of interferon-β to the model did not alter these results. Also age, EDSS and the number of exacerbations in the 2 year period before entry into the study were not significantly associated with the exacerbation rates (all p>0.18).

		Relative exacerbation rate	95% CI	P-value
All-trans retinol concentrations	Low <2.9 µmol/l	1.2	0.8-1.8	0.329#
	Medium 2.9-3.7 µmol/l	1.3	0.8-1.9	0.254#
	High >3.7 µmol/l	1 (reference)	-	-
ARP infection	Yes	1 (reference)	-	-
	No	0.4	0.3-0.6	<0.001
25-OH-D concentrations	Low <50 nmol/l	2.0	1.2-3.4	0.012*
	Medium 50-100 nmol/l	1.3	0.8-2.2	0.281*
	High >100 nmol/l	1 (reference)	-	-

overall p-value: 0.489

* p-value for trend: 0.010

Table 3. Association between exacerbation rate and categorized serum all-trans retinol concentrations according to the multivariable analysis including infection and 25-OH-vitamin D concentrations. ARP infection = at risk period for infection.

Association between vitamin A and vitamin D

Serum concentrations of all-trans retinol and 25-OH-D had a significant linear correlation. Mixed model regression analysis showed that for every doubling of serum 25-OH-D concentration the mean all-trans retinol level increased by 0.59 µmol/l (p<0.001). The correlation however was weak (r=0.15).

Discussion

This case-control study shows that vitamin A concentrations are not significantly lower in MS patients than in healthy controls. Our longitudinal study also shows that vitamin A is not associated with relapse risk in MS patients. We also found that vitamin A and vitamin D were associated in a linear manner.

There are several reasons to hypothesize that vitamin A is involved in MS. In the past, it has been hypothesized that the susceptibility period in multiple sclerosis lies in early childhood[21] and that the element responsible was (a deficiency in) vitamin A[22]. This hypothesis was based mainly on epidemiological evidence, and the mechanisms through which vitamin A deficiency was thought to cause MS were effects on normal CNS myelination and effects on skull and bone growth affecting normal CNS growth. Since knowledge of the functions of vitamin A in immunity has grown in the last decades[2] (inhibiting Th17 cell formation[7] and promoting Treg formation[7,8,12]), the hypothesis that vitamin A might be involved in the development and disease course of MS has become stronger. Recently, it was also found that RXR agonists can stimulate remyelination[23]. Vitamin A has even been suggested as a (supplementary) treatment option in MS[11,15]. However, in the present case-control and longitudinal studies we cannot provide any evidence that vitamin A is really involved in MS.

In the case-control study, we found somewhat lower retinol concentrations in patients than in controls, but this was not significant. Other studies that addressed this topic had conflicting results. One study also did not find lower levels than in controls[24]. Another study found lower levels in MS patients than in controls[25]. Differences with our study were that all patients had a secondary progressive disease course, and that a different technique was used for the measurements (Neeld-Pearson with trifluoroacetic acid instead of HPLC). A third study compared retinol levels in patients and controls, finding significant differences only between subgroups[15]. It should be noted that in that study retinol levels were slightly higher in interferon-β treated patients.

The borderline significance in our results suggests that our groups might have been too small. On the other hand, as figure 1 shows, the mean levels of vitamin A in patients and controls are not far apart, and confidence intervals are wide, suggesting substantial variation within the patient and control group. In our longitudinal study, we also found substantial within-patient variation of retinol levels. We found that vitamin A was also dependent on age, which has been described before by Looker et al. [26] but was not found by Hallfrisch et al.[27]. Because our control group was age- and sex matched, this has not influenced our conclusions.

In the longitudinal study we found no association between vitamin A and exacerbation rate. One recent study using the same technique for retinol assessment, found an inverse association between new lesion formation on MRI and vitamin A levels[14]. We could not confirm an association between vitamin A and clinical disease activity in MS in our study. But

although our longitudinal study with its frequent serum sampling provides a robust way of studying this topic, the fact that we found no association does not totally exclude a role for vitamin A in MS relapses. There might be local function or production of RA in the CNS associated with relapses, that cannot be measured systemically, for example by tissue-specific expression of retinoid receptors or retinaldehyde dehydrogenase (RALDH)[2].

A limitation of our study is that none of the patients in the case-control study used interferon-β. In the longitudinal study, the use of interferon-β did not influence our results. In studies by others on this topic conflicting results have been found: some found a synergistic effect of RA with interferon-β on T suppressor cell augmentation[28], others found the association between vitamin A and MRI outcomes to be non-significant during interferon-β use[14]. We were unable to confirm any of this here.

We found an association between serum levels of vitamin A and D. Vitamin A can be absorbed from food, but its levels can also be increased via a rise in hepatic production as a result of exposure to light[29,30]. Because vitamin D is also a fat-soluble vitamin that can be absorbed from food and can also be synthesized under the influence of sun light, it is not surprising that the concentrations of both vitamins increase and decrease simultaneously. However, we did not find synergistic action in this study: whereas a higher vitamin D level was associated with a lower relapse risk, vitamin A was not.

For our analyses of retinol levels, we used random serum samples and not fasting samples. This is justified because retinol is derived from hepatic and other body stores and is a good measure of vitamin-A status, unlike retinyl-esters, which are absorbed after a vitamin-A rich meal[15].

In recent years, several studies on the relation between vitamin A and MS have been performed, using different techniques and markers, sometimes with conflicting results[13,14,15,24,25,31]. Routine measurement of vitamin A and carotenoids is now generally done using high performance liquid chromatography (HPLC). Lately, the use of plasma RBP as a surrogate marker for retinol is increasing. It should be realized that this is only valid in the absence of infection or when adjusted for CRP levels[13,32].

In conclusion, there are several reasons to hypothesize that vitamin A has a role in the development and disease course of MS. Here we did not observe differences in retinol levels between patients and controls. In addition, in the prospective study on exacerbations, we found no association with disease activity. These results do not feed the perception that vitamin A could be a useful treatment for MS patients [11,15].

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Chapter 9

No evidence for an association of osteopontin plasma levels with disease activity in multiple sclerosis

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Mult Scler, 2014

We read with interest the article by Kivisäkk et al[1]. in which they evaluated the plasma levels of osteopontin in a cohort of patients with multiple sclerosis (MS). Kivisäkk and colleagues measured higher plasma osteopontin levels in MS patients than in controls, but they could not demonstrate any significant association of osteopontin with disease activity.

Osteopontin is a multifunctional molecule that has been proposed as a biomarker for disease activity in MS. It has been hypothesized to be involved in relapses in MS for several reasons: osteopontin has been found to be one of the genes with the most common transcripts in MS lesions. In experimental autoimmune encephalomyelitis (EAE) models, administration of osteopontin induced relapse and inhibited spontaneous recovery. In patients with relapsing–remitting MS, levels of osteopontin were higher during relapses than during remission[2]. One study showed that osteopontin levels were increased 1 month before the appearance of a new lesion on brain magnetic resonance imaging (MRI)[3], and although this study had some methodological shortcomings, its results added to the suggestion of osteopontin as a possible biomarker for MS disease activity.

We have conducted a prospective longitudinal study in which we measured osteopontin plasma levels every 8 weeks in 58 relapsing–remitting MS patients. Patients were followed for a mean of 1.6 years. Osteopontin levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA; IBL International, Hamburg, Germany). The association between osteopontin levels and the relapse risk in the first, second, third and fourth week after sampling were calculated using generalized linear mixed-effects models.

In this prospective study, we found no association between osteopontin levels and relapse risk. We also found no difference in osteopontin levels between patients with active and stable disease and no association of osteopontin with EDSS score. There was also no difference between interferon treated and non-treated patients, and no association with infections.

With this, we confirm the results of the cross-sectional study by Kivisäkk et al. that no association was found between osteopontin and clinical disease activity, even in a prospective study with frequent plasma measurements.

