Multiple sclerosis -the impact of environmental- and lifestyle factors

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Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2021

UNIVERSITY OF BERGEN

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Date of defense: 15.04.2021

Year: 2021

Title: Multiple sclerosis -the impact of environmental- and lifestyle factors

Name: Silje Agnethe Stokke Kvistad

Print: Skipnes Kommunikasjon / University of Bergen

Scientific environment

This thesis was performed at the Norwegian Multiple Sclerosis Competence Centre, Department of Neurology, Haukeland University Hospital and the Department of Clinical Medicine (K1), University of Bergen, Norway.

The work was also influenced by the Hormone Laboratory and the Department of Immunology and Transfusion medicine at Haukeland University Hospital.

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Funded by: University of Bergen

Acknowledgements

First and foremost I would like to express my deepest gratitude to my main supervisor Øivind Torkildsen. Your scientific knowledge, being a living encyclopedia on the field along with your positivity and confidence has been essential for my work. I greatly appreciate your enthusiasm and support and highly value your good advises. Your response-rate is supersonic and your door is always open for interesting discussions. I will forever be thankful to you for introducing me to the great world of science. As well as for including me in the MS group in spite of my changing directions. I greatly admire you as a doctor and as a person.

My sincere gratitude also goes to my co-supervisor Kjell-Morten Myhr. Your high scientific capacity and knowledge has been invaluable. I also appreciate your high energy and humerous remarks brightening the days. Even at your most busy times you have always been available for questions and scientific inspiring ideas. I am truely grateful for being part of your team.

I would like to express my gratitude to the MS patients in the OFAMS study, and all involved personnel, who have made this project possible. I am thankful to all my co-writers for their feedback and contribution; Jūratė Šaltytė Benth, Kristin Amsrud Løken, Trygve Holmøy, Stig Wergeland, Søren J. Bakke, Antonie G. Beiske, Kristian S. Bjerve, Harald Hovdal, Finn Lilleås, Rune Midgard, Tom Pedersen, Annika E. Michelsen, Pål Aukrust and Thor Ueland. I would like to thank Tove Marøy and Hanne Linda Nakkestad at the Neurological research laboratory for their assistance. I am especially thankful to Jūratė Šaltytė Benth for invaluable help with statistical work.

I feel privileged that I have been part of the MS group and got to perform my research at the Norwegian Multiple Sclerosis Competence Centre, homing exclusively warm and amazing people. I would like to thank all my colleagues for fruitful discussions and for brightening my days. A special thanks to Hilde Marie Torgauten, Hilde Norborg and Hanne Marie Bø Lunde for great support during this time. I am thankful to Lars Bø for including me in the team. I admire your positivity and great knowledge. At last I want to thank my dear friend and colleague Kristin Wesnes. I truely value our

scientific, and not so scientific conversations. Thanks for proofreading and improving my thesis. You are walking sunshine.

I am grateful to Ole-Bjørn Tysnes, Jørn Sagen and Einar Klæbo Kristoffersen for giving me the possibility to coordinate my work as a doctor with my MS project. Jørn, your cheerful humming in the corridors can light up any day and your love for what you do is so inspiring. If you were a hormone I`m sure you would be an endorphine. Einar, I am greatly endepted to you for letting me into the amazing world of immunology. Thanks for being so understanding at times when I had to be absent due to my PhD work.

During the years of my project I have worked at different places. I am thankful for the stream of good people I was able to work with, all my great colleagues at the Hormone laboratory and at the Department of Neurology. Especially thanks to Jana Midelfart Hoff and to Solveig Glad for recruiting me to the field of MS.

I am grateful for my work at AIT and for all my wonderful colleagues there, making a scientific inspiring, but also warm and enthusiastic environment. Especially thanks to Einar Klæbo Kristoffersen, Guro Melve, Tor Hervig, Knut Liseth, Silje Helland Kaada, Kristin Gjerde-Hagen, Bjarte Skoe Erikstein, Torunn Apelseth and Elin Opheim. Finer colleagues are nowhere to be found and I feel blessed for working with you all.

I would like to thank my dear friends Anette, Irene, Tien, Gunnhild and Hege Marie for sharing the good times as students and for our everlasting friendships. Special thanks to Hege Marie for our resuscitating dinner-dates and my “sister” Marianne for being my life-west so many times.

Most, I am grateful and deeply thankful to my family. To my mother and father, Olaug and Svein Ole for their invaluable and endless love. For turning up at my door with pancakes and hot cocoa in the worst periods of exams and later for baby-sitting at times I had to work. I truely value your support and unfaltering faith in me. I also want to thank my extended loving family; Randi and Birger, Unni and Steinar, Hans and Kheam, Susanne and Kjell-Arne.

Last, but most importantly I am forever grateful for my wonderful home-team, my dear husband Christopher for being my working day and my evening rest, and my wonderful children Victor, Helena, Sverre and Ella. You are the treasures of my life, making me feel truely blessed every day.

Bergen, November 2020

Silje Agnethe Stokke Kvistad

Contents

Abbreviations

AIM	Apoptosis inhibitor of macrophage
ALCAM	Activated leucocyte adhesion molecule
APC	Antigen Presenting Cell
BAbs	Blocking antibodies
BMI	Body Mass index
CCL21	Chemokine (F-C-motif) ligand 21
CDMS	Clinically definite multiple sclerosis
CIS	Clinical isolated syndrome
CNS	Central Nerve system
CXCL16	Chemokine (C-X-C) ligand 16
CSF	Cerebrospinal fluid
DMT	Disease modifying drugs
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded Disability Status Scale
HLA	Human leucocyte antigen
IFNβ	Interferon Beta
IL1-Ra	Interleukin 1 receptor
IR	Insulin resistance
IL-6	Interleukin 6
MMP-9	Matrix metalloproteinase 9
MS	Multiple sclerosis
MRI	Magnetic resonance imaging
NAbs	Neutralizing antibodies
NEDA	No evidence of disease activity

NMO	Neuromyelitis optica
OCB	Oligoclonal bands
OPN	Osteopontin
OPG	Osteoprotegerin
OR	Odds ratio
PDDS	Patient Determined Disease steps
PPMS	Primary progressive multiple sclerosis
PTX3	Pentraxin 3
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
sTNF-R1	Soluble tumor necrosis factor receptor 1
SPMS	Secondary progressive multiple sclerosis
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alfa
WHO	World Health Organization

Abstract

Background: Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), likely caused by an interaction of genetic and environmental factors. Epstein-Barr virus infection, low serum vitamin D levels, smoking and obesity increase the risk of MS. However, knowledge of their effect on disease activity and progression have been limited.

Objective: The main objective was to explore the role of different environmental and lifestyle factors for MS disease activity. In more detail, we sought to evaluate whether there is an association between tobacco use or body mass index (BMI) and MS disease activity. We also explored the potential of two adipokines, leptin and adiponectin as biomarkers for disease course or interferon-beta (IFNβ) treatment response in MS.

Methods: All data in our studies were based on the OFAMS study, a randomized placebo-controlled multicenter study of 92 patients with relapsing-remitting MS (RRMS) that was conducted between 2004 -2008. The patients were followed for 24 months with repeated magnetic resonance imaging (MRI) of the brain, blood tests and clinical evaluations, 6 months prior to and 18 months during IFNβ-treatment.

For the current thesis, we analyzed serum samples for cotinine, a biomarker for tobacco use, and the adipokines leptin and adiponectin. For the first study, the patients were categorized as tobacco-users and non-tobacco-users according to their serum cotinine level. For the second and third study, patients were categorized based on the World Health Organisation (WHO) classification of BMI into three groups; normal weight patients (BMI< 25 kg/m^2), overweight patients (BMI 25-30 kg/m^2) and obese patients (BMI >30 kg/m^2). All analyses were adjusted for age, gender and BMI.

Results: We did not find any association between tobacco use and MRI activity (paper I). Further, there was no difference between tobacco users and non-tobacco users

regarding baseline Expanded Disability Status Scale (EDSS) score, EDSS-progression or relapse-rate. For tobacco users, there was no correlation between serum cotinine levels and disease activity.

There was no difference in clinical and MRI activity between patients stratified by BMI prior to IFNβ-treatment. During IFNβ-treatment, 80 % of overweight or obese patients had MRI activity compared to 48 % in the group of normal weight patients (p=0.001). The number of patients obtaining NEDA (no evidence of disease activity)-status differed according to BMI; 26 % in the normal weight group compared to only 13 % in the group of overweight and obese patients (p=0.05) (paper II).

There was no association between serum levels of leptin or adiponectin and MRI disease activity (paper III). The serum levels of leptin were lower and the levels of adiponectin higher during IFNβ-treatment compared to the treatment-naïve period, reflecting the anti-inflammatory effect of the drug.

Conclusion: In our studies, we found no direct association between tobacco use or BMI and MS disease activity. During IFNβ-treatment fewer of the overweight and obese patients obtained NEDA-status compared to patients with normal weight, indicating that BMI could affect IFNβ-treatment response. Serum levels of leptin and adiponectin seem not to be suited as biomarkers for disease activity or IFNβ-treatment response in MS.

List of Publications

I. Kvistad SS, Myhr KM, Holmøy T, Benth JŠ, Løken-Amsrud KI, Wergeland S, Beiske AG , Bjerve KS, Hovdal H, Lilleås F, Midgard R, Pedersen T, Bakke SJ, Torkildsen Ø.

No association of tobacco use and disease activity in multiple sclerosis

Neurol Neuroimmunol Neuroinflamm, 2016 Jul 14;3(4):e260.

II. Kvistad SS, Myhr KM, Holmøy T, Benth JŠ, Wergeland S, Beiske AG, Bjerve KS, Hovdal H, Lilleås F, Midgard R, Pedersen T, Bakke SJ, Michelsen AE, Aukrust P, Ueland T, Sagen JV, Torkildsen Ø.

Body mass index influence interferon-beta treatment response in multiple sclerosis.

J Neuroimmunol. 2015 Nov 15;288:92 -7.

III. Kvistad SS, Myhr KM, Holmøy T, Benth JŠ, Wergeland S, Beiske AG, Bjerve KS, Hovdal H, Midgard R, Sagen JV, Torkildsen Ø.

Serum levels of leptin and adiponectin are not associated with disease activity or treatment response in multiple sclerosis

J Neuroimmunol. 2018 Oct 15; 323:73-77.

1. Introduction

Multiple sclerosis (MS) is an immune mediated disease of the central nervous system (CNS) characterized by inflammatory demyelination and axonal degeneration. The disease was first described in 1868 by a French neurologist, Dr. Jean-Martin Charcot [1] emphasizing three typical, but not pathognomonic clinical features of MS; intention tremor, nystagmus and scanning speech [2]. MS primarily affects young adults, typically presenting with subacute episodes of neurological deficits. The symptoms vary greatly according to the area of the demyelinating lesions in the CNS, but typical manifestations are visual disturbances, sensory loss, limb weakness and gait ataxia [3]. The exact cause of MS is still not known, but genetic factors in combination with environmental and/or lifestyle factors are probably involved [4, 5].

1.1 Pathogenesis

The major pathological mechanisms in MS are inflammation, demyelination and axonal degeneration. Initially the disease is predominated by an inflammatory process and presence of immune cells in the CNS [6]. As the disease evolves, the inflammation becomes less prominent and the pathological process consists mostly of neurodegeneration and sclerosis. The main neuropathological features of MS are demyelinated areas in the white and grey matter of the brain and spinal cord known as plaques, associated with axonal loss, and later brain atrophy [6, 7].

1.2 Descriptive epidemiology

The global prevalence of MS is estimated to vary from approximately 50-300 per 100.000 people [3]. A distinctive epidemiological feature of MS is an increasing prevalence with increased distance from the equator. Norway has a high prevalence of MS with around 200 patients per 100,000 and an incidence of about 8-10 per 100,000

[8, 9]. As for many autoimmune diseases there is a clear female to male ratio, and women's hazard of MS is about three times greater than for males [10].

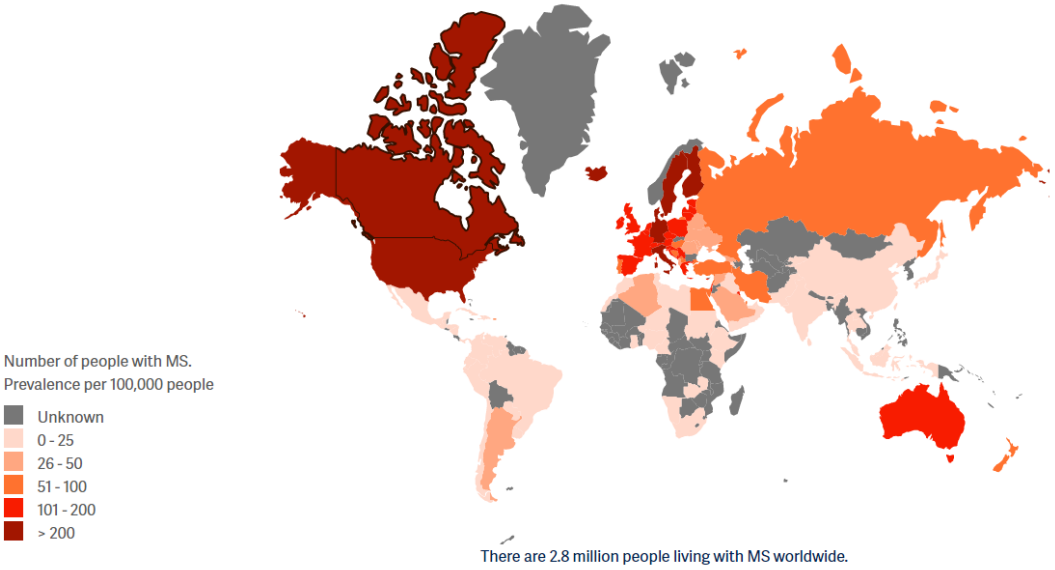

Figure 1. Global prevalence of MS in 2020

The figure illustrates the distribution of MS across the world with prevalence reported per 100.000 population. Reproduced with permission from Multiple Sclerosis International Federation-Atlas of MS-3rd Edition (September 2020).

1.3 Clinical characteristics

The disease onset is for most patients in the early adulthood. The patients typically present with loss of a neurological function depending on the location of the CNS lesion. Common presenting symptoms are; unifocal vision disturbances caused by optical neuritis, sensory symptoms, motor weakness, gait disturbance and bladder problems. The typical disease course consists of repeating subacute episodes of clinical

symptoms followed by symptom remission [3, 11]. The disease severity and relapse frequency is heterogenous and unpredictable at disease onset.

1.4 Diagnosis

MS is diagnosed based on a work-up of anamnestic information, clinical evaluation and additional tests including MRI and cerebrospinal fluid (CSF) analyses [12, 13]. The clinical neurological examination aims at detecting dissemination of disease in time and space. MRI T2-weighted scans show hyper-intensity (white) lesions in the CNS. CSF can be analysed for oligoclonal bands (OCB), indicating abnormal inflammatory response in the brain. Different classification and diagnostic criteria has been proposed over the years, but the current established diagnostic criteria for MS are "the McDonald Criteria", recently revised in 2017 [12].

Relapsing-remitting multiple sclerosis		
Number of clinical attacks	**Number of lesions with objective clinical evidence**	**Additional data needed for a diagnosis of MS**
≥ 2 clinical attacks	≥ 2	None
≥ 2 clinical attacks	1 (and a clear-cut historical evidence of a previous attack involving a lesion in a distinct anatomical location)	None
1 clinical attacks	≥ 2	Dissemination in time demonstrated by an additional clinical attack or by MRI ***OR*** Demonstration of CSF-specific oligoclonal bands.
1 clinical attack (Clinical isolated syndrome (CIS))	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI ***AND*** Dissemination in time demonstrated by an additional clinical attack or by MRI ***OR*** Demonstration of CSF-specific oligoclonal bands.

Primary progressive multiple sclerosis can be diagnosed in patients with:

- 1 year of disability progression (retrospectively or prospectively determined) independent of clinical relapse

Plus two of the following criteria:

- One or more T2-hyperintense lesions characteristic of MS in one or more of the following brain lesions: periventricular, cortical or juxtacortical, or infratentorial
- Two or more T2-hyperintense lesions in the spinal cord
- Presence of CSF-specific oligoclonal bands

Table 1. The revised McDonald criteria 2017 [12].

The 2017 McDonald criteria for diagnosis of MS in patients with an attack at onset (Relapsing-remitting MS) and by progression from onset (Primary progressive MS).