Although these results do not rule out a role for osteopontin in MS pathophysiology, we conclude that as a biomarker for MS disease activity, osteopontin does not seem useful.

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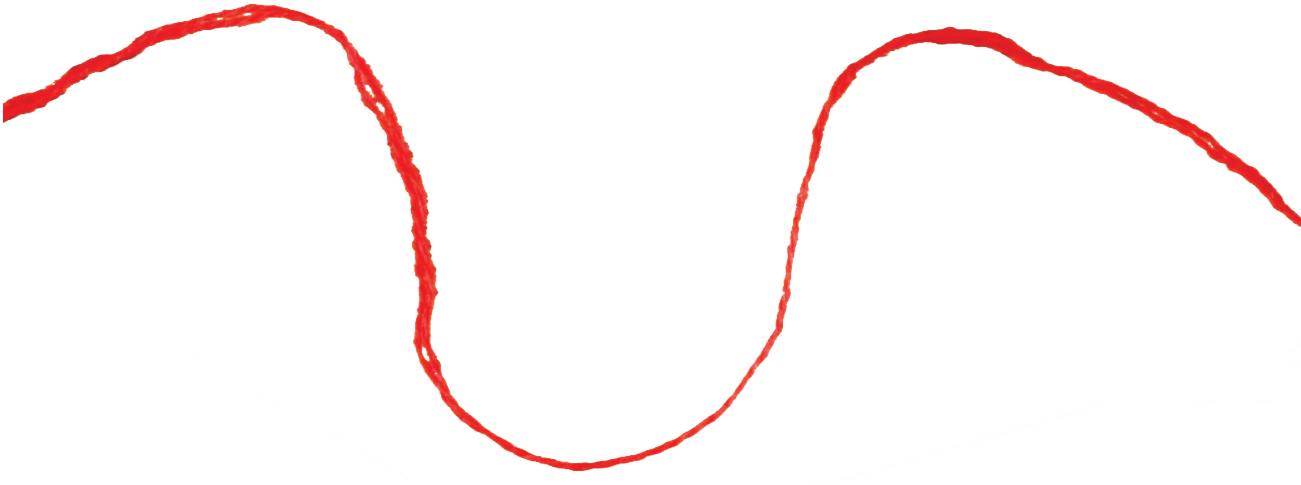
Chapter **10**

General discussion



General discussion

Multiple sclerosis is a devastating disease that strikes young people in the prime of their lives. The fact that MS is also characterized by a large heterogeneity in clinical disease course brings along substantial uncertainty for the patients and their treating physicians. The main aim of this thesis was to find predictors for diagnosis and disease course of MS. In this chapter the main findings are discussed, along with their clinical implications and future perspectives.

Part 1: CIS to MS

Main findings and clinical implications

After a clinically isolated syndrome, not all patients will develop MS; after 20 years, 37% still has monophasic disease according to a study by Fisniku et al.[1]. In our own cohort of CIS patients, with a mean follow-up of almost 4 years, 40-45% has reached clinically definite MS. Because there is no single gold-standard laboratory test to diagnose MS, the diagnosis is primarily clinical, and based on the exclusion of other diagnoses and the demonstration of symptoms and signs attributable to CNS lesions that are disseminated in space ("DIS") and time ("DIT"). International committees of experts have published diagnostic criteria for MS[2], which, over the years, have been revised several times with the aims to allow for a faster and simpler diagnosis[3-6]. The most recent (McDonald 2010) revisions were published in 2011, with as most important change the simplification of the MRI requirements. In **chapter 2**, we applied these criteria to a cohort of 178 CIS patients to validate them, and we found that test characteristics of 2010 criteria were similar to 2005 criteria, but the diagnosis of MS could be made significantly faster with the newest criteria, in some patients even already at baseline, which is a great advantage. Also other groups have tested and validated the newest diagnostic criteria [7-9] showing that they function well in CIS patient populations from around the world. However, it should be noted that although the 2010 criteria are advantageous compared to previous diagnostic criteria, the diagnosis of MS can be made at baseline only in a minority of patients, still leaving the future uncertain for most CIS patients.

In the search for new prognostic markers in CIS patients, we took both a clinical and a proteomics approach.

Because MS patients very often suffer from fatigue, we investigated if this was already a common symptom at the stage of CIS. In **chapter 3**, we show that indeed fatigue is a frequent symptom in CIS patients, with the prevalence and severity of fatigue in our cohort similar to that in MS patients: 46.5% of CIS patients suffered from fatigue. This fatigue was not related to the type or severity of the attack, nor with lesion load on brain MRI. Importantly, we found that fatigue was associated with an increased risk for CDMS, even independent of MRI measures. The pathophysiology of MS-related fatigue is not exactly known; inflammatory cytokines combined with CNS dysfunction, sleep disorders and depression are likely to play a role[10, 11]. We investigated if vitamin D was associated with fatigue in CIS

patients, but it was not. Although one previous study[12] has shown that fatigue sometimes precedes other symptoms in MS and another study[13] described fatigue in early MS, this is the first time that fatigue has been studied separately in CIS patients and that its predictive value for CDMS has been shown. Because MS-related fatigue can be very disabling, we feel that the evaluation of fatigue deserves more attention in the routine work-up of patients with CIS. In clinical practice, it is easy to measure using the FSS questionnaire[14].

In **chapter 4**, we describe a proteomics approach to find biomarkers in the CSF of CIS patients. We studied the CSF of 47 CIS patients and 45 controls, and found peptides relating to 36 proteins to be differentially abundant in CIS patients and controls. Contrary to what we expected, only 1 protein was more abundant in patients than controls (unsurprisingly immunoglobulin-related; Ig kappa chain C region), but 35 proteins were lower in CIS patients than controls. Most of these 35 proteins were related to gray matter integrity or cell-cell signaling in the nervous system. There are two possible explanations for the lower abundance of these proteins in the CSF of patients: the lower abundance of proteins involved in the integrity of the CNS may either precede or even predispose for a first demyelinating attack, or alternatively be the result of the attack and subsequent smoldering chronic disease process. The first explanation is in line with others who describe the phenomenon that gray-matter related proteins are less abundant in the CSF of patients than in controls, in CIS[17-19] but also in more neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington[15, 16]. In a study in Alzheimer's patients at disease onset [20], a depletion of several proteins was found in the serum of patients in the very early stage of the disease; this was hypothesized to reflect the breakdown of neural cell membranes in those individuals destined to phenoconvert from cognitive intactness to Alzheimer's disease. In a similar manner, in early MS it can be hypothesized that a dysfunction in CNS gray matter physiology can be observed as a depletion of proteins essential for normal development and function in the CSF in those destined to undergo a first demyelinating event. The fact that recently discovered new MS-related genes also included several gray-matter related genes might add to the credibility of this hypothesis [21]. The alternative explanation for lower protein abundance in CSF of patients compared to controls, is that the disease process leads to lower levels, for example by means of decreased production as a result of the disease process, or of increased elimination by macrophages because of increased immune activation. An argument in favor of the latter explanation is the fact that most identified proteins were even lower in patients with the highest MRI lesion load, which can be regarded as a measure for inflammation. It is remarkable that we did not find any marker to distinguish the patients who remained monophasic and those who developed clinically definite MS during follow-up, not even when only the fast converters were taken into account. Which factors determine if a patient remains monophasic or goes on to develop MS remain to be elucidated.