1.5 Classification of MS

There are different subgroups or phenotypes of MS depending on the clinical presentation and course. In 1996 the US National Multiple Sclerosis Society (NMSS) Advisory Committee on Clinical Trials in Multiple Sclerosis defined four different forms of MS; relapsing-remitting (RR), secondary progressive (SP), primary progressive (PP), and progressive relapsing (PR) [14]. Since the knowledge of disease pathology and mechanisms has evolved, the classification was revised in 2013 [15]. The new classification has emphasized disease activity, defined as relapses or MRI findings and disease progression. The new term “clinical isolated syndrome” was added as a subgroup and in addition all forms was subcategorized as active or non-active [16].

1.5.1 Relapsing-remitting MS (RRMS)

The most common form of MS is relapsing-remitting MS presenting with repeated clinical relapses. A clinical relapse is defined as; “A monophasic clinical episode with patient-reported symptoms and objective findings typical of multiple sclerosis, reflecting a focal or multifocal inflammatory demyelinating event in the CNS, developing acutely or sub-acutely, with a duration of at least 24 hours, with or without --recovery, and in the absence of fever or infection” [12]. The first single demyelinating episode is termed a clinically isolated syndrome (CIS) [17]. Most patients will have a second episode and be diagnosed with clinically definite multiple sclerosis (CDMS) [18]. The risk of CDMS is associated with number of MRI lesions, the presence of oligoclonal bands in the CSF, and inversely associated with age at CIS onset [17]. Patients with current brain lesions and oligoclonal bands in CSF have an 86 % risk of CDMS after a five year follow-up [18]. For patients diagnosed with RRMS the disease course is individual and unpredictable. Some patients have an aggressive disease with multiple MRI lesions and frequent clinical relapses, while others may not have another relapse for years. RRMS will for many patients develop into a secondary progressive MS (SPMS) in time. SPMS is characterized by a gradually increasing disease load and absence of the typical relapsing-remitting flow. There are no clear clinical, imaging, immunologic or pathologic criteria for the transition point to SPMS, and the diagnosis is often set retrospectively [3, 16].

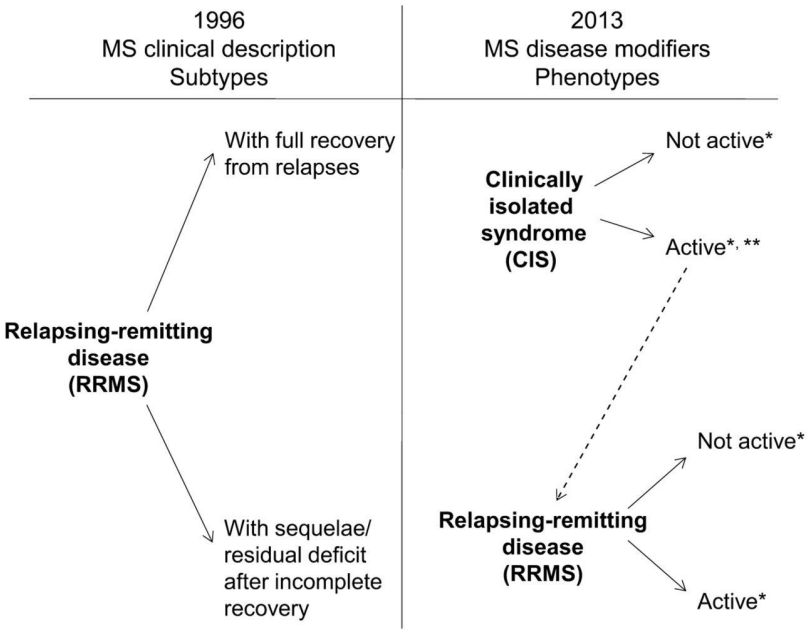

Figure 2. The 1996 and 2013 definition of MS subgroups for relapsing-remitting disease [16].**Activity determined by clinical relapses and/or MRI activity (contrast-enhancing lesions; new or unequivocally enlarging T2 lesions assessed at least annually); if assessments are not available, activity is "indeterminate". ** CIS, if subsequently clinically active and fulfilling current multiple sclerosis (MS) diagnostic criteria, becomes relapsing-remitting MS (RRMS). Permission to reproduce according to Creative Commons Attribution licence 3.0.*

1.5.2 Primary progressive MS (PPMS)

About 10-15 % of MS patients present with a gradually progressive load of symptoms with no clear relapses, and are diagnosed with primary progressive MS (PPMS) [3]. Unlike RRMS, PPMS is often diagnosed in patients that are 50-60 years old, and there is no evident gender ratio [19]. The most common clinical presentation is progressive spastic paraparesis [20]. Since PPMS is mostly dominated by a neurodegenerative

process, and not inflammation, most of the therapeutic choices in MS are ineffective in PPMS [19].

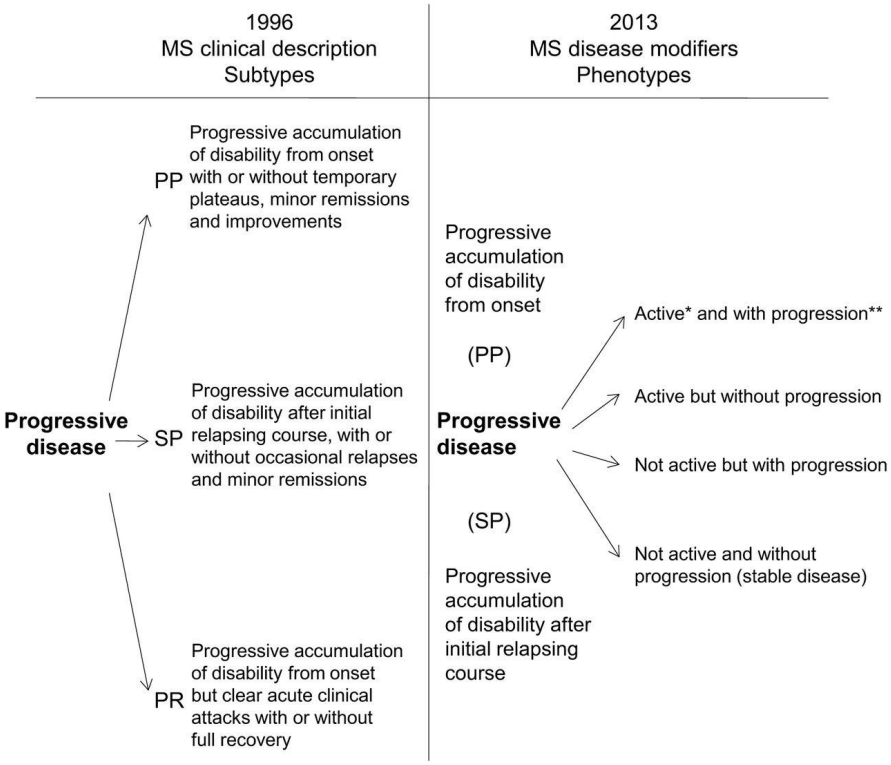

Figure 3. The 1996 and 2013 definition of MS subgroups for progressive disease [16]. ** Activity determined by clinical relapses assessed at least annually and/or MRI activity (contrast-enhancing lesions; new and unequivocally enlarging T2 lesions). **Progression measured by clinical evaluation, assessed at least annually. If assessments are not available, activity and progression are "indeterminate". Permission to reproduce according to Creative Commons Attribution licence 3.0.*

1.6 Treatment

There is no curable treatment for MS, but several immunomodulatory medications can reduce disease activity and disability progression [11, 21]. Most medications have been proven effective in RRMS, but so far few have shown an effect in slowing progression of PPMS [22]. All medications are mainly based on the same principle; to reduce the inflammatory burden by inhibiting the immune response. The medications are referred to as disease modifying therapies (DMT) [3]. Some medications are self-administred either as tablets (fingolimod, dimethyl fumarate, teriflunomide, cladribine, ozanimod, siponimod) or as subcutaneously or intramuscular injections (IFNβ and glatiramer acetate), while others are infused at the hospital (alemtuzumab, natalizumab, rituximab, ocrelizumab). All DMTs except cladribine are administrered as the same dosage for all patients, regardless of BMI. For the distinct choice of medication there are two therapeutic approaches; 1) escalation-strategy starting with a moderately effective medication and escalating to a more effective DMT if the patient presents with new signs of disease activity, and 2) induction-strategy starting with a higly effective therapy at once [3]. A few patients with an aggressive disease and poor response to DMTs may be offered autologous hematopoietic stem cell treatment [23]. During acute relapses the patients may benefit of intravenous high-dosage steroid treatment and in some cases plasma exchange [3].

Many MS patients suffer from different symptoms that affect their quality of life such as fatigue, cognitive impairment, spasticity, pain, impaired ambulation and bowel-, bladder-, and sexual-dysfunction [3]. There is often a need for a multidisciplinary approach including different medications, exercise, physiotherapy and cognitive rehabilitation.

Modifiable environmental factors also seem to play a part in the disease control [24]. So far, vitamin D supplements and smoke cessation is recommended [25]. It is possible that the maintenance of normal body weight and a healthy diet can have a beneficial effect on the disease course via immunological mechanisms, but this need further studies.

For evaluating and optimizing the DMT, the term NEDA -"no evidence of disease activity" has been introduced as an outcome measure in MS [26, 27]. When the term was first introduced, NEDA was defined as a composite of no relapses, no EDSS-progression and no MRI activity (new or enlarging T2-lesions or Gd-enhancing lesions). The description for an ideal treatment goal was based on data collected in clinical trials [28, 29]. With this approach the inflammatory component of the disease was emphasized and not the ongoing neurodegenerative damage. Other parameters was therefore suggested to be added including brain volume, neurophysiological outcomes like cognitive function, and neurofilament levels in blood or CSF [26, 30].The term NEDA-4 was introduced including the absence of MRI brain atrophy [31]. Recently, neurofilament in CSF has been demonstrated as a useful biomarker of disease activity, and are suggested incorporated in the term NEDA-5 [32]. It is likely that the definition of NEDA will evolve in the future including more measurements.

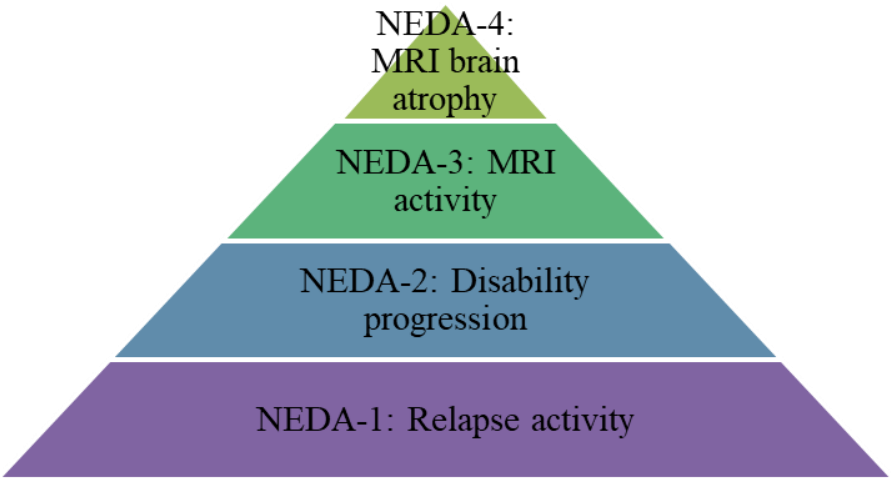

Figure 4. NEDA-4.

Illustration of the optimal therapeutic goal in MS, no evidence of disease activity. For each step in the pyramid the patient has no sign of the disease activity parameter illustrated, hence a more optimal treatment response.

1.7 Prognosis

The MS disease course is unpredictable. Some patients have a “benign MS” with one or a few clinical attacks during the whole disease span, while others experience a large disease load within just a few years. There are, however, some useful prognostic factors. Older age at time of diagnosis, male gender, frequent relapses initially and presence of lesions in the spinal cord are all considered predictors of a higher risk of disability accumulation and progressive disease [33]. A high baseline MRI lesion load and oligoclonal bands in the CSF are factors highly predictive of conversion of CIS to MS [18].

Recent therapeutic developments have substantially improved the long-term prognosis for MS patients, with a possibility to halt or slow down disease progression a longer time period before transition to SPMS. In a recent study, only 10 % of MS patients had an EDSS ≥6, 15 years after the diagnosis, compared to more than 50 % in earlier studies [34].

Autologous hematopoietic stem cell treatment in MS has shown very promising results with >70 % of the patients obtaining NEDA-3-status 10-year post-treatment [35]. Nevertheless, there is still no curable treatment, and even with less disability the patients struggle with other symptoms. This is reflected by the fact that working status among MS patients have changed little over time. In a recent study more than 80 % of the patients were unemployed 10 years after being diagnosed with MS [36].

2. Risk factors for MS

MS is most likely caused by an interaction of genetic and environmental factors [37-39]. Although much is still unknown, the scientific evidence so far supports the hypothesis of a genetic susceptible individual exposed to one or more environmental events that trigger the development of the disease. The role of genetics in MS was early recognized through familial aggregation [40], but the difference in MS prevalence by latitude and the results of migration studies made it evident that also environmental factors are involved [41, 42].

The prevalence of MS has increased over the last decades, to a greater extent than what can solely be explained by better diagnostics [43]. This has encouraged the search of other environmental risk factors in addition to the established factors Epstein-Barr virus (EBV), smoking, obesity and vitamin D. Excessive intake of salt, organic solvents, air pollution, western diet and shift work are factors suggested to possibly increase the risk of MS, whereas alcohol use, excessive physical activity, cytomegalovirus infection, snuff (oral tobacco) and high coffee consumption may possibly reduce the risk [4, 44-53]. As MS is a chronic disease with an unpredictable course, recognizing risk factors and focusing on disease prevention is important.

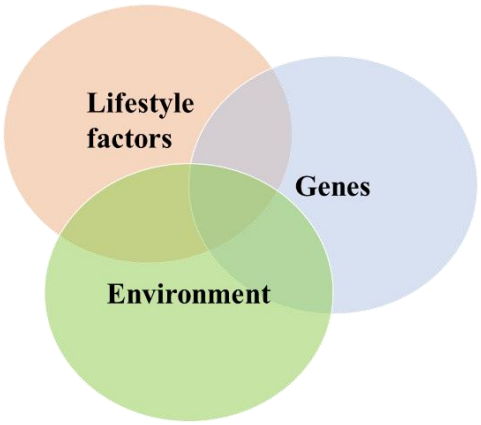

Figure 5. MS is caused by interaction of several factors.

2.1 Genetics

MS is not considered a genetic disease, but the role of genetics in MS were early known through twin studies. The concordance rate for monozygotic twins is around 25-30 % whereas dizygotic twins and other siblings have a risk of 2-5 % [54, 55]. The lifetime risk for individuals with northern European ancestry is about 0,1-0,2 % [54]. The risk of MS is strongest associated with alleles of the human leukocyte antigen (HLA), and genotypes in the HLA locus have been found to account for as much as 30 % of the genetic susceptibility in MS [56, 57]. Changes in amino-acid positions in the peptide-binding groove of the HLA-molecule affect the recognition and binding of antigens [58]. Patients with the genotype DRB1*1501 are three times more likely to develop MS than non-carriers, [3] and for homozygous carriers the risk increases six-fold [59]. Also HLA DRB1 *1303, *0404, *0401 and *1401 are associated with MS, in addition to a great number of other immune-related risk alleles, many connected with the role of regulatory T cells [60]. Genome-wide association studies (GWAS`s) have until now identified more than 200 genetic regions associated with MS outside of the HLA [58, 61]. Interestingly, some environmental and lifestyle factors have shown interactions with genetic risk loci leading to an increased disease risk [4, 62-64].

2.2 Environmental risk factors

Several environmental factors are associated with MS, and many of them seem to affect both MS risk and the disease course. The exact pathogenetic mechanisms for their interplay are plural and not well known, but it is likely that all assess their impact through ways of affecting the immune system [38].