In chapter 5, we put many of the known predictors together to create a prediction model for CDMS in CIS patients. Based on a Cox regression model with stepwise backward selection, the final model consisted of 5 predictors: DIS+DIT2010 (the baseline scan fulfills criteria for dissemination in time and place according to the 2010 revised McDonald criteria), a lesion in the corpus callosum, cerebrospinal fluid oligoclonal bands, fatigue and an abnormal

MRI. With this model, 3 risk groups were created (low, intermediate and high risk) with respective 5-year risks for CDMS of 19.4%, 56.0% and 92.5%. Of note, in this model CSF oligoclonal bands added significant predictive ability to the model, even in addition to the DIS+DIT2010 MRI parameters. In the McDonald 2010 criteria, OCB have lost importance and can no longer be used for the criterion of dissemination in space. But importantly, OCB can add biological information to the mere images of the MRI by indicating central nervous system inflammation, and are also important to exclude other diagnoses[24]. Furthermore, our results indicate that OCB have predictive value, even when used in addition to the 2010 MRI criteria. This argues for the performance of a lumbar puncture in CIS patients, not only to rule out other diseases from the differential diagnosis, but also to predict the risk subsequent for CDMS.

Although some studies have shown that vitamin D was a predictor for CDMS in CIS patients, its predictive value was not strong enough to make it into the final prediction model described in **chapter 5**. Also, in the study on fatigue described in **chapter 3**, we did not find an association between vitamin D levels and a diagnosis of CDMS. Vitamin D is regarded as one of the most important environmental risk factors for MS, with evidence for a role for vitamin D in MS from research in epidemiology[25, 26], genetics[27] and experimental autoimmune encephalitis[28-30]: it is well known that the latitudinal gradient of MS prevalence may be attributed to vitamin D, and that people with lower intake of fatty fish, less childhood sunlight or lower vitamin D levels have a higher risk to develop MS [26, 31, 32]. Furthermore, it is known that vitamin D has immunological properties, being able to shift the immune response to a more regulatory, anti-inflammatory state[33]. Two studies have investigated the predictive value of 25-OH-D in CIS patients before. The first one, in 465 patients[34], found significantly increased risks for new lesions and increased lesion volumes on MRI for patients with low 25-OH-vitamin D levels, but only a borderline increased risk for CDMS. The second study[35] found increased risk only in women with very low 25-OH-vitamin D levels. In our study described in **chapter 3**, we found no association of vitamin D with CDMS (HR 0.9, 95% CI 0.3-2.1), not even when looking only at female patients (data not shown). We don't exactly know when in life low levels of vitamin D affect MS risk (assuming that vitamin D does affect MS risk, and not the other way around). There are indications that this is already in utero or in early childhood[25, 36], but also later in life, affecting the disease course of MS, as several studies, including one described in this thesis (**chapter 6**) have shown. Although the latter are all observational studies, it seems as if vitamin D can affect MS risk and severity continuously through life. If this would be true, one would expect it to also be associated with progression from CIS to MS. Then, our study might have been underpowered to find the association, but as the hazard ratio we found was very close to 1, the influence of vitamin D, if it is real, will probably be only moderate.

In summary, the findings of the first part of this thesis bring us to the hypothesis that possibly, some dysfunction of neuro-axonal physiology predisposes for a first demyelinating event. Patients with monophasic CIS and (future) RRMS can be distinguished at the time of CIS by means of the latest diagnostic criteria, the presence of fatigue, the presence of lesions in the corpus callosum and CSF oligoclonal bands. Maybe with the exception of fa-

tigue, all of these markers can be regarded as (surrogate) markers of increased inflammation in the patients who go on to develop RRMS.

Main findings part 1:

- The 2010 revised criteria are valid and accurate and allow for a faster diagnosis of MS; in a subgroup of patients already after one attack
- Fatigue is common and relatively severy among CIS patients
- Fatigue is an independent predictor for CDMS in CIS patients
- Multiple gray matter proteins are lower abundant in the CSF of CIS patients than in the CSF of controls
- No CSF proteins differentiate between CIS patients who do and do not develop MS
- Vitamin D does not seem to be a strong predictor for CDMS in CIS patients
- With a clinical prediction model based on 5 widely available clinical parameters, 3 risk groups for CDMS can be distinguished

Clinical implications part 1:

- The McDonald 2010 criteria are validated
- Fatigue in CIS patients deserves more attention
- Although the clinical prediction model described in this thesis needs validation, CSF OCB, corpus callosum lesion and fatigue can be measured in addition to the McDonald 2010 criteria to give additional information regarding the risk for CDMS in CIS patients.

Part 2: RRMS to next attack

Main findings and clinical implications

In about 80% of patients with MS, the disease starts with a relapsing-remitting course. In addition to the short-term disability associated with the relapses, they are known to be the cause of residual disability in 50% of cases[37]. Therefore, it is important to find factors associated with relapses.

Because vitamin D is one of the main environmental factors implicated in MS, as described before, we investigated the association of serum 25-OH-vitamin D levels and relapse risk in relapsing-remitting MS patients, described in **chapter 6**. Serum samples of 73 patients were collected frequently (every 8 weeks), which is advantageous because of the known seasonal fluctuation of 25-OH-D levels. 25-OH-D levels were divided into three categories: low (<50 nmol/L), medium (50–100 nmol/L), and high (>100 nmol/L) levels. It was shown that lower 25-OH-D levels were significantly associated with a higher exacerbation risk in RRMS patients. In the low 25-OH-D category, the risk for an exacerbation was 2 times higher than in the high 25-OH-D category. This association was log linear without a threshold effect. A doubling of serum 25-OH-D concentrations lowered the exacerbation risk by 27%. Other prospective studies have confirmed these findings[38, 39], although the latter found significantly less T2 lesions with increasing 25-OH-D levels, but only a trend in relapse risk decrease[39].

There are many reasons to assume that there is in fact a beneficial effect of sufficient vitamin D levels on MS disease course: the association we found was significant, with a linear dose-response effect, it has biological plausibility, and is consistent with results from other studies. However, the possibility that an association between vitamin D levels and MS disease course is caused by reverse causality (the possibility that the higher relapse rate is not caused by lower vitamin D levels, but that the low vitamin D levels are caused by increased disability that prevents patients from spending time outdoors) cannot be ruled out completely. In the study described in **chapter 6**, several facts argue against such a phenomenon. First, for every week the influence of the vitamin D levels during the preceding 4 weeks on exacerbations was calculated. Secondly, adjustment for disability (EDSS) did not alter the inverse association between 25-OH-D levels and exacerbation risk. Furthermore, only the serum samples taken at the regular 8-weekly visits were used in the sinusoidal model, and samples taken during exacerbation visits were not evaluated. Still, clinical intervention studies are needed to further investigate the relationship between vitamin D supplementation and disease course in MS. So far, only few intervention studies on vitamin D in MS patients have been performed. A recent meta-analysis including 5 randomized controlled trials evaluating any form or dose of vitamin D on relapse risk, with a total of 129 treated patients and 125 controls, showed no significant association of treatment with vitamin D and relapse risk (OR 0,98, 95%CI 0,45-2,16) [40]. However, the included studies were limited by small sample sizes and included different formulations and durations of vitamin D treatment. Larger and more prolonged high-quality randomized controlled trials are needed to address this issue. In **chapter 7**, we investigated if vitamin D was also associated with the postpartum relapse

in pregnant MS patients. As is known, the relapse rate of patients decreases during pregnancy, but the risk for a relapse increases again after delivery. It is not known exactly known what causes this postpartum relapse, but it is hypothesized that there is a shift from a predominantly Th1 cell pro-inflammatory immunologic state to a more anti-inflammatory Th2 profile during pregnancy, reducing disease activity. After delivery, the immunologic state shifts back into the more pro-inflammatory profile resulting in the higher postpartum relapse risk[41-43]. A major factor in these changes in immune tolerance is thought to be the change in hormone levels during pregnancy, especially a strong increase in the levels of estrogens and cortisol. Other possible contributing factors include the increased secretion of anti-inflammatory cytokines, peptides, proteins and hormones by the fetal-placental unit[44], the downregulation during pregnancy of inflammation-related genes that are abnormally upregulated in MS[45], or microchimerism[46]. We hypothesized that vitamin D might also play a role here, but we could not find any association of vitamin D with postpartum relapse risk. Possibly, an effect of vitamin D is small, and is overridden by much stronger hormonal influences. Importantly, we found that serum 25-OH-D concentrations changed under the influence of pregnancy, and did so equally in patients and controls; we did not find any significant difference between 25-OH-D levels of patients and controls. Furthermore, we found that 25-OH-D levels were associated with better general health, social functioning and mental health in healthy pregnant women, but not in MS patients. This might be explained by the fact that pregnancy generally ameliorates MS. Therefore, MS patients already feel better during pregnancy than before[12], and may not notice a relatively small beneficial effect of vitamin D.