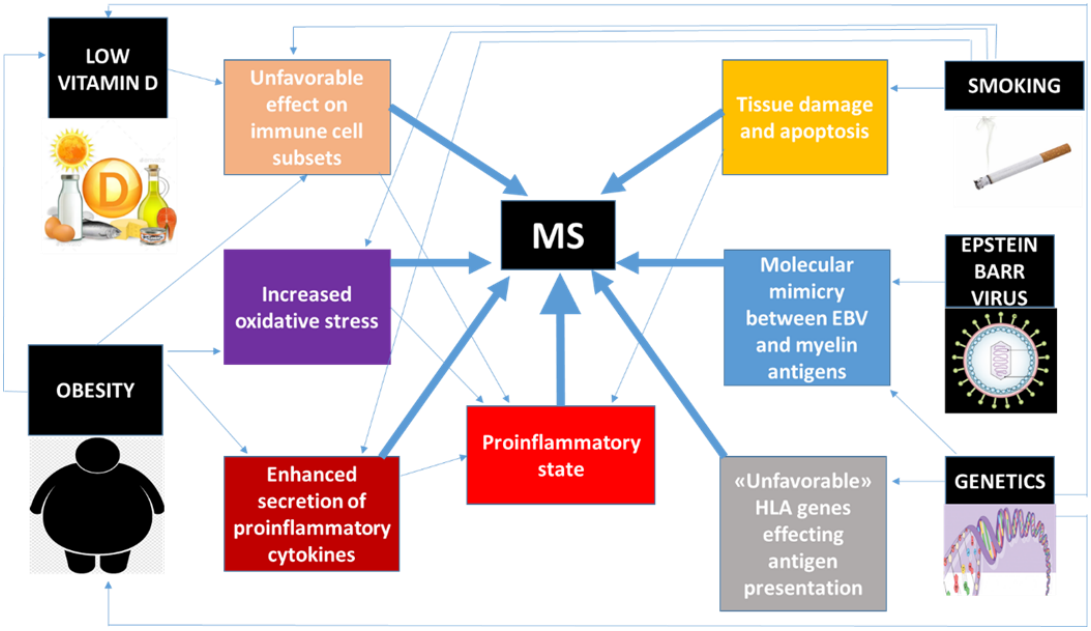

Figure 6. Overview of different risk factor for MS.

Genetic and environmental factors affect the risk of MS. Some of the possible pathogenetic mechanisms are illustrated.

2.2.1 Epstein-Barr virus

Epstein-Barr virus (EBV) and infectious mononucleose was the first, and has since been the strongest environmental factor known to be associated with MS [65, 66]. Many studies indicate that EBV exposure is required for the development of MS, and this was emphasized in a longitudinal study following seronegative military personnel, demonstrating that all became EBV seropositive before MS onset [67, 68]. EBV is consistently associated with MS risk across different regions and ethnic groups [69]. Higher Epstein-Barr nuclear antigen-1 antibody serum levels are associated with an increased MS risk [70, 71]. The antibody levels in serum may also affect the MRI disease activity, [72, 73] although the results of different studies are conflicting [74-76]. The immunopathological mechanism connecting EBV and MS are not well understood. A study exploring brain tissue described evidence of EBV-infection in

brain-infiltrating B-cells, and hypothesized that EBV-infected autoreactive B-cells enter and accumulate in the CNS, where they produce antibodies and stimulate autoreactive T-cells [77]. Other explanations may be molecular mimicry between EBV and myelin antigens or a general inflammation caused by EBV leading to bystander damage in CNS [66, 78].

2.2.2 Vitamin D

Vitamin D was suspected early on to play a part in MS due to the increasing MS incidence with increased latitude, as sunlight exposure is the main factor for vitamin D serum levels, along with a diet of fatty fish. Over the years, a large body of evidence suggests a likely role for vitamin D in MS [79]. A large study of American soldiers showed an association between 25(OH)D serum levels and the risk of MS, where individuals with the highest serum levels had a 62 % lower risk [80]. Similar findings were also reported in a Swedish study [81]. The serum level in newborns seem to influence the risk of MS in later life, with lower MS risk for newborns with higher 25(OH)D levels [82]. Several studies show the benefit of vitamin D supplementation to prevent MS disease later [83-85]. Also genetic factors influencing vitamin D metabolism has been associated with MS risk [86].

Furthermore, vitamin D levels seem to affect the disease course in MS. Higher serum levels are associated with decreased MRI activity, [87-89] and a lower number of relapses [87, 90]. The role of vitamin D may be explained by its immunemodulatory capacity to increase and stimulate regulatory T-cells and promote an anti-inflammatory serum cytokine profile, priming the immune system towards an anti-inflammatory state [91, 92]. Several studies have described a beneficial immunological response of vitamin D supplementation for MS patients [25, 93, 94]. However, two recent randomized studies did not find a clear effect of highdose Vitamin D3 supplementation as add-on to DMTs on disease progression, although supplementation had beneficial effects regarding MRI activity [95, 96]. Daily vitamin D supplementation is advised for all MS patients [25].

2.2.3 Tobacco smoking

Smoking is a well established environmental risk factors for MS [65, 97-99]. Although a widespread habit worldwide, smoking is more prevalent among MS patients than in the general population [100, 101]. Three large prospective studies on women all found that smoking of more than 15 cigarettes a day was associated with an increased risk of MS, with a relative risk in the studies varying from 1.4 to 1.7 [102, 103]. A Swedish study found similar results for men [104]. Further, several case-control studies have reported an association [105-108], although a few found contradictive results [109, 110]. The serum levels of cotinine, a nicotine metabolite and biomarker for tobacco use, are also associated with MS risk [99]. A large meta-analysis concluded that smoking increase the MS risk by more than 50 % [111]. The association was stronger for men and for current smokers than for previous smokers, and in addition there is a dose-response relationship between smoking habits and MS [111, 112]. Passive smoking also increases the risk of MS, with an increased risk the longer the duration of exposure [113].

Tobacco use is associated with a more severe disease course in MS [114-116]. In CIS, smoking has been shown to increase the MRI activity and cause an earlier conversion to CDMS [117, 118]. A number of studies report a more rapid disease progression in smoking patients, and the progression is positively correlated to cigarette consumption [115, 119-121]. A study found that smokers reached EDSS scores of 4 and 6 significantly faster than non-smokers [115]. Interestingly, for ex-smokers the disease progression-rate was not higher than for the non-smokers. The conversion from RRMS to SPMS has also been reported to be promoted in smokers [122]. A study that followed 179 MS patients for more than five years found a 3.6 times higher risk of convertion to SPMS among smokers compared to never-smokers [122]. Although most studies confirm an association with disease progression, there have been contradictory results [123]. In a Dutch survey of 364 MS patients, smoking habits did not influence disease progression, and an American study found no association between serum cotinine levels and MS disease progression [124, 125].

The mechanism of how smoking increases MS disease progression and conversion to SPMS is not clear, but possible explanations could be increased inflammation and disease activity or degenerative processes [122, 126]. Relatively few studies have explored the role of tobacco use in inflammatory disease activity in MS, and the results have been conflicting [119, 125, 127-129]. Some studies report no association between smoking and relapse rate, while two studies of patients with CIS found increased MRI activity among smokers [118, 119, 128, 130]. Smoking is reported to increase the lesion load and brain atrophy in MS patients, and serum levels of neurofilament, a biomarker of neuroaxonal injury is also increased in smoking MS patients [131-136].

The pathogenetic mechanisms of smoking in autoimmune diseases are numerous [137, 138]. Tobacco smoking has a profound effect on the immune system, by its many ways of stimulating towards a pro-inflammatory immune state in addition to several immune suppressive effects [126, 139, 140]. The components of smoke have an impact on the immune-cell subsets causing elevated circulatory T-cells and augmented auto-reactive B-cell activity and a dysfunction of antigen-presenting cells (APC) [139, 141]. In addition, pro-inflammatory cytokines are increased. This may lead to CNS inflammation and a direct toxicity on neurons. Recent research results indicate that smoking lead to a dysregulation in gene expression in peripheral blood cells which may contribute to CNS inflammation [137]. A study showed that two systems affected by smoking, the renin-angiotensin system and indoleamine 2, 3-dioxygenase activity lead to reduced number of regulatory T-cells in MS [142]. Tobacco smoking further affects the antigen exposure due to tissue hypoxia and cellular necrosis. There may also be a direct injury on the blood brain barrier (BBB), whereas nitric oxide contribute to BBB dysfunction [137]. Tobacco smokers have increased serum levels of metalloproteinase-9 that are known to promote migration of autoreactive immune cells across the BBB [143]. Nicotine has been shown to increase the BBB permeability, but may also cause beneficial effects in MS by inhibiting immune responses, hence causing an anti-inflammatory effect [44]. Some studies also report that nicotine may possess a protective effect in Experimental autoimmune encephalomyelitis (EAE) [144-146]. It is thereby likely that the main acting agent causing the CNS damage in MS is not

primarily nicotine, but other components in tobacco. Corroborating this, a study on the use of oral tobacco (snuff), found a dose-dependent negative association between the risk of MS and oral tobacco [147], indicating that chronic lung irritation from smoking, and not nicotine in itself could be the cause of the association. This theory fits well with reports suggesting sources of lung irritation as air pollution and organic solvents to increase the risk of MS [47, 50, 148-151]. Results from animal studies also emphasize the importance of the mode of delivery as autoreactive T-cells were found to be activated in the lungs in EAE mice [152].

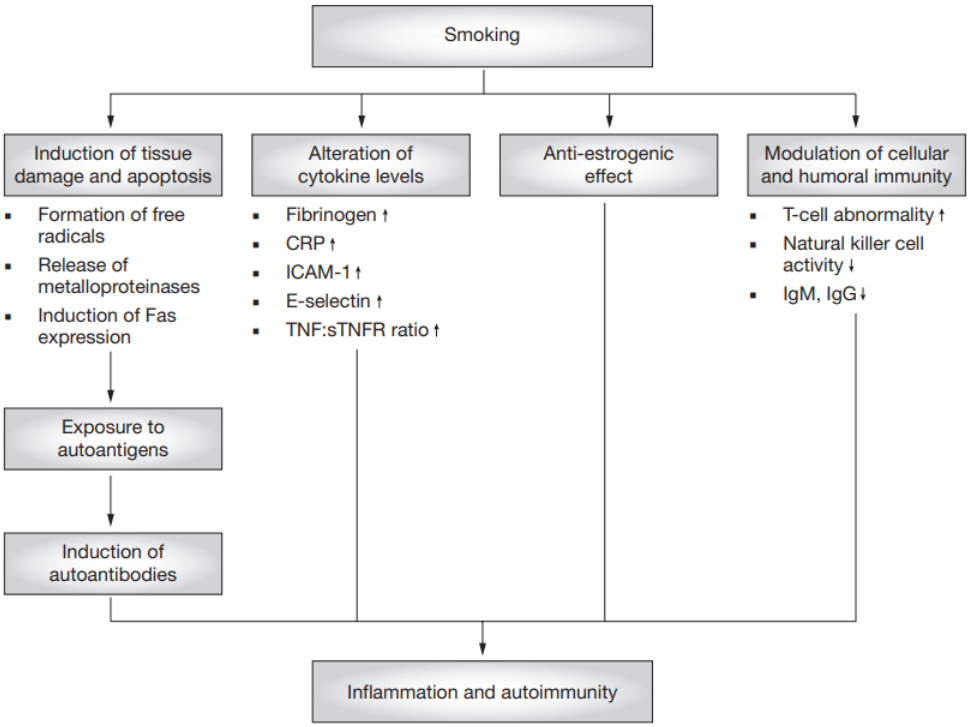

Figure 7. The effect of smoking.

Illustration of several possible mechanisms of how smoking may affect the immune system and contribute to autoimmunity. Reproduced with permission from Springer nature, Copyright © 1969, Nature Publishing Group [153].

2.2.4 Obesity

The World Health Organization (WHO) defines obesity as an excessive fat accumulation that presents a risk to health. Individuals with body mass index (BMI) >25-30 are considered to be overweight, and individuals with BMI >30 are obese. Obesity is associated with cardiovascular diseases, cancers and autoimmune diseases [154, 155]. The epidemic of obesity is one of the worlds` greatest public health challenges. More than 1.9 billion people were estimated as overweight and obese in 2015, and the numbers are increasing [156]. Both high and low prevalence of obesity among MS patients are reported [157-159], but a recent meta-analysis describes in general lower mean BMI in MS patients than in healthy controls [160].

Figure 8. The WHO classification of BMI

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Obesity in youth is associated with increased risk of MS [161]. An American study on two large cohorts of nurses and a Swedish case control study (EIMS=Epidemiological Investigation of MS) both found a 2-fold increased MS risk for individuals with higher BMI (>27 and ≥30 kg/m2) at age 18-20 years [162, 163]. Other studies have shown an

association between obesity in childhood and MS. In a large Danish study of more than 300.000 school children, higher BMI in childhood and early adolscence was associated with an increased MS risk, especially in girls [164]. A recent German study found that obesity in childhood was associated with a twofold odds of MS in both genders, [165] and another study in California describe similar results [166]. Birth weight is, however, not associated with MS risk [164]. A study using recalled body size instead of objectively measured BMI also confirmed a positive association between obesity and MS risk [167]. Only one early study found a possible inverse association between high BMI and MS risk, but this might have been associated to recall bias [168].

The link between obesity and MS risk may be via vitamin D, which is fat soluble. Obese individuals have more vitamin D sequestered in their fatty tissue making less available in the blood [169-171]. It is currently debated to which extent obesity itself increases MS risk, or whether obesity mainly is a proxy for low serum vitamin D levels or other factors related to overweight and obesity. Findings in a recent study provide evidence for both an independent and a causal effect between vitamin D level and obesity as risk factors in MS [172]. Inherited genetic variations influencing BMI have been found to be associated with MS, supporting a causal effect of increased BMI on susceptibility to MS [173, 174]. There is also an interaction between obesity and HLA antigen MS risk variants, and a study demonstrated a sevenfold increase in MS risk of obese MS patients with the HLA-DRB1*15 allele compared to non-carriers [64].

Relatively few studies have addressed the role of obesity for the MS disease course. In an American study, obesity among female MS patients was associated with a higher risk of a relapsing disease course at disease onset [175], but in a more recent study, BMI did not influence the risk of relapses [176]. However, a non-significant trend of higher annual relapse rate among overweight and obese children with MS have been reported [177].

A study found that higher BMI was associated with higher EDSS and another study found the same result for female patients [178, 179]. Another study showed higher disability, assessed by EDSS score, in MS patients with insulin resistance and adiposity

[180]. Studies have also found that BMI and dyslipidemi are associated with MS disability and disease progression [181-183]. Patients with increased waist circumference have been reported of a 47 % increased odds of severe disability, assessed by Patient Determined Disease steps (PDDS) score [184]. However, a previous study did not find any association between BMI and disability for MS patients using PDDS score [157].

MS patients with higher BMI are reported to have a greater reduction in brain volume, [185] and a study reported of worse performance in cognitive tests compared to normal weight patients [186]. Obese MS patients are also found to be more depressed, have lower functional capacity and worse self-rated health status [187, 188].

The link between MS and obesity is still unclear, but may be attributed to mainly two immunological responses: a chronic systemic inflammation and activation of a humoral immune response triggering production of autoantibodies. White fat tissue has an endocrine function secreting several proinflammatory cytokines like tumor necrosis factor α (TNFα) and interleukin-6 (IL-6) leading to a systemic low grade inflammation in obese individuals. This peripheral inflammation may directly disrupt the BBB causing neuroinflammation [189]. The increased serum levels of cytokines, insulin and saturated fatty acids can also activate different cells of the CNS. An activation of microglia cells and astrocytes leads to further increased levels of proinflammatory cytokines like IL-6 and TNFα and reactive oxygen species (ROS) [190, 191].

Apoptosis inhibitor of macrophage (AIM) is a macrophage-derived blood protein, increased in obese individuals, that induces release of fatty acids and chemokines from adipocytes leading to increased infiltration of M1-macrophages to fatty tissue. This causes polarization towards an unfavourable M1-macrophage profile that subsequently leads to an utter inflammation [192]. The increased release of fatty acid caused by AIM also activates the NLRP3-inflammasome that mediates caspase-1 activation and secretion of proinflammatory cytokines IL-1β and IL-18. This inflammasome and the cytokines released is thought to be involved in MS pathogenesis, and is found to have

a critical role for the EAE development [193, 194]. AIM form immune complexes with IgM and has also a role in the production of IgG autoantibodies [155].

Obesity causes induction of Th17-cells, a T-cell subset involved in the pathogenesis of autoimmune diseases. Th17-cell activities are shown to effect MS mostly through the secretion of proinflammatory IL-17, but also through IL-21 that effect the infiltration of lymphocytes in MS lesions, and IL-22 that promotes BBB damage [195, 196]. In addition to cytokines, fat tissue secretes a number of adipokines that can affect the MS pathogenesis in several ways.