Although the study has some limitations (including small sample sizes at some time points and lack of dose information on vitamin D supplementation in some patients), we conclude that there was no association of 25-OH-D with postpartum relapse nor with quality of life in MS patients, and that vitamin D levels did not differ between healthy pregnant women and pregnant MS patients. Therefore, with regard to QOL and postpartum relapse, we feel that there is no evidence for a need to advise MS patients to take more vitamin D supplements during pregnancy than healthy pregnant women. However, in all pregnant women, vitamin D sufficiency should be maintained, for reasons of bone health, general health and the possibility that maternal vitamin D may influence the risk of the fetus to develop MS.

Another fat-soluble vitamin that has been hypothesized to play a role in MS is vitamin A. Vitamin A has been shown to inhibit Th17 cell formation and promote Treg formation[47-49]. Furthermore, it was recently found that agonists for RXR receptors (a vitamin A receptor) can stimulate remyelination[50]. Vitamin A has even been suggested as a (supplementary) treatment option in MS[51, 52]. In **chapter 8** we describe a case-control study on vitamin A levels and a longitudinal study with frequent serum sampling where we investigated the association of vitamin A with MS relapses. We did not find significant differences between vitamin A levels in patients and controls, nor did we find associations of vitamin A and relapse risk in RRMS patients. Vitamin A and vitamin D were correlated in a linear manner. One recent study using the same technique for retinol assessment, found an inverse association between vitamin A levels and new lesion formation on MRI, but not with clinical disease

activity[53]. Although our longitudinal study with its frequent serum sampling provides a robust way of studying this topic, the fact that we found no association does not totally exclude a role for vitamin A in MS relapses. There might be local function or production of RA in the CNS associated with relapses, that cannot be measured systemically, for example by tissue-specific expression of retinoid receptors or retinaldehyde dehydrogenase (RALDH) [54]. Limitations of our study include a small sample size for the case-control study and the lack of interferon-β using patients in the longitudinal study; others have described conflicting findings: one study found synergistic effects of retinoic acid with interferon-β on T suppressor cell augmentation[55], another found the association between vitamin A and MRI outcomes to be non-significant during interferon-β use[53]. This was something we could not address. Although vitamin A has immunological properties and although conflicting results have been published on this topic in recent years, the results of our study do not feed the perception that vitamin A could be a useful treatment for MS patients.

In **chapter 9**, we describe a study that investigated the association of osteopontin levels and MS relapses. In 2001 it was discovered that the osteopontin gene was one of the most common transcripts in MS lesions[56]. Later studies showed that administration of osteopontin inhibited remission and induced relapse in EAE models, and in MS patients levels of osteopontin were higher during relapses than during remission[57, 58]. One study showed that osteopontin levels were increased 1 month before the appearance of a new lesion on brain magnetic resonance imaging (MRI)[59]. However, we and others could nog confirm these findings[60]. We also found no difference in osteopontin levels between patients with

Main findings part 2:

- Low vitamin D levels are associated with higher relapse risk
- There is no evidence for an association of vitamin D with postpartum relapse risk
- Vitamin A is not associated with relapse risk
- Vitamin A levels of MS patients and controls do not differ significantly
- Osteopontin is not associated with relapse risk

Clinical implications part 2:

- In patients with relapsing-remitting MS, vitamin D deficiency should be prevented, but there is not enough evidence to start vitamin D treatment in MS patients
- Pregnant MS patients do not need to take more vitamin D supplements than healthy controls

active and stable disease and no association of osteopontin with EDSS score, treatment, or infections. Although these results do not rule out a role for osteopontin in MS pathophysiology, we concluded that as a biomarker for MS disease activity, osteopontin does not seem useful.

Future perspectives

MS is a complex disease resulting from an interplay of genetic and environmental factors. Its etiology and pathophysiology are still not completely clear.

In order to better understand such a complex disease and to identify prognostic factors and biomarkers that could ultimately lead to the discovery of new treatments, natural history studies are of great importance. From the Rotterdam MS center ErasMS, the PROUD study (Predicting the OUtcome of a Demyelinating event) is coordinated. This study, performed at ErasMS and several regional hospitals, includes all patients with a clinically isolated syndrome within 6 months of symptom onset, if they sign for informed consent. Clinical assessment, blood and CSF samples and MRI scans are taken at baseline, and patients are followed prospectively. Because the identification of reliable prognostic factors or biomarkers might need large numbers of patients and many years of follow up, (international) collaboration is indispensable. Recently, guidelines have been published for the collection of samples[61], control groups[62] and the reporting of biomarker study results[63] to simplify and accelerate such collaboration and the publication of findings. Focus of international collaboration is now also on the influence of genetics on clinical outcome; both the association of known MS-related genes with outcome, and new GWAS analyses related to pooled outcome parameters. Not only for biomarker research, but also to study MS genetics and gene-environment interactions, the collection of data and samples from large groups of patients in a transparent and uniform way in order to simplify the integration of data from multiple centers is key.

One of the topics presented in this thesis, and one of the most studied environmental factors associated with MS is vitamin D. As vitamin D is cheap and relatively safe, it would make an attractive addition to the current treatment options in MS. However, data from high-quality randomized controlled trials are lacking. As mentioned before, only few intervention studies have been performed so far, including only a small number of patients[40]. Because of the increasing observational evidence that higher vitamin D levels are beneficial for MS disease course, and because vitamin D is cheap and widely available, some argue for the treatment of MS patients with vitamin D in spite of the absence of evidence from treatment trials, and some patients and treating physicians even already use and prescribe it. Although one study in 25 patients that were followed for 1 year using up to 40.000 IU vitamin D per day showed no serious side effects[64], there is no information about the long-term effect, and vitamin D can certainly cause side effects when used in high dosages, most importantly hypercalcemia[65]. For these reasons, there is a strong call for intervention studies on vitamin D in MS patients. At ErasMS, the VIDEO study (VItamin D treatment Effect on retinal nerve fiber loss after Optic neuritis) is being performed. This is randomized, placebo-con-

trolled an intervention study on the effect of cholecalciferol in patients with optic neuritis. The main outcome measure is the thickness of the retinal nerve fiber layer as measured by OCT, which is known to decrease after optic neuritis. Difficulties in studying vitamin D treatment in MS/CIS patients include the fact that it is not known what the optimum dose or serum concentration is, and difficulties to obtain distinct treatment- and placebo groups: it is inevitable that 25-OH-D levels vary between patients, due to the fact that sunlight and fish intake are major sources. Furthermore, it is not ethical to keep people at very low vitamin D levels, so some patients in the placebo groups will have to be treated with low dose vitamin D as well. Finally, as we also notice in the VIDEO-study, people hesitate to take part in such a study, because from the many publications in the media it seems as if the beneficial effect of vitamin D on MS has already been proven and patients do not want to risk ending up in the placebo group. However, in spite of these difficulties, it is important that this kind of studies are being performed. Until evidence from treatment trials becomes available, vitamin D insufficiency should be treated but high dose vitamin D treatment should be discouraged.