Obesity may be linked to a diet consisting of much saturated fatty acids, often referred to as a "Western diet". This diet influence the immune system, causing a proinflammatory state in autoimmune diseases, and is suggested to be a risk factor for MS [49, 197]. Possible pathogenetic mechanisms involve dysbiosis in the gut microflora, modulation of components in the inflammatory cascade and interaction with regulatory T-cells [198, 199]. Animal dietary intervention studies have demonstrated the effect of diet in EAE. Calorie restricted mice had less disease severity and reduced demyelination and axonal damage, [200-202] whereas fat-diet fed mice had a more severe EAE with increased neuroinflammation, oxidative stress and CNS infiltration [195, 203, 204]. There has also been some studies indicating the beneficial effects of a healthier diet on MS disease activity and course [205, 206].

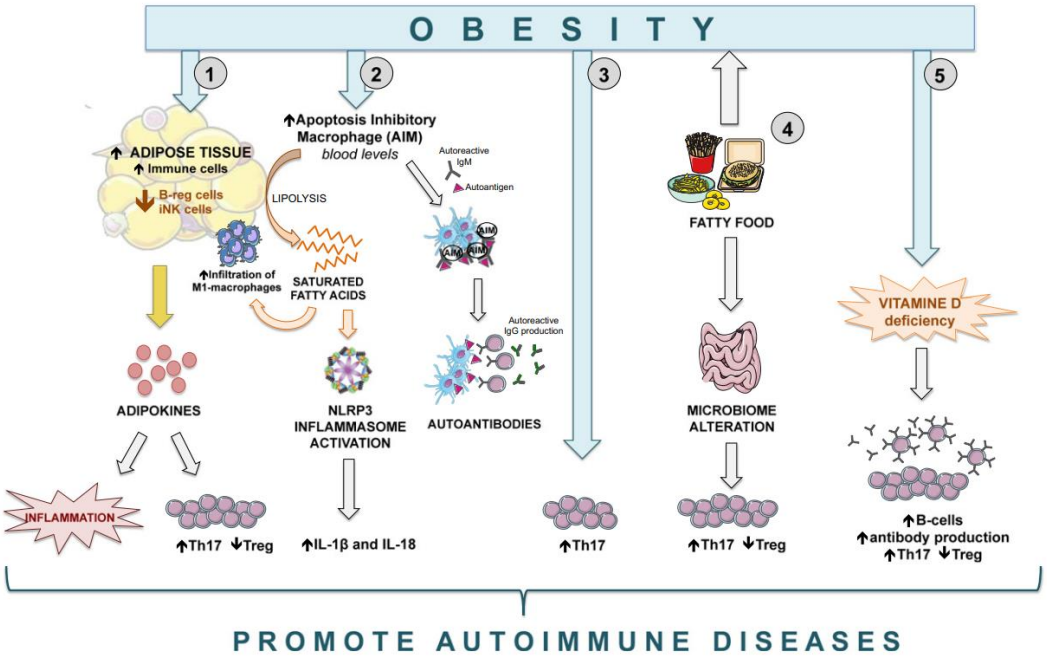

Figure 9. The effect of obesity in autoimmune diseases.

The figure illustrates the main mechanisms suggested for how obesity may promote autoimmune diseases. Reprinted by permission from Elsevier: Autoimmunity Reviews ©. 2014 [155].

2.3 Potential biomarkers of disease activity in MS

A biomarker can be defined as " a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [207]. Since MS is a heterogenous disease with an unpredictable disease course, there is an evident need for biomarkers. Potential biomarkers could improve disease diagnosis, help predicting disease course and optimizing treatment. Multiple biomarkers have been explored in both serum and CSF [208, 209]. The biomarkers currently in use in MS is MRI white matter lesions, oligoclonal bands in the CSF and JC viral titers for natalizumab

treatment [208]. A possible upcoming biomarker for clinical use is serum neurofilament [210]. In this thesis we explored the potential of other possible biomarkers.

2.3.1 Adipokines

Adipokines are cytokines (cell signalling proteins) or hormones secreted by the fatty tissue shown to play a part in the regulation of the immune system [211]. Dysregulation of the production and secretion of adipokines contribute to the chronic low grade inflammation in obesity [212]. Most adipokines drive the immune system towards a pro-inflammatory state, but some possess anti-inflammatory traits. The counterbalance between the serum levels of the different adipokines may contribute to the pathogenesis of different obesity-linked complications [212]. Leptin and adiponectin are the most abundant adipokines produced by adipocytes [211].

Leptin

Leptin is a proinflammatory adipokine with a potential role in the pathogenesis of several autoimmune disorders [213, 214]. The main function of leptin is regulation of metabolism by modulating feeding behaviour through the CNS, promoting satiety and stimulating energy expenditure. Being produced by adipocytes, it signals the body`s energy stores and functions as a negative feedback adipostat [215]. Serum leptin levels correlate with adipose mass and are 2-3 times higher in woman than in men [211, 215, 216].

Leptin stimulates the proliferation and activation of monocytes by inducing production of cytokines like TNF-α, IL-6 and IL-1, and also stimulates the production of CC-chemokine ligands like CCL3, CCL4 and CCL5 by macrophages [211]. Neutrophil chemotaxis and production of ROS are promoted in addition to activation of natural killer cells (NK cells) [211]. The maturation and survival of dendritic cells are also enhanced by leptin. Further, leptin modifies T-cell immunity by effecting T-cells and mononuclear cells to increase the production of Th1-type cytokines IL-2 and IFNγ and

suppress the production of the Th2-type cytokine IL-4, hence polarizing T-cells towards a Th1-cell type [217].

A meta-analysis of leptin levels in MS patients concluded that serum levels of leptin are higher in MS patients than in healthy controls [218]. In a recent study there was a positive association between serum leptin concentration and the risk of MS [219]. Also different gene variants for the LEP gene are found to be associated with susceptibility to MS [220]. Leptin serum levels are found to be inversely associated with the number of regulatory T-cells in MS patients, [221, 222] and are positively correlated to proinflammatory mediators [222]. Several studies have explored the role of serum leptin level as a biomarker in MS, with conflicting results. A study found increased levels before clinical relapses, and decreased levels during IFNβ-treatment [223], while others have described higher levels during remission state [224, 225]. A small study of 45 patients with RRMS found a greater risk of clinical relapses and MRI disease activity the upcoming year in patients with higher serum leptin levels, suggesting it to be a potential biomarker for disease course in MS [226]. MS patients have also been reported to have higher levels of leptin in CSF than healthy controls, and this has been suggested an essential role for worsening central inflammation in MS [179, 221].

Interestingly, leptin deficient mice (ob/ob mice) are resistant for development of EAE, but become susceptible to the disease after leptin administration [227]. Endothelial leptin receptor knockout mice are also found to have reduced disease burden [228]. The leptin receptor expression is upregulated in hippocampus of EAE mice, and the endothelial leptin signalling are shown to enhance BBB dysfunction in EAE [228]. Serum leptin levels affect EAE as they are found to be increased before the clinical onset of EAE in mice, and starvation delayed the disease onset and attenuated the symptoms of EAE [229].

Leptin affects the immune system, and it is suggested that blocking the serum levels of leptin may be beneficial for the reduction of autoimmune reactivity [214]. Therapeutic leptin antagonism are found effective in preventing and treating immunity-related

disorders in mice [230], and could be a possible future treatment for autoimmune diseases in humans [230, 231].

Adiponectin

Adiponectin is mainly an anti-inflammatory adipokine. The immune functions include suppression of macrophage activity, inducing the production of anti-inflammatory cytokines like IL-10, IL-1 receptor antagonist (IL-1RA) by dendritic cells, monocytes and macrophages, and suppression of interferon-γ production [211]. This adipokine is, however, also found to promote the activation of dendritic cells and to affect the T-cells towards a pro-inflammatory Th1 and Th17 polarization [211]. In humans the serum concentration of adiponectin is about 5-10 mg per ml, much higher compared to leptin circulating in concentrations of a few nanograms per ml [211]. The circulating levels are affected by several factors like gender, age and lifestyle [211]. Adiponectin circulates in three main isoforms, low-molecular weight (LMW) trimers, medium-molecular-weight (MMW) hexamers, and high molecular-weight (HMW) multimers and their biological activity is somewhat different [232].

The serum levels of adiponectin in MS patients have been explored with conflicting results. A small study found no difference in serum levels between MS patients and healthy controls, [233] but several other studies have found decreased levels in both adults and children with MS [234-237]. One recent study reported higher serum levels in MS patients and an altered oligomerizaton state, with an increase of the high molecular weight oligomers [238]. The serum levels of adiponectin is not found to increase the risk of MS [239], but some genetic variants of the ADIPOQ genes are found to be associatied with susceptibility to PPMS [220].

For children with MS, higher serum levels of adiponectin are reported to be associated with a lower risk of relapses [235]. However, a recent study of 99 MS patients found that patients with higher serum adiponectin levels at baseline had a higher risk of disease progression, assessed by multiple sclerosis severity score [238]. Another study following MS patients over a 2-year period found no association between adiponectin serum levels and disease activity [240].

EAE is more aggressive in mice with adiponectin deficiency, causing greater CNS inflammation, demyelination and axon injury. The disease load can be enhanced by adiponectin treatment [241]. The cause of this effect may be through inhibiting the differentiation of Th17-cells, a proinflammatory linage of T- cells related to the pathogenesis of most autoimmune diseases [242].

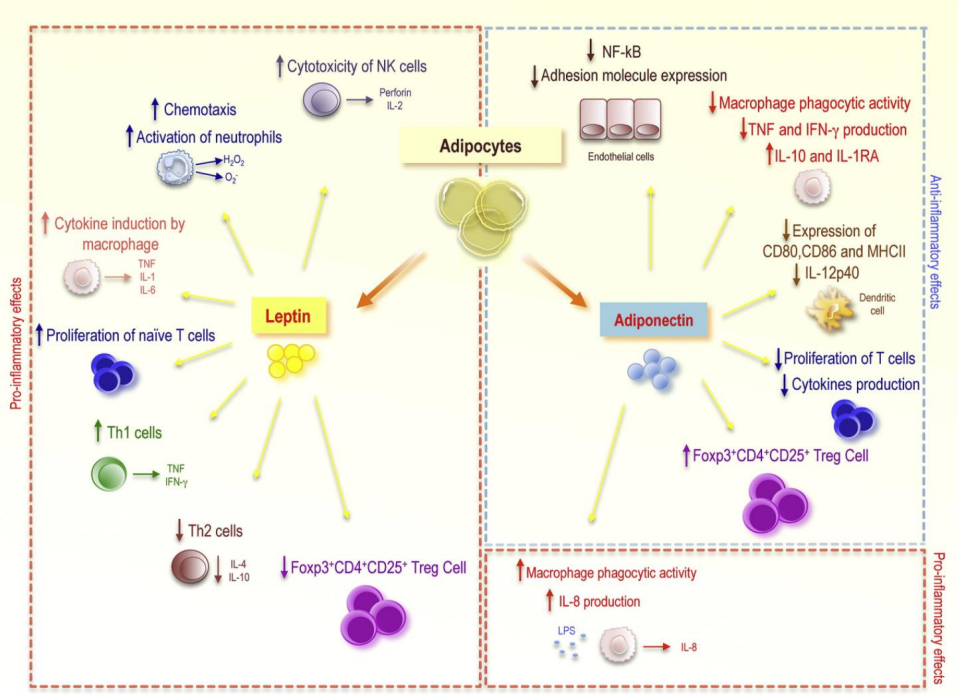

Figure 10. The effect of leptin and adiponectin in innate and adaptive immunity.

The figure illustrates some of the mechanisms of leptin and adiponectin in immunity. Leptin increases the cytotoxic ability of NK-cells, activates neutrophil cells, enhances macrophage secretion of proinflammatory cytokines, increases the T-cell proliferation and promotes a Th1-cell profile. Adiponectin decreases the phagocytic activity of macrophages, lower the expression of HLA II receptors on dendritic cells and lower proliferation of T-cells, except for an increase in T-regulatory cells. However adiponectin may also increase the activity of macrophages contributing to a proinflammatory effect. Reprinted with permission from Elsevier, Biochimie © 2012 [243].

Other adipokines

The role of several adipokines have been explored in MS. Serum levels of visfatin, resistin and adipsin are found to be increased in MS patients [236, 240]. A study reported of a correlation between plasma levels of adipsin, EDSS score, and MRI lesions and suggested a role of adipsin in the accumulation of neurological disability [240]. Chemerin works as a chemoattractant for antigen-presenting cells (APC), and is shown to be expressed in white-matter lesions of MS. It is involved in the migration of peripheral cells into the CNS and may influence the CNS inflammation in MS [244, 245].

2.3.2 Inflammation markers

The chronic CNS inflammation leading to axonal damage and demyelination is a distinct pathological characteristic of MS. As the inflammatory process in MS changes according to natural fluctuation of the disease, or as a response to treatment, the serum levels of different cytokines and interleukins is likely to differ. The levels of inflammatory markers may also change in accordance to the interactions of environmental factors like smoking, obesity or vitamin D [246]. Since the disease course in MS is unpredictable, and few biomarkers are available so far, the search for potential candidates is ongoing. Different inflammation markers in serum may be possible biomarkers in MS for prediction of disease course and treatment response.

Osteoprotegerin (OPG): Osteoprotegerin is a soluble secreted protein and member of the TNF-receptor superfamily. It is important in the regulation of bone metabolism. Receptor activator of NF-κB (RANK) is expressed on osteoclasts and needs the binding of RANKL for induction of bone resorption [247-249]. OPG can bind to RANKL and prevent it from engaging to its receptor RANK, hence inhibit bone resorption. The OPG/RANKL/RANK system also play a part in regulation of immune responses, with a main function in controlling the number of regulatory T-cells and the formation of

self-tolerance in T-cells [247]. Different immune cell subsets express these proteins. OPG is expressed in B-cells, RANKL is expressed by active T-cells and macrophages, and RANK is expressed by macrophages and dendritic cells [247]. The RANKL/RANK/OPG system has a role in MS. A study found that MS patients have lower levels of OPG in CSF at disease onset compared to healthy controls [250]. Another study found an inverse association between serum levels of OPG and MRI disease activity in MS [251]. Interestingly a study showed that mice that lacked RANKL in T cells were unable to evolve EAE, suggesting pharmacological RANKL inhibition as a potential therapeutic target in MS [252].

Soluble tumor necrosis factor receptor 1 (sTNF-R1): This cytokine receptor is characterized by its ability to bind tumor necrosis factor (TNF) and belongs to the TNF-α receptor superfamily [251]. It modulates immune responses by its ability to bind and neutralize the proinflammatory cytokines TNF-α and TNF-β. The serum level of this receptor has been explored as a possible biomarker for IFNβ-treatment response in MS [253]. A study described that MS patients with a greater increase in the serum level of sTNF-R1 had a better MRI response and less clinical relapses, suggesting serum measurement as a possible method for identifying the patients most likely to benefit mostly of IFNβ-treatment [253].

Chemokine (C-X-C motif) ligand 16 (CXCL16): This small cytokine belongs to the CXC chemokine family, and has a role in mediating the innate immune response by attracting activated T-cells and NKT-cells. It is expressed on antigen presenting cells during inflammation [251, 254]. A study suggested CXCL16 as a possible biomarker in MS, demonstrating that the serum levels was inversely associated with MRI activity [251]. CXCL16 is important in EAE with a role in the recruitment of inflammatory mononuclear cells into the CNS [255].

Chemokine (C-C motif) ligand 21 (CCL21): This is a lymphoid cytokine normally produced by stroma and endothelium of lymph nodes and spleen [256]. It binds to the receptor CCR7, expressed in dendritic cells, T-cells and activated B-cells, and is important for the migration of T-cells and dendritic cells to secondary lymphoid organs [257]. A study showed that intrathecal production of CCL21 was elevated in MS patients, but not in patients with non-MS-type optic neuritis, suggesting that it play a role in CNS inflammation [258]. This is consistent with findings in studies of EAE indicating that CCL21 play a part in the development of autoimmune inflammation [259] and is important for the development of EAE [260].

Transforming growth factor (TGF) β1: This is a growth factor and multifunctional cytokine that is involved in cellular processes like cell growth, apoptosis, cell differentiation and extracellular matrix synthesis [261]. It plays a part in CNS inflammation, causing activation of microglia, but also possesses immunosuppressive properties. A study showed that the administration of TGF-β to mice had a protective effect in EAE [262]. Another study found that TGF-β administration enhanced remyelination in EAE, and even stimulated human oligodendrocyte maturation suggesting a possible therapeutic potential in MS [263].

Pentraxin (PTX3): PTX3 is an acute phase protein that belongs to the group of long pentraxins and is produced by macrophages, neutrophils, endothelial cells and dendritic cells in response to cytokines like IL-1 and TNF-α [264]. The roles in immunity are plentiful including complement regulation, opsonization, removal of apoptotic cells and control of neutrophil migration [264]. The serum levels of PTX3 are higher in patients with autoimmune diseases than healthy controls [265]. Patients with MS are also found to have higher plasma levels, with lower levels during remission than in relapse phases [266]. PTX3 seem not to be of major significance during EAE. The disease severity was no different in PTX3-deficient mice than wild-type mice, and administration of PTX3 did not alter the disease course [267].

Matrix metalloproteinase (MMP-9): These enzymes are essential for the degradation of various tissue extracellular matrix proteins. They have several roles during inflammation, including the regulation of barrier function and activity of inflammatory cytokines and chemokines [268]. In MS, MMP-9 are involved in damaging the BBB leading to leukocyte extravasation into the brain. An upregulation of MMP-9 is associated with a worse disease course in EAE, and mice that lack MMP-9 have proven to be less susceptible for the induction of EAE [269, 270]. Serum MMP-9 levels are found to be elevated in RRMS patients and found to be related to relapses and MRI activity [271, 272].

Interleukin-1 receptor antagonist (IL-1Ra): IL-1Ra belongs to the IL-1 cytokine family and is secreted by immune cells, epithelial cells and adipocytes [273]. Acting mainly as a regulation of inflammation through binding and blocking of IL-1 receptors, it seems to have a role in terminating the inflammatory response. IL-1RA genes are found to be upregulated in MS patients [274]. The anti-inflammatory effects of this cytokine are shown to play a part in EAE, whereas administration of IL-1Ra delayed the onset of EAE and reduced disease severity in mice [275, 276]. A study showed increased serum levels of IL-1Ra in MS patients during IFNβ-treatment, [277] and a positive association between vitamin D levels and serum levels of IL-1Ra has also been found [278].

Osteopontin (OPN): OPN is an extracellular matrix protein that is expressed in different immune cells like T-cells, dendritic cells, macrophages and natural killer cells. The role in inflammatory processes is prominent, causing production of IL-12, IL-17 and IFNγ, whereas it inhibits the expression of IL-10. Further, it mediates cell migration, adhesion and function as a Th1-cytokine [279]. Several studies have explored the potential of OPN as a biomarker in MS. A meta-analysis of 22 studies concluded that MS patients have higher levels of OPN in CSF and blood compared to controls. Further, patients with active disease had higher levels in CSF compared to

patients with stable disease, [280] and also plasma levels are found to be higher during relapses [281]. A study found that plasma OPN levels were higher in patients with SPMS compared to RRMS patients [281]. This proinflammatory cytokine is important in EAE, and OPN-deficient mice are resistant to progressive EAE [282].

Activated leucocyte cell adhesion molecule (ALCAM): ALCAM belongs to the immunoglobulin superfamily and is expressed in epithelial cells, endothelia and neurons. It is involved in immune responses where it is responsible for stabilization of the immunological synapse, activation and proliferation of T-cells and leukocyte migration [283]. This adhesion molecule has been shown to promote B-cell extravasation and migration across BBB in MS, and the expression of ALCAM is upregulated in both active MS and EAE brain lesions [283]. An increased number of B-cells that express this molecule are found in both the serum and the brain of MS patients. The blocking of ALCAM causes a less severe EAE disease course, and therapeutic antagonism may be a future treatment in MS [284].

3. Study rationale and aims

3.1 Rationale

Tobacco use and obesity are lifestyle factors known to increase the risk of MS. They may also influence the disease course, but studies conducted so far have shown conflicting results. Lifestyle factors are modifiable, and knowledge of their role in MS can be of great importance, offering patients the opportunity to influence the disease by lifestyle choices.

3.2 Aims

The aim of this project was to:

1) Explore the effect of tobacco use in patients with RRMS to determine whether there is an association between tobacco use and MRI disease activity, relapse rate and EDSS progression.

2) Examine the association between BMI and disease activity in patients with RRMS to determine if obesity is associated with a more aggressive MS disease course.

3) Examine if adiponectin and leptin are associated with MRI disease activity in MS, and their potential as future biomarkers for IFNβ-treatment response.

4. Methodology

4.1 Source of data: The OFAMS study

4.1.1 Study design

The OFAMS (ω-3 fatty acid treatment in multiple sclerosis) study was a multicenter, randomized, double-blind, placebo-controlled trial for investigating whether ω-3 fatty acids reduced MRI and clinical disease activity in patients with MS [285]. The study population consisted of 92 Norwegian patients with RRMS according to the McDonald criteria [286]. Patients were recruited from 13 different neurology departments in Norway between December 2004 and July 2006. The inclusion criteria were age 18–55 years and Expanded Disability Status Scale (EDSS) score ≤5. All patients had active disease with ≥1 relapse, or new T1-weighted gadolinium-enhancing (T1Gd+) or T2-weighted (T2) lesions on MRI in the year prior to inclusion. Patients who used or had used any previous immunomodulatory treatment for MS were excluded, as well as patients where initiation of medical treatment could not be delayed.

4.1.2 Interventions and follow-up

Patients were randomized to receive either Triomar™ capsules (Pronova Biocare, Sandefjord, Norway) containing 1350 mg of eicosapentaenoic acid (EPA) and 850 mg of docosahexaenoic acid (DHA) or placebo (corn oil) at baseline. From month six all patients received subcutaneous injections of IFNβ three times weekly. The total follow-up period was 24 months; six treatment-naïve months (without DMT) and 18 months with IFNβ-treatment.

A total of six patients were lost to follow up, one at baseline, three at study month two, one at study month 12 and one at study month 18. Further, five of the patients discontinued treatment.

The result of the study showed no difference between the group of patients receiving omega-3 or corn oil, hence the cohort has later been pooled for several additional analyses [73, 89, 251, 278, 287-291].

The studies in this thesis are all based on the OFAMS study. The varying 86-88 patients in the cohort of the three studies had all completed at least 12 months of the OFAMS-study. Patient characteristics are illustrated in Table 1.

Patients, n (%)	88 (100)
Female	57 (64.7)
Male	31 (35.2)
Caucasian, n (%)	88 (100.0)
HLA-DRB1*15-positive[a], n (%)	58 (66.7)
Proportion of T1Gd+ lesions, % (SEM)	52 (5.4)
Age, median years (range)	39 (19-58)
Years since diagnosis, median years (range)	1 (0-13)
Years since first symptom, median (range)	3 (0-23)
EDSS score at inclusion, median (range)	2.0 (0.0-4.9)

The first paper included 87 of the patients and the second paper included 86 of the patients.

a) Not available in 4 patients, T1Gd+=Gadolinium enhancing T1 lesions, EDSS=Expanded disability status scale.

Table 2. Baseline demographic characteristics of the patients in the cohort.

4.1.3 Measurements

All patients were followed with repeated evaluations and measurements during the studies as illustrated in Table 3.

	Month												
	Baseline	1	2	3	4	5	6	7	8	9	12	18	24
	NO TREAMENT							INTERFERON β1a TREATMENT					
Weight and height	•												
MRI examination	•	•	•	•	•	•	•	•	•	•	•		•
EDSS score	•						•				•	•	•
Serum-inflammation markers and 25-hydroxyvitamin D	•	•		•			•	•		•	•	•	•
Serum-cotinine	•						•				•	•	•
Serum leptin and adiponectin	•	•		•			•	•		•	•	•	•

MRI = magnetic resonance imaging; EDSS = Expanded Disability Status Scale. Relapses were recorded throughout the study period

Table 3: Different examinations and different time points.

Outcome measures in the studies were combined unique activity, (new MRI T1-Gd enhancing lesion, new or enlarging T2-lesion, or both), number of relapses, increased disability assessed by EDSS score and NEDA-3-status (no evidence of disease activity).

Relapse: A relapse was defined as the appearance of new or worsening of old neurological symptoms or signs, in the absence of fever, persisting for more than 48 hours and causing objective changes on neurological examination.

Disease progression: An increase of ≥1 point in EDSS score recorded at a clinical visit and sustained for > 6 months was defined as disease progression.

MRI: Cerebral MRI was performed according to a standardized protocol comprising T2-weighted and T1-weighted gadolinium enhancing (T1Gd+) scan, using a standard head coil with a 1,5 Tesla MRI unit. The guidelines for use of MRI in MS trials were followed [292]. Blinded assessments of the results were conducted by 2 experienced neuroradiologists. The sum of T1Gd+lesions and new or enlarging T2 lesions was denoted as combined unique activity (CUA).

NEDA: Patients with optimal treatment outcome, meaning no disease activity, for the follow-up period obtained NEDA-status. NEDA was defined as a composite consisting of absence of relapses, no sustained disability progression assessed by EDSS scoring and no MRI activity (new T1Gd+ or new/enlarging T2-lesions) for the given period [27]. As more demands for an optimal treatment response have evolved, including MRI brain atrophy and CSF-findings, the NEDA-status definition used in our studies is currently referred to as NEDA-3 status.

BMI: Height and weight were registered for all patients at inclusion, and BMI was calculated as weight/(height)^2. The patients were categorized based on the WHO classification of obesity into three groups; Normal weight patients, (BMI < 25 kg/m^2); Overweight patients (BMI 25–30 kg/m^2); Obese patients (BMI >30 kg/m^2). For some analyses, overweight- and obese patients were combined into one group.

Laboratory tests: The serum samples were collected by venepuncture during the planned clinical visits. All were collected at day time between 07-15 p.m, and without any special patient preparations. The samples were shipped in a frozen state and stored at -80 ºC until post-study analyzes. Some analyses were performed in relation to the original OFAMS-study, while others were analysed at later time points as new studies

based on the OFAMS cohort evolved. Lab technicians were blinded for any information regarding the patients.

-*HLA-DRB1 typing:* The HLA-DRB1 status was determined by DNA sequencing. Serum was analyzed at the Department of Immunology and Transfusion Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway with the SeCore DRB1 Locus sequencing kit (Invitrogen, Carlsbad, CA, USA). Patients carrying at least one DRBI*15 allele were considered HLA-DRB1*15 positive.

-*Neutralizing antibodies against IFNβ (NAb):* NAb were detected with a myoxvirus-resistant protein A messenger ribonucleic acid induction assay at the Department of Neurology, Haukeland University Hospital, Bergen, Norway. Samples with titer >20 were classified as positive.

-*Inflammation markers:* A selection of ten inflammation markers were chosen based on knowledge regarding their function in immunity and assumed relevance in MS pathogenesis. The serum concentrations were analysed with enzyme immunoassay kits (EIA) from R&D Systems (Stillwater, MN, USA) at the Research Institute of Internal Medicine, Oslo University Hospital, Rikshospitalet, Oslo, Norway; osteoprotegerin (OPG), soluble tumor necrosis factor receptor 1 (sTNFR1), the chemokines CXCL16 and CCL21, transforming growth factor (TGF)β1, pentraxin 3 (PTX3), matrix metalloproteinase 9 (MMP-9), interleukin-1 receptor antagonist (IL-1Ra), osteopontin (OPN), and activated leukocyte cell adhesion molecule(ALCAM) [251]. Consecutive patient samples were analysed in neighbouring wells on the same plate. The intra-and inter-assay coefficient of variation were <10 % for all EIAs.

-*Vitamin D:* The 25-hydroxyvitamin D [25(OH) D3 and 25(OH) D2] was measured with a radioimmunoassay kit (RIA-kit, ImmunoDiagnostic Systems, Boldon, UK) at the Department of Medical Biochemistry, St. Olavs Hospital, Trondheim, Norway. The coefficient of variation was 5.4 % and 6.3 % at 29 and 112 nmol/L [89].

-*Cotinine:* Serum levels of cotinine was measured using liquid chromatography tandem mass spectrometry (LCMS) at Bevital AS (Bergen, Norway). All serum samples for the same patient were performed simultaneously. The lower limit of detection was 1 nmol/l, the within-day coefficient of variation was 2.0 - 6.6 % and the between-day CV was 3.9 %.

Cotinine is a nicotine metabolite and the mostly applied biomarker for tobacco use with a high sensitivity (96-97 %) and specificity (99-100 %) [293]. The metabolite has a half-life of 15-40 hours, reflecting tobacco exposure the last 3-5 days. Serum cotinine levels >85 nmol/l is consistent with recent tobacco use, and the cut point mostly used for distinguishing tobacco users from non-tobacco users in the general population [293, 294]. An advantage of cotinine compared to other tobacco-biomarkers is that the optimal cut-point is little affected by the prevalence of smoking in the chosen population [295].

We categorized the patients into tobacco users and non-tobacco users according to serum cotinine levels. Patients with serum cotinine level ≥85 nmol/l in ≥60 % of the samples were considered tobacco-users.

-*Adipokines:* Serum-leptin and adiponectin were measured by radioimmunoassay kit (Merc Millipore Corporation, Hormonlaboratoriet, OUS, Norway). All samples from the same patients were analysed simultaneously, with consecutive samples in neighbouring wells on the same plate. The total coefficient of variation for leptin was <10%, and for adiponectin <12 %.

4.1.4 Therapy

The patients in our studies were followed for six months without any DMT, and then 18 months during interferon-beta-1a (Rebif) treatment. IFNβ was administered as subcutaneous injections of 44 μg 3 times per week. The dosage was the same for all patients. At the time of the OFAMS study, IFNβ was a primary first-line treatment choice for MS. The study design without DMT for 6 months would not have been

ethical acceptable today. But at the time when the study was performed, knowledge about the importance of early treatment start was limited. In addition, an application to the The Norwegian Health Economics Administration for approval of treatment start was needed, and the processing time typical 1-4 months. Thus the study design was approved by the ethical committee.

The PRISM study had demonstrated the effectiveness of subcutaneous IFNβ-1a treatment in RRMS patients regarding relapse rate, MRI outcomes and disability [296, 297]. There was also convincing long-term effect, however, in a follow-up study as much as 23 % developed persistent neutralizing antibodies and this was associated with reduced efficacy [298]. The molecular mechanisms of action for IFNβ are multiple and not fully clear. The anti-inflammatory effect of the drug is caused by inhibition of T-cell proliferation, an altered cytokine response leading to a shift in T-cell subsets from a Th1-phenotype towards a more favourable Th2-type, and reduced T-cell migration [11]. The lymphocyte migration through the BBB is decreased by alterations in cellular adhesion molecule expression and decreased matrix metalloproteinases. Further, there is an enhancement of anti-inflammatory cytokines, and the expression of HLA II molecules are decreased causing less activation of lymphocytes [299]. As more efficient DMTs for MS have evolved and become available, IFNβ is no longer a common choice of therapy in Norway, but may still be considered in some cases [3].

Several factors may interfere with the efficacy of IFNβ-treatment including noncompliance and development of antibodies [300]. The development of antibodies is a common phenomenon for biological medications. Several patients develop binding antibodies (BAbs), and a smaller proportion of patients develop neutralizing antibodies (NAbs) that interfere with the biological and therapeutic effect of the drug. The immunogenic potential of IFNβ varies from 28-47 %, and is independent of gender, age and disease duration [301, 302]. For most patients the evolvement of NAbs will occur during the first 18 months of therapy, and analyzing for NAbs during IFNβ-treatment is recommended in an European guideline [302]. Obese patients have been shown to have a decreased ability to produce IFN-α and IFN-β in response to viral infections, and this is caused by an increased SOCS3 basal mRNA expression in peripheral blood mononuclear cells, a protein that negatively regulates type I

interferons [303]. This could possibly present a cause for a poorer response to IFNβ-treatment in obese. However, a study did not find that the expression of this protein was different between responders and non-responders to IFNβ [304].

The IFNβ serum level can be quantified by an ELISA method, but therapeutic drug monitoring for assessing the individuals drug concentration in relation to therapeutic response is not used for IFNβ-treatment as the drug has a short serum half-life [305]. However, for some patients the biological activity of IFNβ can be assessed by evaluating different IFN-stimulated genes such as myxovirus-resistance protein A (McA), b2-microglobulin and oligo-adenylate-synthetase. Especially measurement of McA and NAbs are shown to help predicting the risk of new relapses [306]. An evaluation of the therapeutic response based on both biological, clinical and MRI follow-up is advised for an early detection of non-responders [307]. However, measurement of the IFNβ biological activity by McA quantification is mostly used in clinically challenging cases as the test is not available in most laboratories [302].