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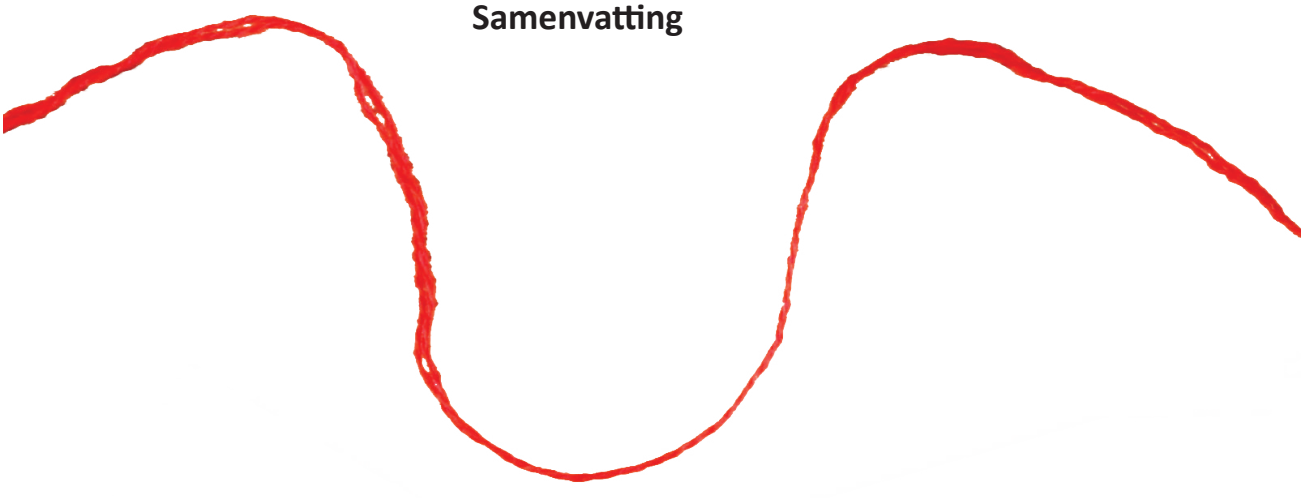
Chapter 11

Summary
Samenvatting



Summary

Multiple sclerosis (MS) is a complex disease that is characterized by a large heterogeneity in radiological and pathological findings but also in clinical disease course and treatment response. For reasons of diagnosis, treatment, patient counselling as well as insight into the pathology of MS, prognostic factors are needed.

This thesis aimed to identify predictive factors for 'the next attack', both in patients with a first demyelinating event, for whom a second attack means getting the diagnosis of clinically definite MS, and in patients with relapsing-remitting MS.

In **chapter 1**, an overview of the known prognostic factors or risk factors for MS and for MS relapses is given.

Part 1 of the thesis focuses on the prediction of the next, disease defining attack in patients with a clinically isolated syndrome suggestive of MS (CIS). First, in **chapter 2**, the latest revised McDonald criteria for the diagnosis of MS are applied to a cohort of CIS patients to test their accuracy. We show that the newest diagnostic criteria have similar test characteristics as the older criteria, but the diagnosis can be made significantly faster, which is a great advantage.

In the search for new predictive markers in CIS patients, we took both a clinical and a biomarker approach. First, we were interested in fatigue. Fatigue is one of the most common and disabling features of MS, but little is known about its prevalence and severity in patients with CIS. Therefore, we investigated the prevalence and severity of fatigue in a cohort of CIS patients, and we also investigated its predictive value for a subsequent diagnosis of CDMS. Fatigue was measured using the Fatigue Severity Scale (FSS, see appendix). We observed that, already at the stage of CIS, fatigue was common (a prevalence of 46.5%) and relatively severe (mean FSS 4.3). Furthermore, the presence of fatigue at time of CIS was found to be an independent predictor for CDMS, in a model correcting for sex, age, neuroanatomical localization of CIS, 25-OH-vitamin D, anxiety, depression, MRI dissemination in space and gadolinium enhancement; the hazard ratio for patients suffering from fatigue was 4.5 (95% CI 1.9 to 10.6). This is described in **chapter 3**.

Next, in **chapter 4**, we investigated the cerebrospinal fluid of CIS patients using high-resolution proteomics techniques, with the aims to find markers that could distinguish between patients and controls, and markers that could distinguish between monophasic CIS patients and patients who would go on to develop MS. We performed a proteomics analysis in CSF of 47 CIS patients and 45 controls, and identified a total of 3159 peptides, relating to 485 proteins. Only 1 protein was significantly more abundant in CSF of CIS patients than in controls: Ig kappa chain C region. Thirty-five proteins were lower in CIS patients than controls, most of them with functions in nervous system development and function. This lower abundance in CIS patients may either precede or even predispose for a first demyelinating attack (e.g. by some disturbed neuro-axonal physiology that shows as a lower abundance of proteins in

the CSF, which predisposes for a first demyelinating event), or be the result of it (for example due to an increased immune activation in patients, which might cause a higher rate of elimination of proteins from the CSF by macrophages). We observed no significant differences in specific peptide abundance levels between patients who did and did not reach a diagnosis of clinically definite MS.

In **chapter 5**, multiple predictors for CDMS in CIS patients are integrated into a predictive model. A multivariate Cox regression model was created after univariate screening of candidate predictors, based on baseline demographic, clinical, MRI, serum and CSF parameters of 431 CIS patients. The model was then further simplified using stepwise backward selection. A simple scoring system was then constructed giving equal weight to all predictors. The final model consisted of the following 5 variables: DIS+DIT2010 (the baseline scan fulfills criteria for dissemination in time and place according to the 2010 revised McDonald criteria), corpus callosum lesion, cerebrospinal fluid oligoclonal bands, fatigue and abnormal MRI. Three risk groups were created: low risk (0-1 risk factor present), intermediate risk (2-3 risk factors) and high risk (4-5 risk factors). The 5-year risk for CDMS in the low-risk group was 19.4% versus 56.0% in the intermediate-risk group and 92.5% in the high-risk group.

In the second part of this thesis, factors associated with relapse risk in patients with relapsing-remitting MS are discussed.

Because there was growing evidence for a role of vitamin D in MS, but little was known about its association with clinical disease course in MS patients, this was investigated in **chapter 6**. In a prospective longitudinal study in 73 patients with relapsing-remitting MS, blood samples for 25-OH-D measurements were taken every 8 weeks. Associations between 25-OH-D levels and exacerbation rates were assessed using Poisson regression (generalized estimating equations) with the individual serum levels as time-dependent variable. During a mean follow-up of 1.7 years, 58 patients experienced a total of 139 exacerbations. Monthly moving averages of 25-OH-D levels were categorized into low (<50 nmol/L), medium (50–100 nmol/L), and high (>100 nmol/L) levels. It was found that exacerbation risk decreased significantly with higher serum vitamin D levels: respective relative exacerbation rates for the medium and high level category as compared to the low-level category were 0.7 and 0.5 (p value for trend: p=0.007). With each doubling of the serum 25-OH-D concentration the exacerbation rate decreased by 27% (95% confidence interval 8%–42%, p=0.008). These results suggest a beneficial effect of vitamin D on disease course in MS, although the possibility of reverse causality cannot be ruled out completely.

After finding that vitamin D levels were associated with relapse risk, we also set out to investigate if vitamin D was associated with the postpartum relapse risk. In the study described in **chapter 7**, vitamin D levels were measured during pregnancy in 43 pregnant RRMS patients and 21 pregnant healthy women, and the association of pregnancy vitamin D levels and the risk for a postpartum relapse and with pregnancy quality of life was investigated. It was found pregnancy-25-OH-D levels of patients and controls were not significantly different,

and lower 25-OH-D concentrations were not associated with postpartum relapse risk. In controls, but not patients, higher 25-OH-D concentrations were correlated with better general health, social functioning and mental health, but not with vitality. We concluded that, with regard to QOL and postpartum relapse, we found no arguments for advising pregnant MS patients to take more vitamin-D supplements than healthy women.

In **chapter 8**, a possible association of vitamin A levels and relapse rate was investigated. Vitamin A is a multifunctional vitamin that can inhibit the formation of Th17 cells, which are probably involved in the development of relapses in MS. Furthermore, it promotes Treg formation. Therefore, vitamin A can be hypothesized to be lower in patients than in healthy controls, and to decrease relapse risk in RRMS patients. We measured all-trans-retinol levels in a case-control study in 31 RRMS patients and 29 matched controls. Furthermore, in a prospective longitudinal study in 73 RRMS patients, serum samples for all-trans-retinol measurements were taken every eight weeks. Associations between all-trans-retinol concentrations and relapse rates were calculated using Poisson regression. Mean vitamin A levels were lower in patients (2.16 µmol/l) than in controls (2.44µmol/l) but with borderline significance (p=0.05). In the longitudinal study, all-trans retinol levels were categorized into tertiles: a low (<2.9 µmol/l), medium (2.9-3.7 µmol/l) and high level (>3.7 µmol/l). We found that relapse rates were not associated with serum all-trans retinol levels (p>0.2), in univariate nor in multivariate analysis. Serum concentrations of all-trans-retinol and 25-OH-vitamin D were positively correlated, although this correlation was weak (r=0.15). It was concluded that we did not find evidence for a role for vitamin A in the disease course of RRMS.

In **chapter 9**, the association of osteopontin levels and relapse risk was investigated. Osteopontin is a multifunctional molecule that has been proposed as a biomarker for disease activity in MS, based on results from pathology studies, animal experiments and studies on osteopontin levels in RRMS patients. In a prospective longitudinal study, samples for osteopontin measurement were taken every 8 weeks in 58 relapsing–remitting MS patients. Osteopontin levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA). The association between osteopontin levels and the relapse risk in the first, second, third and fourth week after sampling were calculated using generalized linear mixed-effects models. We found no association between osteopontin levels and relapse risk. We also found no difference in osteopontin levels between patients with active and stable disease and no association of osteopontin with EDSS score. There was also no difference between interferon treated and non-treated patients, and no association with infections. Although these results do not rule out a role for osteopontin in MS pathophysiology, we concluded that as a biomarker for MS disease activity, osteopontin does not seem useful.

In summary, patients with monophasic CIS and (future) RRMS can be distinguished at the time of CIS by means of the latest diagnostic criteria, the presence of fatigue, the presence of lesions in the corpus callosum and CSF oligoclonal bands. In patients with RRMS, vitamin D levels are associated with relapse risk. In order to better understand a complex disease such as MS, and to identify prognostic factors and biomarkers that could ultimately lead to the discovery of new treatments, natural history studies are of great importance. Not only

for biomarker research, but also to study MS genetics and gene-environment interactions, the collection of data and samples from large groups of patients in a transparent and uniform way in order to simplify the integration of data from multiple centers is key.

Samenvatting

Multiple sclerose (MS) is een complexe ziekte die wordt gekenmerkt door een grote heterogeniteit, zowel in radiologische en pathologische bevindingen als in klinisch ziektebeloop en respons op therapie. Voor de diagnose, behandeling, patiëntenvoorlichting en inzicht in de pathologie van MS zijn voorspellende factoren van groot belang.

Dit proefschrift had tot doel om voorspellende factoren voor 'de volgende aanval' te identificeren, zowel in patiënten met een eerste aanval van demyelinisatie, voor wie een tweede aanval het krijgen van de diagnose klinisch definitief MS betekent, als in patiënten met relapsing-remitting MS.

In **hoofdstuk 1** wordt een overzicht gegeven van de bestaande voorspellende factoren of risicofactoren voor MS en MS-aanvallen.

Deel 1 van dit proefschrift gaat over het voorspellen van de volgende, ziekte-definiërende aanval in patiënten met een zogenaamd klinisch geïsoleerd syndroom verdacht voor MS (CIS). Eerst, in **hoofdstuk 2**, zijn de nieuwste herziene McDonald-criteria voor de diagnose MS toegepast op een cohort van CIS-patiënten om hun testeigenschappen te onderzoeken. We tonen aan dat de nieuwste diagnostische criteria ongeveer gelijke testeigenschappen hebben als de oude criteria, maar dat de diagnose significant eerder gesteld kan worden, wat een groot voordeel is.

Op zoek naar nieuwe voorspellende markers in CIS-patiënten hebben we zowel naar klinische kenmerken als naar biomarkers gekeken. Eerst hebben we vermoeidheid onderzocht; dit wordt beschreven in **hoofdstuk 3**. Vermoeidheid is een van de meest voorkomende en meest invaliderende kenmerken van MS, maar er is niet veel bekend over het voorkomen en de ernst ervan in patiënten met CIS. Daarom hebben we de prevalentie en ernst van vermoeidheid in een cohort van CIS-patiënten getest, en ook de voorspellende waarde van vermoeidheid voor het krijgen van de diagnose klinisch definitief MS onderzocht. Vermoeidheid werd gemeten met de Fatigue Severity Scale (FSS: deze loopt van 1 tot 7, zie bijlage). We vonden dat vermoeidheid veel voorkwam, zelfs al op moment van CIS, met een prevalentie van 46.5%, en dat het relatief ernstig was (gemiddelde FSS 4.3). Bovendien was de aanwezigheid van vermoeidheid op het moment van CIS voorspellend voor de diagnose klinisch definitief MS, in een model dat corrigeerde voor leeftijd, geslacht, neuro-anatomische lokalisatie van de klachten, vitamine D, angst, depressie en MRI-afwijkingen. De hazard ratio voor vermoeide patiënten om later MS te krijgen was 4.5 (95% CI 1.9 tot 10.6).

In **hoofdstuk 4** onderzochten we vervolgens de hersenvloeistof van CIS-patiënten met behulp van hoogwaardige proteomics-technieken, op zoek naar markers die patiënten en controles zouden kunnen onderscheiden, en markers die een onderscheid zouden kunnen maken tussen patiënten met monofasisch CIS en patiënten die later MS krijgen. Er werd een proteomics-analyse gedaan van de liquor van 47 CIS-patiënten en 45 controles, en daarbij werden 3.159 peptides geïdentificeerd, die gerelateerd waren aan 485 eiwitten. Slechts 1

van deze eiwitten was significant verhoogd in de liquor van patiënten ten opzicht van controles: Ig kappa chain C region. Vijfendertig eiwitten waren lager in de liquor van patiënten dan controles, en dit waren voor het grootste gedeelte eiwitten met functies in de ontwikkeling en functie van het centraal zenuwstelsel. Deze lagere aanwezigheid van eiwitten in CIS-patiënten zou ofwel 1. vooraf kunnen gaan aan de eerste aanval van demyelinisatie, ofwel 2. er het gevolg van zijn. Een mogelijke verklaring voor de eerste optie zou kunnen zijn dat een verstoorde neuro-axonale fysiologie, zichtbaar als een lagere aanwezigheid van eiwitten, predisponeert voor een CIS. Voor de tweede optie zou een mogelijkheid zijn dat door een verhoogde immuunactivatie in patiënten een verhoogde eliminatie van eiwitten uit de liquor, bijvoorbeeld door macrofagen, wordt veroorzaakt. We vonden geen significante verschillen in eiwitten tussen patiënten die wel en niet definitief MS kregen.