4.1.5 Ethical approval and patient consent

The OFAMS study protocol was approved by the Regional Committee for Medical and Health Research Ethics in Western Norway and the Norwegian Medicines Agency. The study was undertaken in accordance with the Declaration of Helsinki and the European Medicines Agency Note for Guidance on Good Clinical Practice. All patients received written and oral information about the study and signed consent to enrollment.

4.2 Statistics

Paper I

This study included 87 of the original 92 OFAMS patients. A total of nine MRI scans (one during study months 1-6 and eight during study months 7-24) were missing. Seven

blood samples for serum-cotinine analysis were missing for months 0-6 and 39 for months 7-24.

The association between tobacco use and MRI activity was assessed by a logistic regression model for hierarchical data, for the total study period, the six months prior to treatment and the 18 months with IFNβ-treatment. All analyses were adjusted for gender, age, BMI and HLA-DRB1*15 status. The differences in number of relapses and baseline EDSS scores among tobacco-users and non-tobacco users were assessed by Independent samples t-test.

Paper II

This study included 86 of the original 92 OFAMS patients. A total of nine MRI scans (one during study months 1-6 and eight during study months 7-24) were missing. The association between BMI and MRI activity before and during IFNβ-treatment was assessed by logistic regression model for hierarchical data. The association between BMI and inflammation markers was estimated by a linear mixed model with random effects for patients and fixed effects for BMI. The results were reported as regression coefficients. Log–transformed inflammation marker values were employed in the models since most values were not normally distributed. The association between seasonally adjusted vitamin D status and BMI was assessed by estimating a linear mixed model with random effects for patients and fixed effects for vitamin D.

Explorative analyses of MRI activity within different categories of BMI status as well as NEDA-status were assessed by z-test for proportions and independent samples t-test. For multiple comparisons, one-way analysis of variance (ANOVA), followed by Fishers' least significant difference method was used. All analyses was adjusted for age and HLA-DRB1*15 status.

Paper III

This study included 88 of the original 92 OFAMS patients. A total of nine MRI scans (one during study months 1-6 and eight during study months 7-24) were missing. Six

blood samples for serum leptin and adiponectin were missing for months 0-6 and 22-24 blood samples for months 7-24 (24 for leptin).

The inter- and intra-patient variance was estimated by a linear mixed model (LMM) with random intercepts for patients. Mean serum levels before and during treatment were compared by including fixed effects for period into LMM. The associations between baseline levels of adipokines and BMI, relapses, NEDA and EDSS score were assessed by linear regression.

Generalized linear mixed models with random intercepts for patients were used to estimate the association between MRI activity and adipokines. Associations between time profile in adipokines, NEDA-status, EDSS progression at baseline and relapses were estimated by LMM. All models were adjusted for gender, age and BMI.

For all analyses results with p values <0.05 were considered statistically significant.

The analyses for all papers were performed in SAS v 9.4 and SPSS v 22 or 25.

5. Summary of results

5.1 "No association of tobacco use and disease activity in multiple sclerosis".

Kvistad SS, Myhr KM, Holmøy T, Benth JŠ, Løken-Amsrud KI, Wergeland S, Beiske AG , Bjerve KS, Hovdal H, Lilleås F, Midgard R, Pedersen T, Bakke SJ, Torkildsen O. Neurol Neuroimmunol Neuroinflamm, 2016;3:e260.

The aim of the study was to explore the effect of tobacco use for disease activity in MS. A cohort of 87 RRMS patients originally included in a randomized placebo-controlled trial of omega-3 fatty acids in MS were followed for two years, 6 months before and 18 months during IFNβ-treatment with repeated MRI examinations, clinical evaluations and collection of serum samples. Cotinine, a serum biomarker of tobacco use was measured at five different time points. The association between tobacco use and MRI activity was assessed by logistic regression.

There was no association between tobacco use assessed by cotinine level and MRI disease activity. We found that 53 (61 %) of the 87 patients had cotinine levels ≥ 85 nmol/l in ≥60% of the measurements and were classified as tobacco users. For these patients there were no association between cotinine levels and MRI activity during the follow-up period, i.e. there were no difference in MRI activity between patients likely to be heavy smokers and light smokers. The results were consistent for the six treatment-naïve months (receiving only ω-3 fatty acids or placebo) and the 18 months during IFNβ-treatment. There was no difference between tobacco users and non-tobacco users in mean baseline EDSS score or EDSS progression during follow-up. The number of patients with clinical relapses in the study period did not differ between tobacco users and non-tobacco users. Adjustment for gender, age, BMI and HLA-DRB1*15 status did not change our results.

5.2 “Body mass index influence interferon-beta treatment response in multiple sclerosis”.

Kvistad SS, Myhr KM, Holmøy T, Šaltytė Benth J, Wergeland S, Beiske AG, Bjerve KS, Hovdal H, Lilleås F, Midgard R, Pedersen T, Bakke SJ, Michelsen AE, Aukrust P, Ueland T, Sagen JV, Torkildsen Ø.J Neuroimmunol. 2015; 288:92-7.

The aim of this study was to explore the association between BMI and disease activity in MS. A cohort of 86 RRMS patients originally included in the OFAMS trial were followed for two years, 6 months before and 18 months during IFNβ-treatment. The follow-up included repeated MRI examinations, clinical evaluations with EDSS score and serum samples. Height and weight were measured at baseline and the patients were categorized according to WHO classification as normal weight patients (BMI < 25 kg/m²), overweight patients (BMI 25–30 kg/m²) and obese patients (BMI >30 kg/m²). The serum samples were analyzed for vitamin D and 10 different inflammation markers at baseline and after 1, 3, 6, 7, 9, 12, 18 and 24 months. The association between BMI and MRI activity was assessed by logistic regression and by independent samples t-test. The association between BMI and inflammation markers and vitamin D was assessed by estimating a linear mixed model.
The mean BMI in the cohort was 25.7 kg/m2 and the cohort consisted of 46 normal weight, 23 overweight and 17 obese patients. Of the patients with normal weight, 24 patients (52 %) had no MRI activity (CUA) during IFNβ- treatment compared to 8 (20 %) of the combined overweight and obese patient-groups. Combining no MRI activity, no clinical relapses and no EDSS progression, 26 % of the patients with normal weight obtained NEDA-3 status during the treatment period compared to only 13 % of the overweight and obese patients. There was no association between BMI and MRI activity for the 6 months prior to IFNβ-treatment and this was consistent after adjustment for gender, age and HLA-DRB1*15 status.

We found a positive correlation with serum IL-1Ra and an inverse correlation with PTX3, but no correlation between BMI and seasonally adjusted vitamin D levels.

5.3 “Serum levels of leptin and adiponectin are not associated with disease activity or treatment response in multiple sclerosis”.

Kvistad SS, Myhr KM, Holmøy T, Šaltytė Benth J, Wergeland S, Beiske AG, Bjerve KS, Hovdal H, Midgard R, Sagen JV, Torkildsen Ø. J Neuroimmunol. 2018;323:73-77.

The aim of this study was to examine the association between serum levels of two adipokines (leptin and adiponectin) and MRI disease activity in MS. A cohort of 88 RRMS patients originally included in a randomized placebo-controlled trial of omega-3 fatty acids in MS (the OFAMS study) were followed for two years, 6 months prior to and 18 months during IFNβ-treatment, with repeated MRI examinations, clinical evaluations and serum measurements of adipokines. The association between serum levels of adipokines and MRI activity was assessed by logistic regression analyses.

There was no association between the serum levels of leptin or adiponectin and MS disease activity. We found a great inter-individual variation in the serum levels of leptin and adiponectin, but the levels were remarkable stable for each patient throughout the study period. The minimum serum-leptin level was 50 pmol/l and the maximum was 6250 pmol/l. The minimum serum-adiponectin level was 0.8 mg/l and the maximum was 31.8 mg/l. About 90 % of the total variation in serum levels was caused by between-patients variations.

We observed no association between serum leptin or adiponectin level at baseline and EDSS score. Patients with relapses or EDSS progression did not differ in serum levels of leptin or adiponectin at baseline or during the study period compared to patients without new disease activity.

Further, there was no difference in the serum levels of adipokines between the patients that obtained NEDA-3 status and patients that had disease activity. The serum leptin level was higher and serum adiponectin level lower before than during IFNβ-treatment as expected by the anti-inflammatory effect of the drug.

6. Discussion

6.1 The contribution of the findings

The exact pathogenesis of MS is not known, but the disease is most likely induced by a complex interplay of genetic and environmental factors affecting the immune system. Obesity and smoking both possess a number of different pathogenetic mechanisms promoting inflammation and possibly driving the immune response towards autoimmunity. Both are established risk factors for MS, and they have also been associated with MS disease course. However, little is known of how obesity and tobacco use may effect the MS disease activity. The papers in this thesis provide more information on these questions.

The MS disease course is unpredictable, and the severity of the disease varies greatly between patients. So far, there are no available biomarkers distinguishing patients regarding prognosis and preferred treatment choice, and the search for biomarker candidates is thus ongoing. The last study in this thesis explored the potential of two adipokines as biomarkers for IFNβ-treatment response in MS.

Smoking and MS disease activity

Although smoking is one of the most studied environmental factors in MS, few studies had addressed the effect of tobacco use on MS disease activity at the time of our study. The need for more knowledge was evident. Two previous studies did not find any association between smoking and relapse rate in MS [119, 128], and a third study reporting of no association between MRI activity in MS and serum cotinine levels had recently been published [125]. In line with these results, we did not find any association between MS disease activity and tobacco use.

Later, a large Danish study of 834 RRMS patients has been published, reporting 20 % increase in relapse rate for smoking RRMS patients during IFNβ-treatment. The risk was dose dependent, and each pack of cigarettes per day increased the number of relapses by about 27 % [129]. The same Danish study group also recently reported of an increase in relapse rate in MS patients who smoked during natalizumab treatment [127]. The results of these studies differ from our findings, and several factors may contribute to this discrepancy. The first study was questionnaire-based, and the patients classified their smoking behaviour as "occasional smoking" or "regularly smoking". The term "regularly smoking" was not explicitly defined. As smoking is considered a "bad habit", it is likely that the patients had a tendency to underreport their own smoking behaviour, and that some smokers are misclassified as non-smokers. This assumption is supported by the fact that only 29 % of the patients in this study were smokers compared to 61 % in our study, although it is most likely that Norwegian and Danish MS patients have somewhat similar smoking habits. It is possible that the patients classified as smokers in their cohort was mainly heavy smokers, whereas the smokers in our cohort probably consisted of both light smokers and heavy smokers. In our study, the patients were classified according to serum cotinine levels, and it is possible that some of the patients used oral tobacco and were misclassified as smokers. This could affect the results as oral tobacco has been reported of a possible protective effect in MS [44]. However, the use of oral tobacco was small in Norway at the time of this study constituting below 20 % of the total proportion of tobacco consumption, and it is therefore likely that the cotinine levels in our samples mainly reflect smoking [308]. The different results may also be caused by the number of study patients. It is possible that the large Danish study observed a difference that we did not detect due to a small cohort and thus a greater possibility of type II error.

In the second Danish study, the patients had a very active RRMS disease with a mean of one relapse a year, contrary to our cohort with relatively few relapses overall. Thus, our cohort consists of RRMS patients with a more moderate disease activity. For MS patients with a more aggravated disease and a larger inflammatory component, smoking may have a larger influence on the relapse rate.

An association between tobacco use and MS disease activity would fit well with the knowledge that smoking likely affects disease progression in MS [115, 120, 122]. However, the effect on disease progression may also be mediated through other mechanisms since studies have shown more brain atrophy and higher levels of serum neurofilament in smoking MS patients [131, 136]. To conclude, the results on this topic are conflicting, and there is a need for more studies regarding smoking and disease activity in MS. Larger prospective studies involving both questionnaires regarding smoking behaviour and the use of other nicotine products, and cotinine analyses could potentially be clarifying.

Author, year	**N**	**Design**	**Follow up time**	**Conclusion**	**Limitations**	**Ref**
Pittas et al, 2009	N=198	Prospective	Median 2,5 years	Smoking was not associated with relapse rate.	-Cohort size	[119]
Weiland et al, 2014	N=2290	Retrospective, longitudinal	1 year	No difference in 12-month self-reported doctor-diagnosed relapse rate according to smoking status.	-Recall bias due to self-reported relapses.	[128]
Munger et al, 2015	N=468	Prospective, longitudinal	5 years	Smoking (assessed by serum cotinine) was not associated with relapse rate.	-Smoking assessed by serum cotinine levels,- possible misclassifications and possible effect of other nicotine products.	[125]
Kvistad et al., 2015	N=87	Prospective, longitudinal	2 years	Smoking (assessed by serum cotinine) was not associated with relapse rate.	-Small cohort -Smoking assessed by serum cotinine levels,- possible misclassifications	

					and possible effect of other nicotine products	
Peterson et al, 2018	N=834	Retrospective, observational, cohort	>2 years	Higher relapse rate in smokers vs. non-smokers, Incidence rate ratio: 1.20 (p=0.027). IRR increase of 27 % per pack of cigarettes per day.	-Recall bias -Vague definition of "regular smoking" in questionnaire, possibly leading to misclassification of smokers	[129]
Peterson et al, 2018	N=355	Retrospective, observational, cohort	2 years	Higher relapse rate in smokers vs. non-smokers. Smoking one pack of cigarettes per day ass. with 38 % increased relapse rate.	-Recall bias -Vague definition of "regularly smoking" in questionnaire, possibly leading to misclassification of smokers	[127]

Table 4: Studies exploring smoking and disease activity in MS.

Smoking and MS disease progression

Several reports have found an effect of smoking on lesion load and brain atrophy on MRI [120, 131]. Further, several studies have reported of an association between smoking and EDSS progression prior to and after our publication [115, 120, 309, 310]. In our study, there was a non-significant trend of disease progression in smokers, where 37 % of the tobacco users had EDSS-progression compared to 21 % of the non-tobacco users. We had a relative short follow-up period of only 24 months and it is possible that our results could have been different with a longer follow-up time. Further, we had a relatively small cohort with overall small changes in EDSS score, and the findings could have been different in a larger cohort or if patients with a more severe MS had been included.

Obesity and MS disease activity

We found no association between BMI and disease activity for the treatment-naïve period. Obesity causes a systemic low grade inflammation and may influence autoimmune diseases in a number of ways [155]. However, there are few reports on how obesity may affect MS disease activity. An American study explored the effect of comorbidities in MS and found that obese women had a more relapsing disease course at onset [175], and a recent report found a trend of higher annual relapse rate in obese MS children [177]. In a study of pediatric-onset MS there was no association between BMI at the time of diagnosis and disease activity [311], and a prospective study of 141 RRMS patients found no association between BMI and the risk of relapses [176]. These latter reports are in coherence with our results. So far, no other studies have explored the association between BMI and MRI disease activity.

Obesity has the last decades been established as a risk factor for MS, based on multiple studies reporting an association between obesity in youth and MS risk [162-164, 167, 172]. The pathogenetic mechanisms are not known, but it is hypothesized that different ways of influencing the immune system are involved. Since obesity affects the MS risk, it is likely that similar immune mechanisms also play a role for the disease activity and evolvement. In our cohort there were relatively few relapses and the number of patients that were overweight and obese were relatively small, and therefore our results cannot exclude a possible association.

There is a need for further studies exploring the association of obesity for MS disease activity, preferably in a larger cohort with a higher proportion of obese patients. Our cohort is probably most representable for patients with a moderate severe RRMS, and the possibility of an association should also be explored in a cohort of MS patients with more severe MS disease.

Obesity and IFNβ-treatment response

We found that fewer overweight or obese patients attained a NEDA-3 status compared to patients with normal weight. Potential explanations could be that the low-grade inflammation observed in obesity may cause a more potent disease or a suboptimal treatment response. There could also be differences related to the administration and dosage regime of the IFNβ-treatment. To examine the possibility of a more potent disease, caused by inflammatory mechanisms, we checked for an association with ten serum inflammation markers that had previously been analysed in this cohort [251]. The serum levels of the inflammation markers were not different among the overweight and obese patients and could not explain our results. We also performed another analyses to explore the serum levels of two adipokines in the cohort. The results of this study showed no association between the adipokines and MS disease activity. There was no difference in the serum level of leptin or adiponectin between the patients that obtained NEDA-status and the patients with disease activity, suggesting that the result of our primary study reflects a suboptimal IFNβ-treatment response in obese patients.