In **hoofdstuk 5** worden meerdere voorspellers voor MS in CIS-patiënten samengevoegd in een voorspellend model. Er werd een multivariaat Cox-regressiemodel gecreëerd na univariate screening van kandidaatvoorspellers, gebaseerd op baseline demografische, klinische, MRI-, serum- en liquorparameters van 431 CIS-patiënten. Het model werd verder vereenvoudigd met behulp van stepwise backward selection. Er werd een eenvoudig scoringssysteem gemaakt dat gelijk gewicht gaf aan alle variabelen. Het uiteindelijke model bestond uit de volgende 5 variabelen: DIS+DIT2010 (de baseline scan voldoet aan de criteria voor spreiding in plaats en tijd volgens de 2010 McDonald criteria), corpus callosum laesie, oligoclonale banden in de liquor, vermoeidheid en een abnormale MRI-scan. Op basis hiervan werden drie risicogroepen onderscheiden: laag risico (0-1 risicofactor), middelmatig risico (2-3 risicofactoren) en hoog risico (4-5 risicofactoren). De 5-jaarsrisico's op klinisch definitief MS in de verschillende groepen liepen uiteen van 19% in de laag-risicogroep ten opzichte van 56% in de middelmatige groep en 93% in de hoog-risicogroep. Na verdere validatie kan dit een praktisch hulpmiddel zijn bij het voorlichten van patiënten en het besluiten tot starten met immuunmodulerende therapie.

In het tweede deel van dit proefschrift worden factoren beschreven die geassocieerd zijn met het risico op een aanval in patiënten met relapsing-remitting MS.

Er was groeiend bewijs voor een rol voor vitamine D in MS, maar er was maar weinig bekend over de associatie van vitamine D met het beloop van de ziekte. Dit hebben we daarom onderzocht in **hoofdstuk 6**. In een prospectieve longitudinale studie in 73 patiënten met relapsing-remitting MS werden bloedmonsters voor 25-OH-D-bepaling elke 8 weken afgenomen. De associatie tussen 25-OH-D-concentraties en aanvalsrisico werd berekend met behulp van Poisson-regressie met de individuele serumconcentraties als tijdsafhankelijke variabele. Tijdens een gemiddelde follow-up van 1.7 jaar kregen 58 patiënten in totaal 139 MS-aanvallen. Gemiddelde 25-OH-D-concentraties werden in drie categorieën ingedeeld: laag (<50nmol/l), gemiddeld (50-100 nmol/l) en hoog (>100 nmol/l). We vonden dat het risico op een aanval significant afnam met hogere serumconcentraties van vitamine D: de respectievelijke aanvalsfrequenties voor de gemiddelde en hoge categorie ten opzichte van de lage vitamine D-categorie waren 0.7 en 0.5 (p-waarde voor trend: p=0.007). Met elke

verdubbeling van de serum 25-OH-D-concentratie nam de aanvalsfrequentie af met 27%. (95% betrouwbaarheidsinterval 8-42%, p=0.008). Deze resultaten suggereren een gunstig effect van vitamine D op het ziektebeloop van MS, maar reverse causality kan niet helemaal worden uitgesloten.

Na de bevinding dat vitamine D-levels geassocieerd waren met ziektebeloop, hebben we onderzocht of vitamine D ook geassocieerd was met het postpartum-aanvalsrisico. In de studie beschreven in **hoofdstuk 7** werden vitamine D-concentraties gemeten tijdens de zwangerschap in 43 zwangere RRMS-patiënten en 21 gezonde zwangere vrouwen, en werd de associatie van zwangerschaps-vitamine D-concentraties met het risico op een aanval na de bevalling en met kwaliteit van leven tijdens de zwangerschap onderzocht. Zwangerschaps-vitamine D-concentraties bleken niet te verschillen tussen zwangere patiënten en controles, en lagere vitamine D-waarden waren niet geassocieerd met het postpartumrisico op een aanval van MS. In controles, maar niet in patiënten, waren hogere vitamine D-waarden geassocieerd met betere algemene gezondheid, sociaal functioneren en mentale gezondheid, maar niet met vitaliteit. We concludeerden dat er, met betrekking tot postpartum aanval en kwaliteit van leven, geen argumenten zijn gevonden om zwangere MS-patiënten meer vitamine D-supplementen te adviseren dan gezonde zwangeren.

In **hoofdstuk 8** werd een mogelijke associatie van vitamine A met aanvalsrisico onderzocht. Vitamine A is een multifunctionele vitamine die de aanmaak van Th17-cellen kan onderdrukken; deze zijn mogelijk betrokken bij het ontstaan van MS-aanvallen. Bovendien stimuleert vitamine A de vorming van regulatoire T-cellen (Treg). Daarom kan worden verondersteld dat vitamine A lager is in patiënten dan in controles, en dat vitamine A het aanvalsrisico in RRMS-patiënten kan verlagen. We hebben all-trans-retinolconcentraties gemeten in een case-controlstudie in 31 RRMS-patiënten en 29 gematchte controles. Verder hebben we in een prospectieve longitudinale studie in 73 RRMS-patiënten vitamine A gemeten in monsters die elke 8 weken zijn afgenomen. Gemiddelde vitamine A-concentraties waren lager in patiënten (2.16 µmol/l) dan in controles (2.44µmol/l) maar dit verschil was net niet significant (p=0.05). In de longitudinale studie werden all-trans-retinolconcentraties in tertielen ingedeeld: laag (<2.9 µmol/l), gemiddeld (2.9-3.7 µmol/l) en hoog (>3.7 µmol/l). We vonden dat de aanvalsfrequentie niet was geassocieerd met serum all-trans-retinolconcentraties (p>0.2), in univariate noch in multivariate analyse. Serumconcentraties van vitamine A en D waren gecorreleerd, maar met een zwakke correlatie (r=0.15). We concludeerden dat er evidentie is voor een rol van vitamine A in het ziektebeloop van MS.

In **hoofdstuk 9** beschrijven we een onderzoek naar het verband tussen osteopontinconcentraties en aanvalsrisico. Osteopontin is een multifunctioneel molecuul, dat gesuggereerd werd als biomarker voor ziekteactiviteit in MS, gebaseerd op pathologiestudies, dierexperimenteel onderzoek en studies naar osteopontinlevels in RRMS-patiënten. In een prospectieve longitudinale studie werden bloedmonsters voor osteopontinonderzoek elke 8 weken afgenomen bij 58 RRMS-patiënten. Osteopontinconcentraties werden bepaald met een commercieel verkrijgbare enzyme-linked immunosorbent assay (ELISA). De associatie tussen osteopontinlevels en aanvalsrisico in de eerste, tweede, derde en vierde week na

bloedafname werd berekend met behulp van generalized linear mixed-effects models. We vonden geen associatie tussen osteopontin en het risico op een aanval. We vonden ook geen verschil in osteopontin tussen patiënten met actieve en stabiele ziekte, en geen verband met EDSS-score. Ook was er geen verschil tussen patiënten behandeld met interferon en onbehandelde patiënten, en geen verband met infecties. Hoewel deze resultaten een rol voor osteopontin in de pathofysiologie van MS niet uitsluiten, concludeerden we dat osteopontin als marker voor ziekteactiviteit in MS-patiënten niet nuttig lijkt.

Samenvattend kunnen patiënten met monofasisch CIS en patiënten met (toekomstig) MS onderscheiden worden op het moment van CIS met behulp van de nieuwste diagnostische criteria, de aanwezigheid van vermoeidheid, de aanwezigheid van corpus-callosumlaesies en oligoclonale banden in de liquor. In patiënten met RRMS zijn vitamine D-concentraties geassocieerd met het risico op een aanval. Om een complexe ziekte als MS beter te kunnen begrijpen, en om voorspellende factoren te identificeren die uiteindelijk zouden kunnen leiden tot de ontdekking van nieuwe behandelingen zijn studies van het natuurlijk beloop van de ziekte van groot belang. Om samenwerken tussen meerdere centra te vereenvoudigen is het noodzakelijk dat data en samples van grote groepen patiënten op een transparante en eenduidige manier worden verzameld, niet alleen voor biomarkeronderzoek maar ook voor het onderzoek naar genetica en gen-omgevingsinteracties.