For routine IFNβ-treatment, all patients receive the same dose independent of their BMI. Most studies of IFNβ-treatment have not addressed BMI as a treatment-related factor [312-314], but different doses of IFNβ-treatment has been explored in some studies, with conflicting results. However, none of these studies adjusted for BMI [297, 315]. So far, only one study has explored the effect of BMI in IFNβ-treatment, but since the patients had SPMS there was no effect of IFNβ-treatment overall [316].

Obesity affects drug pharmacokinetics and bioavailability, but knowledge of the effect for subcutaneous injections is scarce [317]. However, some studies describe lower drug concentration after subcutaneous injection in obese patients [318-320]. Obese patients have a decreased subcutaneous blood flow as a result of a decrease in capillary density in subcutaneous tissue that may cause a suboptimal drug absorption [321]. The lower drug concentration may also be explained by the fact that obese patients have a larger distribution volume [321]. Other factors that can lead to less biological effect of IFNβ

include noncompliance and Nabs. The development of NAbs did not differ between normal weight patients and obese patients in our cohort.

After our publications, two reports have found a suboptimal treatment response in obese MS patients. In 2019, a large report described that obese children with MS experienced almost twice as many relapses during treatment with IFNβ and glatiramer acetat, and that the switch rate to a second-line DMT was about 50 % higher in obese than normal-weight children [165]. Another report of pediatric MS patients which compared patients from the US and seven other countries, showed that the patients from US had higher BMI, more frequent relapses during IFNβ-treatment and more often switched to another DMT [322].

All DMTs except cladribine are administered as the same dosage for all patients regardless of BMI. Few studies have explored the role of BMI for these treatments [323]. A recent study found that high BMI was associated with a lower natalizumab receptor occupancy on leukocytes and wearing-off symptoms during natalizumab treatment [324].

All together, these papers emphasize the need for investigating the role of obesity for pharmacokinetics in IFNβ-treatment and also other DMTs, and that there most likely should be BMI-adjusted dosing regimens. Another option would be to strive for a closer evaluation of bioactivity of the drugs, possible by the biomarker for monitoring IFNβ-treated patients, -MxA mRNA [306].

Obesity and inflammation markers

Obesity causes a low-grade systemic inflammation. We studied the association between BMI and 10 different inflammation markers, and found that two of the markers were associated with BMI. Pentraxin-3 (PTX3) is an inflammatory protein with several immunological functions. PTX3 serum levels reflect the grade of inflammation and tissue damage, and has been suggested as a biomarker in autoimmune diseases. In our study we found that PTX3 was inversely associated with

BMI, a finding in coherence with another report [325]. A study have described plasma PTX3 levels of MS patients to be higher during relapses [266]. Moreover, some studies indicate that PTX3 has no major role in the neuroinflammation of MS [267, 326]. Bases on our results, any association between obesity and MS activity does not seem to involve PTX3-mechanisms.

Interleukin 1 receptor antagonist (IL1-Ra) is a soluble inhibitor of the proinflammatory cytokines IL1-α and IL1-β, known to play a role in MS and EAE [327]. Administration of IL-1Ra are shown to have a protective effect in EAE contributing to a milder disease course, [276] and serum levels of IL-1Ra correlate with disease activity and treatment in MS [328, 329]. We found a positive correlation between the serum level of IL-1Ra and BMI, in coherence with other studies [330, 331]. Since higher levels of IL-1Ra are found to be beneficial in MS, our results suggest that any negative influence of obesity for MS is not caused by IL-1Ra related mechanisms.

Obesity and vitamin D

We did not find any correlation between serum 25-hydroxyvitamin D and BMI. This finding is somewhat unexpected since vitamin D is a fat soluble vitamin that can be sequestered in the fatty tissue, causing lower serum vitamin D levels [332, 333]. Other mechanisms for lower vitamin D levels in obese are less sun exposure and volumetric dilution [333]. The association of low serum 25(OH)D level with obesity have been found in studies of both adults and children, and serum 25(OH)D level is reported to be 20 % lower in obese that normal weight individuals [169, 170, 332-334]. However, there are also studies of no correlation [335, 336]. Most studies reporting of a correlation consist of a high proportion of patients with BMI >30. Our cohort had relatively few obese patients, and it is possible that our findings would have been different if more of the patients were obese or morbidly obese (BMI>35).

Adipokines and MS disease activity

In our cohort, fewer of the overweight and obese patients obtained NEDA-status. We sought to further explore on this finding in the last paper of this thesis, checking whether there could be a more profound inflammation and different levels of adipokines in the obese patients. Leptin and adiponectin are fatty hormones with important roles in the regulation of the immune system, contributing to the obesity driven systemic inflammation. Most studies have found increased serum leptin levels and decreased adiponectin levels in MS patients compared to healthy controls, and it is therefore assumed that they somehow affect the MS disease [218, 236]. But studies regarding their role in MS disease activity are conflicting [223-226, 238, 240]. These adipokines are found to possess important effects in EAE, as leptin deficient mice are resistant for developing EAE, and adiponectin administration improve the EAE disease severity [227, 241]. Based on the effect of leptin in EAE animal models, therapies for limiting the bioavailability of leptin has been suggested [337]. In our study, however, there was no association between MS disease activity and the serum level of these adipokines. The serum levels did not differ for patients with higher EDSS score at baseline or between patients that did or did not obtain NEDA-status. Our study suggests that these adipokines do not have profound roles associated with MS disease activity, and indicates that the therapeutic potential of medications influencing serum level of these adipokines is most likely poor.

Adipokines as biomarkers

Leptin and adiponectin are suggested biomarkers in MS, and a number of studies have explored the serum fluctuations in MS, with largely heterogenous results. For serum leptin, higher levels initially were found to predict a more aggravated disease the upcoming year, but in another report high levels initially were associated with a milder disease, while other studies have found that the levels were higher during remission state [224-226, 235]. For adiponectin, higher serum levels in children with MS are found to be associated with a lower risk of relapses, but another study found opposite

results where higher baseline levels were associated with disease progression [235, 238]. Yet another study found no association between serum adiponectin levels and MS disease activity [240].

We did not find any association between relapses or MRI disease activity and the serum levels of leptin or adiponectin. Further, the serum levels did not differ among patients with or without EDSS-progression or for patients that did or did not obtain NEDA-status. However, during IFNβ-treatment the serum leptin levels dropped and the adiponectin levels increased for all patients reflecting the anti-inflammatory effect of the drug.

For both leptin and adiponectin there were major differences in the serum levels between patients, but the levels for each patient were relatively stable for all eight measurements during follow up. About 90 % of the total variation in serum levels of the adipokines could be attributed to between-patient variations. According to these results, one could presume that if the serum levels of these adipokines had a role in the disease activity or course, it would be highly detectable in this cohort given the large differences between patients. Our results indicate that neither of these adipokines have potential as biomarkers for disease activity or IFNβ-treatment response in MS.

Conclusions

In this thesis we explored the role of tobacco use and obesity in MS. After our studies were conducted, more reports have been published on this topic. Smoking and BMI have independently been shown to be negatively associated with global disability and depression in MS [338]. Both factors together have also shown an even worse prognosis, and obese MS patients that smoked had an increased risk of SPMS [339].

Smoking and obesity are modifiable lifestyle factors, opposed to other fixed factors affecting the disease course, such as genetics. The effect of lifestyle changes can make a great impact on the disease. A study showed that patients who stopped smoking before or after the disease onset had the same disease progression as never smokers

[115]. It has also been demonstrated that patients who stopped smoking at the time of the diagnosis developed SPMS eight years later than the patients that continued to smoke [340]. There is a need of more studies to gain information on lifestyle factors, to guide patients and remind healthcare providers to engage and motivate for lifestyle-based behaviour changes [341]. The papers in this thesis all contribute with information and may cause more focus on the field.

6.2 Methodological considerations and limitations

6.2.1 Patient population

The OFAMS cohort is a well-defined cohort with a thoroughly follow-up over two years. It comprised 92 RRMS patients with a mean age of 39 years and a female: male ratio of about 2:1. All patients had active disease with ≥1 relapse or new T1-weighted gadolinium-enhancing (T1Gd+) or T2-weighted (T2) lesions on MRI in the year prior to inclusion. Although the patients had an active MS disease, they were followed for six months without DMT. At the time this study was conducted it was not unusual to wait some months before starting treatment and the study had ethical approval, but this kind of treatment delay would probably not be permitted in a study nowadays. Patients that demanded immediate disease modifying treatment were, however, excluded from the study. This suggests that our results may be generalized to RRMS patients with moderately active disease.

The number of relapses in this cohort was relatively low, especially for the period during IFNβ-treatment, meaning it could be difficult to detect possible associations between some factors and relapses. In our studies we have been aware of this weakness, and although we have checked for an association with relapses, our main assessment has been MRI disease activity.

A total of 26 of the patients had an EDSS progression during the study period. Although EDSS score is an established measure to evaluate disease progression and treatment response, it is not very sensitive for smaller changes early in the disease course. For our results regarding disease progression, the follow-up period is probably too short. As an example, smoking has been shown to affect disease progression in several studies, but we were not able to find this in our study, although we found a non-significant trend. This might have been due to short follow-up time.

Our studies consisted of 86-88 RRMS patients. The small population size is a weakness increasing the likelihood for type II error, i.e. falsely acceptance of a null hypothesis of no association when there actual is an association.

The patients had multiple MRI examinations, but brain atrophy was not examined. Studies have shown an association between brain atrophy and both smoking and obesity [131, 185]. It is possible that increased brain atrophy due to neurodegeneration is the cause of increased disease progression in smoking MS patients, and not a more active inflammatory disease. This theory would fit well with our results, hence to examine MRI brain atrophy and possibly also analyzing serum neurofilament as an assessment of neuroaxonal injury would have been interesting and could have strengthen our results.

The original study was a randomized controlled study, a strength regarding the patient inclusion and stringent data collection. However, it is a weakness that all studies in this thesis were not original studies assembled according to a hypothesis, but retrospective studies based on data from the first study. This implicates that measurements and data are not optimally collected for our study purposes. For our study on tobacco use we classified the patients according to serum cotinine levels as the patients were not asked about smoking habits in the original OFAMS-study.

The patient cohort from the OFAMS study has been the objective of a number of studies, searching for different associations with MS disease activity. So far, studies have been published on vitamin A, D, E, different inflammation markers, α-linolenic

acid and EBV- antibodies in addition to the studies included in this thesis [73, 89, 251, 278, 287-291]. For this material a large number of tests have been carried out without pre-planned hypothesis and there may be a possibility for type 1-errors, hence finding a false association. In our second study we explored the association between BMI and ten different inflammation markers, and for PTX3 and IL1-Ra the association would still have been significant after bonferroni correction.

6.2.2. Confounders

We adjusted for HLA-DRB1*15 status and gender in our studies. HLA-DRB1*15 is the major risk allele for MS, and is reported to interact with both smoking and obesity, greatly increasing the risk of the disease [63, 64]. As for most autoimmune diseases, the prevalence of MS is greater among women. There might be gender differences in the disease caused by effect of gonadal hormones on the immune system, and differences in response to immunotherapy [10].

We did not adjust for NAbs in these studies. However, 27 of the 88 patients developed NAbs during IFNβ-treatment. This is a confounder that could potentially affect our results, and it is a weakness that this was not adjusted for and discussed in our reports. However, we have re-analysed our main results adjusting for NAbs. This did not alter any of our results. In the second paper, we found that 19 of the normal weight patients, 8 of the overweight patients and no obese patients had NAbs. Interestingly, four of the patients obtaining NEDA-status had NAbs; three normal weight patients and one overweight patient. Thus NAbs did not explain the difference in NEDA-status between normal weight patients and overweight-and obese patients.

Serum vitamin D level was not adjusted for in any of our studies, even though an association between serum vitamin D levels and MRI-activity has been confirmed in this cohort [89]. The serum levels of 25(OH) D are found to be reduced in smokers, and passive smoking is also associated with lower vitamin D levels [342, 343]. Obese individuals tend to have lower vitamin D levels than individuals with normal weight.

In our second study we therefore explored the correlation between BMI and serum vitamin D level in this cohort, but the correlation was weak.

Another potential confounding factor in our study is dietary habits. A typical "Western diet" with much saturated fatty acids may cause a proinflammatory effect on the immune system and also affect BMI. For this cohort we had no information regarding dietary behaviour.

Serum cholesterol profile are reported to be associated with the MS disease course [176, 181, 182]. Further, the lipid profile and function are found to be altered in MS, with a more profound effect in patients with low BMI, possibly contributing to disease progression [344]. Hence, this may have been a confounding factor in our BMI study. A recent study describes changes in serum cholesterol in relation to IFNβ-treatment, and a greater decrease in HDL cholesterol was associated with less long-time brain atrophy, suggesting it to be a biomarker for treatment response in MS [345]. The measurement of serum cholesterol levels and adjusting for this in our cohort could have been an interesting supplement analysis, possibly strengthening our results.

Physical activity has a role in MS, as excessive physical activity has been shown to decrease the MS risk regardless of BMI [45]. For children with MS, more physical activity was associated with a lower annual relapse rate [346]. We had no information about the grade of physical activity for the individual patients in our cohort, and this may be a possible confounder for our results.

Body composition and fat distribution may have been a confounding factor in our BMI studies. High weight may in some cases reflect high muscle mass, and it is the amount of fat tissue that is likely to affect the low grade systemic inflammation that could be the connection between obesity and MS. In our study it could have been a strength to add measure of waist circumference and body composition, presumably by bio-impedance measurement or dual energy X-ray absorptiometry, in addition to height and weight.

6.2.2 Serum analysis and laboratory testing

All studies are based on analyses of blood samples, collected in the original OFAMS-study. Samples were collected in different treatment centers and transported in a frozen condition to Haukeland University hospital for cryopreservation. The samples were stored at -80°C until analysis. The analyses were performed simultaneously for all samples from each patient. The lab technicians were blinded for treatment, as well as MRI activity. However, there are a risk of pre-analytic, analytic and post-analytic variations and errors.

Furthermore, the samples were not originally collected for the tests of cotinine and adipokines. The serum leptin levels have a high grade of diurnal variation, and optimally all samples should have been collected at the same time of the day. Since all the samples in the study were collected at daytime between 07.00-15.00 p.m, this is probably not a great limitation in our study.

For adiponectin we did not analyze the different isoforms. Since they possess some different biological effects, it might have been interesting to check for the association of the different isoforms with MS disease activity.

Cotinine is a nicotine metabolite that reflects the tobacco use the last 3-5 days. The fact that we used this biomarker and not patient questionnaires to classify tobacco users is a weakness. Patients that may have smoked regularly, but recently quit will according to our method be classified as non-tobacco users, even though cigarette smoking may have long-lasting consequences on the immune system. If so, it might have affected our results. Then again, questionnaires also possess weaknesses, and since smoking is known to be a bad habit, people tend to underreport the actual amount. A combination of self-reported data validated by serum cotinine measurements would have been the optimal solution.

The use of snuff and other nicotine products may have been confounders in our tobacco study as nicotine is shown to have a protective effect in inflammatory diseases by dampening inflammation. If some of the patients had high serum cotinine level because of the use of snuff this could have affected our results. At the time of our study the

proportion of smokeless tobacco consumption in Norway was low, and we therefore assume that the cotinine levels predominantly reflect smoking [347].

7. Conclusions

Paper I: We did not find any association between serum cotinine level and MRI disease activity. Our results indicate that tobacco use do not influence short-term MS disease activity.

Paper II: Fewer of the overweight and obese patients in our study obtained NEDA-3 status after the 18 months of IFNβ-treatment compared to the normal weight patients, suggesting that BMI may affect IFNβ-treatment response. This indicates that the treatment should be individualized according to weight, opposed to the standard treatment regime of one dose for all.

Paper III: We did not find any association between the adipokines leptin and adiponectin and clinical- and MRI disease activity, indicating that these adipokines are not suitable as biomarkers for disease activity in RRMS.