Appendix 1 - Fatigue Severity Scale

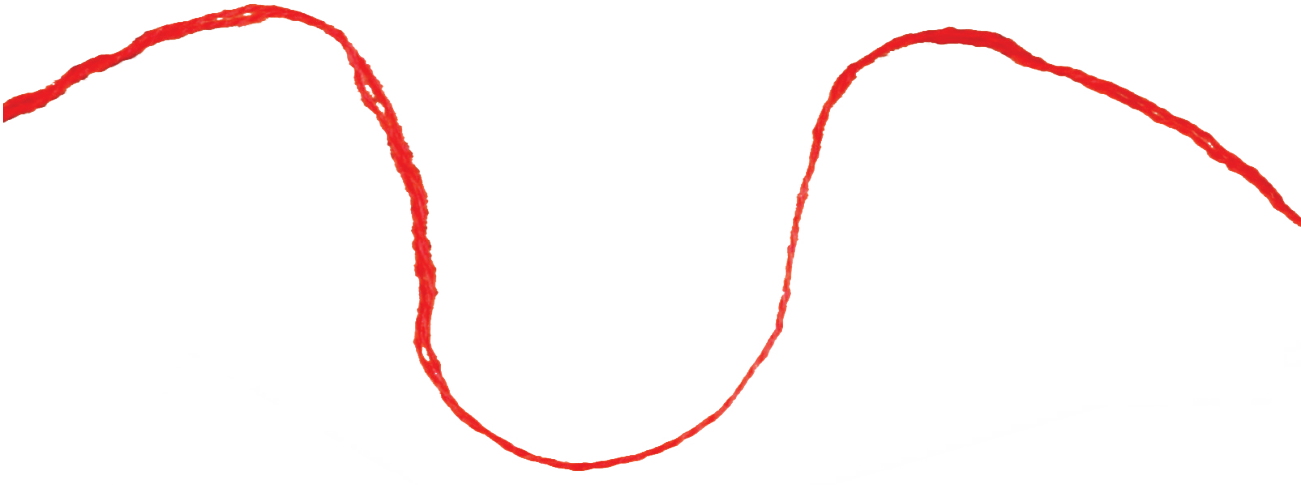
Vermoeidheid

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 de 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

Chapter **12**

Epilogue



List of abbreviations

CDMS	clinically definite multiple sclerosis
CIS	clinically isolated syndrome
CNS	central nervous system
CSF	cerebrospinal fluid
DIS	dissemination in space
DIT	dissemination in time
EBV	Epstein-Barr virus
EDSS	expanded disability status scale
FSS	fatigue severity scale
MRI	magnetic resonance imaging
MS	multiple sclerosis
OCB	oligoclonal bands
PPMS	primary progressive multiple sclerosis
RRMS	relapsing-remitting multiple sclerosis
SPMS	secondary progressive multiple sclerosis

Dankwoord

Voor het onderzoek leidend tot dit proefschrift zijn we van Amsterdam naar Rotterdam verhuisd. Een grote stap, die door veel Amsterdammers met argwaan werd aanschouwd. Maar Rotterdam heeft ons met open armen ontvangen. We hebben ontzettend veel fijne, leuke mensen om ons heen gekregen. Vooral in de vervelende periode na de geboorte van Jippe heb ik me erg gesteund gevoeld. Er zijn dan ook een heleboel mensen die ik wil bedanken.

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About the author

Tessel Runia was born on March 4th 1981 in Wageningen. She graduated in 1999 from the Stedelijk Gymnasium in Breda, and proceeded to study architecture at Delft University of Technology. In 2001 she moved to Amsterdam to study medicine at the University of Amsterdam. After obtaining her medical degree in 2008, Tessel started working at the Department of Neurology at the Onze Lieve Vrouwe Gasthuis in Amsterdam. In 2009 she started her PhD research at the Rotterdam MS Center ErasMS under supervision of Prof. R.Q. Hintzen, and succeeded Dr. Naghmeh Jafari as coordinator of the prospective PROUD-study (PRedicting the OUtcome of a Demyelinating event). From April 2014 onwards she works as resident in neurology at the Erasmus MC University Hospital in Rotterdam (head: Prof. P.A.E. Sillevis Smitt). In 2015, she will move to San Francisco, CA, USA, for 6 months with her husband Hans and their two children, to work on a research project on the influence of microbiota on the adaptive immune response in MS at the Baranzini lab at UCSF MS Center.

List of publications

Runia TF, Neuteboom RF, De Groot CJM, De Rijke YB, Hintzen RQ. The influence of vitamin D on postpartum relapse and quality of life in pregnant multiple sclerosis patients. Eur J Neurol. 2014

Runia TF, Jafari N, Siepman DA, Hintzen RQ. Fatigue at time of CIS is an independent predictor of a subsequent diagnosis of multiple sclerosis. J Neurol Neurosurg Psychiatry. 2014

Runia TF, Van Meurs M, Nasserinejad K, Hintzen RQ. No evidence for an association of osteopontin plasma levels with disease activity in multiple sclerosis. Mult Scler. 2014; 20(12):1670-1.

Runia TF, Hintzen RQ. The role of vitamin D in MS. Tijdschr Neurol Neurochir. 2014;115: 26-30.

Runia TF, Hop WC, de Rijke YB, Hintzen RQ. Vitamin A is not associated with exacerbations in multiple sclerosis. Mult Scler Relat Disord. 2014; 3(1): 34-39.

Runia TF, Jafari N, Hintzen RQ. Application of the 2010 revised criteria for the diagnosis of multiple sclerosis to patients with clinically isolated syndromes. Eur J Neurol. 2013;20(12):1510-6.

Runia TF, van Pelt-Gravesteijn ED, Hintzen RQ. Recent gains in clinical multiple sclerosis research. CNS Neurol Disord Drug Targets. 2012;11(5):497-505.

Runia TF, Hop WC, de Rijke YB, Buljevac D, Hintzen RQ. Lower serum vitamin D levels are associated with a higher relapse risk in multiple sclerosis. Neurology. 2012;79(3):261-6.

PhD portfolio

1. PhD training		
Courses	Year	Workload (ECTS)
Writing successful grant proposals	2009	0.8
Basiscursus Regelgeving Klinisch Onderzoek (BROK)	2010	0.9
Infections and inflammations of the central nervous system; Imaging and clinical symptoms	2010	0.6
Biostatistics for clinicians	2010	1.0
Biostatistical methods I: basic principles	2011	5.7
Biostatistical methods II: popular regression models	2011	4.3
Biomedical English writing and communication	2011	4.0
Introduction to clinical and public health genomics	2011	2.0
SNP course VIII	2011	1.5
EDSS course	2012	0.2
Neuro-immunology course	2010, 2012	1.0
Seminars and workshops		
Basiscursus MS	2009	0.4
MSMS symposium	2012	0.2
Oral presentations		
Meeting of the Dutch MS Research Foundation	2012	1.4
Wetenschappelijke vergadering NVN	2014	1.0
ErasMS symposium; Environmental factors in MS	2012	1.4
Poster presentations		
ECTRIMS (8 posters)	2010 - 2014	8.0
Wetenschappelijke vergadering NVN (2 posters)	2011	1.5
Meeting of the Dutch MS Research Foundation	2011	1.0
(Inter)national conferences		
Congress of the European Committee for Treatment and Research in Multiple Sclerosis (ECTRIMS)	2011, 2012, 2014	3.0
Meeting of the Dutch MS Research Foundation	2009 - 2013	1.4
Wetenschappelijke vergadering NVN	2011, 2014	0.8
2. Teaching		
Lecture 'Optic neuritis, MS and NMO', Rotterdam Eye Hospital	2012	0.4
Lecture 'Pregnancy and MS', Research Master Programme Infection & Immunity (MolMed), Rotterdam	2013	1.0
Total ECTS		43.5