8. Further perspectives

Several studies have contributed to a greater understanding of the relationship between environmental and lifestyle factors, and MS. Even though the cause of MS is still unknown, strong evidence supports an involvement of both genetic and environmental factors. Low serum vitamin D levels, smoking and obesity likely increase the risk of MS and may also effect the disease course. Interestingly, these factors are all modifiable. Relatives of MS patients can influence their MS risk by striving for a normal body weight, avoiding or quit smoking and by taking vitamin D supplements. For MS patients, vitamin D supplementation and smoking cessation are recommended.

In this thesis, we have contributed with knowledge regarding lifestyle factors and MS disease activity. Although smoking seems to affect the MS disease progression, it might not directly affect MRI activity or relapse rate. Possibly, effect on neurodegenerative processes is the main mechanism of how smoking effect the MS disease course, thus explaining our negative results.

Obesity and tobacco use have several negative health effects. Although we did not find a direct association with MS disease activity, these factors possess a number of other negative effects in MS by influencing disease progression and comorbidity. As both are lifestyle factors, patients may look to our results for a reason to maintain a bad habit. We are aware of our responsibility to always proclaim the uncertainty of our results, and to reflect on our finding in a bigger picture.

The results of our second and third study indicated a suboptimal IFNβ-treatment response in obese patients. IFNβ- treatment has, so far, been administered as a one-dose fits all regime, as for most DMTs. Our study may have drawn attention to this problem. More recently, a similar study has been conducted for pediatric patients supporting our findings. We hope that in the future, the role of obesity for pharmacokinetics in DMT and other treatments in MS will be attended, and that there will be individually adapted treatment protocols and BMI-adjusted dosing recommendations where needed. It is likely that obese patients may attain a better

treatment response if the dose is administered according to weight, and this can be of great importance for the patients, avoiding increased disease burden.

Since the individual disease course and treatment response is somewhat unpredictable, there is a need for biomarkers in MS. We have explored the role of leptin and adiponectin and did not find these to be suitable markers. Our study indicates that their role in the disease pathogenesis may not be profound, and that they are not promising treatment targets in MS. Considering the impact of leptin in animal studies, the results of our study was rather disappointing, and there is still a need for more studies on the role of adipokines in autoimmune disease pathogenesis and course.

Many studies are based on the OFAMS cohort, as all papers in this thesis. Recently a 10-years follow-up study was conducted in this cohort. It may contribute to important information on how environmental factors effect the long-term disease course in MS.

All together the impact of environmental factors in MS continue to be an important research field that need further studies in the years to come.

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No association of tobacco use and disease activity in multiple sclerosis

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ABSTRACT

Objective: To study whether tobacco use is associated with MRI and clinical disease activity in patients with multiple sclerosis (MS).

Methods: Prospective cohort study of 87 patients with relapsing-remitting MS originally included in a randomized placebo-controlled trial of omega-3 fatty acids in MS (the OFAMS Study). Serum levels of cotinine (biomarker of tobacco use) were analyzed at baseline and every 6 months for 2 years. MRI activity was assessed at baseline and monthly for 9 months and after 12 and 24 months.

Results: Fifty-three patients (61%) had serum cotinine levels ≥85 nmol/L on ≥60% of the measurements and were considered tobacco users and 34 (39%) had cotinine levels <85 nmol/L, consistent with non–tobacco use. There was no association between tobacco use and the occurrence of new gadolinium-enhancing T1 lesions, new or enlarging T2 lesions, or their aggregate (combined unique activity). Furthermore, there was no association between cotinine levels and MRI activity for the tobacco users, and tobacco users did not have more relapses or Expanded Disability Status Scale progression.

Conclusion: Our results indicate that tobacco use does not directly influence MRI activity or relapse rate in MS. This may implicate that the reported association between smoking and MS disease progression could be mediated through other mechanisms. ***Neurol Neuroimmunol Neuroinflamm* 2016;3:e260; doi: 10.1212/NXI.0000000000000260**

GLOSSARY

BMI = body mass index; **CI** = confidence interval; **EDSS** = Expanded Disability Status Scale; **HLA** = human leukocyte antigen; **IFN-β-1a** = interferon beta-1a; **MS** = multiple sclerosis; **OR** = odds ratio; **RRMS** = relapsing-remitting multiple sclerosis; **SPMS** = secondary progressive multiple sclerosis; **T1Gd** = T1-weighted gadolinium-enhanced.

Multiple sclerosis (MS) is an inflammatory disease of the CNS. The etiology is unknown, but there seems to be an interaction between genetic and environmental risk factors.[1] Several human leukocyte antigen (HLA) subtypes are known to increase the risk of MS,[2] and several environmental factors such as low serum vitamin D levels, Epstein-Barr virus infection, and smoking are associated with MS.[3,4]

Smoking is a known risk factor for developing MS.[3,5–8] Smokers with clinically isolated syndrome have been reported to have earlier progression to clinically definitive MS than nonsmokers,[9] and smoking has been suggested to decrease the time for disease conversion from

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Funding information and disclosures are provided at the end of the article. Go to Neurology.org/nn for full disclosure forms. The Article Processing Charge was paid by the University of Bergen.

relapsing-remitting MS (RRMS) to secondary progressive MS (SPMS).[10–14] It has been suggested that smoking influences the clinical progression in MS and several studies have explored this, with conflicting results.[12,15,16] One study has reported that smoking cessation decreases the risk of Expanded Disability Status Scale (EDSS) progression,[14] but a recent report did not find any association between tobacco use and MS activity or progression over a 5-year follow-up.[17]

To address the effect of tobacco use in established MS, we examined the associations among serum cotinine levels, MRI, and clinical disease activity in a 2-year longitudinal study of 87 HLA-DRB1*15–typed patients with RRMS.

METHODS

Standard protocol approvals, registrations, and patient consents. The study was approved by the Regional Committee for Medical and Health Research Ethics in Western Norway Regional Health Authority, and all participants gave written informed consent.

Study participants and design. The study design has been presented previously.[18–20] In brief, this was a cohort study of 87 patients with RRMS according to the McDonald criteria originally included in a randomized placebo-controlled trial of omega-3 fatty acids at 13 Norwegian MS centers from December 2004 until July 2008 (the OFAMS [ω-3 Fatty Acid Treatment in Multiple Sclerosis] Study). Included patients had ≥1 clinical relapse, a new T1-weighted gadolinium-enhanced MRI (T1Gd)-positive lesion or enlarging T2 lesions in the last 12 months before enrollment. The patients were followed for 24 months with thorough examinations including serum samples, MRI scans, and clinical scorings. MRI scans were performed at baseline and monthly for 9 months and then after 12 and 24 months according to a standardized protocol.[18] The sum of T1Gd+ lesions and new or enlarging T2 lesions was denoted as combined unique activity. Clinical data were recorded by experienced neurologists, including EDSS scores every 6 months and clinical relapses throughout the study period. The patients did not use any immune modulatory drugs at inclusion, but from month 6, all patients started subcutaneous injections with 44 μg of interferon beta-1a (IFN-β-1a) (Rebif; Merck KGaA, Darmstadt, Germany) 3 times weekly. All patients were randomized to receive omega-3 fatty acids (Triomar; Pronova Biocare AS, Sandefjord, Norway) or placebo (corn oil) daily throughout the study period. Since no effects from omega-3 fatty acid supplementation on MS disease activity were detected in any part of the study, all patients were pooled in the current analysis.

Measurements. Cotinine is a nicotine metabolite with a half-life of 15 to 40 hours. It is the most widely used biomarker for recent tobacco use and has a high sensitivity (96%–97%) and specificity (99%–100%).[21] The serum levels reflect tobacco exposure during the prior 3 to 5 days. Serum samples were stored at −80°C until analysis, which was performed simultaneously for all samples from each patient. The laboratory technicians were blinded for the clinical and radiologic status of the patients. Serum levels of cotinine were measured using liquid chromatography tandem mass spectrometry at Bevital AS (Bergen, Norway). The method was highly sensitive and specific with a lower limit of detection at 1 nmol/L. The within-day coefficient of variation was 2.0% to 6.6% and the between-day coefficient of variation was 3.9%. We analyzed serum samples from patients at baseline and months 6, 12, 18, and 24.

Cotinine levels >85 nmol/L are indicative of recent tobacco use[22] and are regarded as the optimal cutpoint widely used for distinguishing tobacco users from non–tobacco users in the general population.[21] Cotinine has the advantage over other tobacco biomarkers that the optimal cutpoints are little affected by the prevalence of smoking in the population sampled.[23] This is also the cutoff value recommended by Bevital AS, the laboratory where the serum analyses were performed.[24]

The patients were categorized into 2 groups according to serum cotinine level. Patients with serum cotinine level ≥85 nmol/L in ≥60% of the samples were considered tobacco users and patients with serum cotinine levels <85 nmol/L in ≥60% of the samples were considered non–tobacco users.

The HLA-DRB1*15 analyses have been described previously.[18,20,25]

Missing values. A total of 9 MRI scans (1 during study months 1–6 and 8 during study months 7–24) were missing. Seven blood samples for serum cotinine analysis were missing for months 0 to 6 and 39 were missing for months 7 to 24.

Statistics. Data were described as frequencies and percentages or means and SDs, as appropriate. The differences in number of relapses and baseline EDSS scores among smokers and nonsmokers were assessed using independent-samples *t* test.

The association between tobacco use and MRI activity was assessed using a logistic regression model for hierarchical data. The model contained fixed effect for smoking status and random effects for intercepts correctly adjusting the estimates for intrapatient correlations. The model was estimated for total study period as well as for periods before and during IFN-β-1a treatment. All models were also adjusted for sex, age, body mass index (BMI), and HLA-DRB1*15 status. The association between cotinine levels and MRI activity among tobacco users (serum cotinine ≥85 nmol/L) was also assessed by a logistic regression model for hierarchical data with fixed effect for cotinine level and random effects for intercepts.

All tests were 2-sided. Results with p values <0.05 were considered statistically significant. The analyses were performed in SAS version 9.4 (SAS Institute, Cary, NC) and SPSS version 22 (IBM Corp., Armonk, NY).

RESULTS

Cotinine levels and MRI disease activity. Of 87 patients, 53 (61%) had cotinine levels >85 nmol/L in ≥60% of the samples and were considered tobacco users, and 34 (39%) were considered non–tobacco users. Most patients had consistent cotinine levels on all samples, but 11 (13%) had consistent levels in 75% of the measurements and 4 (5%) in 60% of the measurements. There was no association between tobacco use and MRI activity, for new T1Gd+ lesions (odds ratio [OR] = 0.76; 95% confidence interval [CI] 0.41–1.43; $p = 0.39$), for new or enlarging T2 lesions (OR = 0.81; 95% CI 0.42–1.56; $p = 0.52$), and for combined unique activity (OR = 0.81; 95% CI 0.43–1.53; $p = 0.51$) for the total study period. The result was consistent for the 6 months before and 18 months

during IFN-β-1a treatment. Adjusting for sex, age, BMI, and HLA-DRB1*15 status did not influence our results (table). Similarly, when only analyzing tobacco users by logistic regression model for hierarchical data, there was no association between cotinine levels and MRI activity during the 2-year follow-up (OR = 0.98; 95% CI 0.94–1.04; $p = 0.52$).

Cotinine levels and clinical disease activity. A total of 42 relapses were recorded in 23 patients, of which 14 occurred during the first 6 study months. There was no difference in number of relapses between the tobacco users and non–tobacco users, with a mean number of relapses of 0.42 (SD = 0.77) for tobacco users and 0.59 (SD = 1.18) for non–tobacco users ($p = 0.41$).

There was no difference in baseline EDSS score between tobacco users (mean of 1.92, SD = 0.85) and non–tobacco users (mean of 1.85, SD = 0.84) ($p = 0.7$). During the study period, 26 patients progressed ≥1 EDSS point. A total of 19 (37%) of the tobacco users had EDSS progression compared to 7 (21%) of the non–tobacco users, but the difference was not significant ($p = 0.14$).

DISCUSSION In this cohort of patients with MS, we could not detect any association between tobacco use, assessed by serum cotinine levels, and MRI or clinical disease activity.

This study was performed in a well-characterized cohort of patients with RRMS prospectively followed for 2 years with repeated and paired MRI scans and measurements of cotinine levels both before and during IFN-β-1a treatment. The results were adjusted for sex, age, BMI, and HLA-DRB1*15 status. All MRI, biochemical, and clinical assessments were performed with strict and standardized procedures.

There are some limitations to our study. We used serum cotinine levels as a proxy for smoking behavior, but being a marker for tobacco use, the levels will also be high among snuff users or users of nicotine gum, and one study has suggested possible protective effects of these considering MS risk.[26] The total proportion of smokeless tobacco of all tobacco consumption in Norway during the study period was, however, less than 20% and even lower among women.[27] It is therefore reasonable to assume that the cotinine levels in our samples mainly reflect smoking. The patients were classified as tobacco users or non–tobacco users based on cotinine level in ≥60% of the measurements, and there is a risk of misclassification, possibly mostly for light smokers since the half-life of serum cotinine is 15 to 40 hours. However, we had serum measurements at 5 different time points and most patients had consistent serum cotinine levels on all measurements, indicating that this is not a major issue. It has also been demonstrated that serum cotinine levels are well correlated with patient-reported smoking behavior.[28] The follow-up period of 24 months is relatively short and our results might have been different with longer follow-up. However, our findings are in coherence with those of a recently published study with a follow-up period of 5 years.[17] Also, the lack of any of the results being even close to significant toward influence of tobacco use on MRI disease activity make it unlikely that there is any association. Our MRI findings were supported by no association between cotinine levels and clinical disease activity. The follow-up period was short and few relapses were reported, thus the sensitivity for clinical activity is low. However, our main outcome was MRI activity, which is a sensitive and well-known assessment for subclinical disease activity.

Table Odds ratios for MRI disease activity associated with tobacco use (serum cotinine ≥85 nmol/L) in patients with relapsing-remitting multiple sclerosis

	Total study period (n = 87)		Before IFN-β treatment, months 1–6		During IFN-β treatment, months 7–24	
	Odds ratio (95% CI)	p Value	Odds ratio (95% CI)	p Value	Odds ratio (95% CI)	p Value
MRI measure						
New T1Gd+ lesions	0.76 (0.41–1.43)	0.39	0.80 (0.34–1.87)	0.61	0.56 (0.19–1.63)	0.28
New T2 lesions	0.81 (0.42–1.56)	0.52	0.74 (0.30–1.81)	0.74	0.85 (0.35–2.08)	0.72
CUA	0.81 (0.43–1.53)	0.51	0.75 (0.31–1.82)	0.52	0.80 (0.32–1.98)	0.62
Adjusted for sex, age, BMI, and HLA-DRB1*15 status (n = 81)						
New T1Gd+ lesions	0.76 (0.39–1.50)	0.43	0.72 (0.28–1.81)	0.47	0.68 (0.21–2.19)	0.51
New T2 lesions	0.87 (0.43–1.75)	0.69	0.65 (0.26–1.68)	0.37	1.04 (0.40–2.72)	0.94
CUA	0.84 (0.42–1.68)	0.63	0.63 (0.25–1.61)	0.33	0.98 (0.37–2.61)	0.97

Abbreviations: BMI = body mass index; CI = confidence interval; CUA = combined unique activity; IFN-β = interferon beta; T1Gd = T1-weighted gadolinium-enhanced.

The lack of any association between smoking and EDSS progression in our study could be attributable to the relatively short follow-up time of 24 months, and the fact that few patients experienced EDSS progression during the study period. A number of earlier studies have reported increased risk of disease progression among smokers.[14,15] The mechanism for this association is largely unknown, but one possibility could be that smoking increases the inflammatory activity in MS. It has been demonstrated in the experimental autoimmune encephalitis model of MS that proinflammatory T cells are activated in the lungs before they attack the brain.[29] Although our negative results may implicate that this mechanism does not have a major role in patients with established MS, they do not exclude this possibility, or that smoking has an important role during the initiation phase of the disease. Smoking is one of the most attractive and studied environmental risk factors for MS. The first reports on an association between MS and smoking were published in the 1990s,[30,31] and since then, a number of studies have confirmed smoking as a risk factor for MS.[5–7,26,32] Even passive smoking has been associated with MS.[33] Recent studies have also described the association between cotinine levels in serum and the risk of MS.[34,35] Previous reports have found an association with clinical disease and earlier progression from clinically isolated syndrome to RRMS and from RRMS to SPMS among smokers.[10–13,36] However, others have not been able to confirm an association between smoking and the risk of MS or an earlier progression to SPMS in smokers.[16,37] Only a few studies have examined how smoking influences disease activity in MS. One study explored the association between MRI lesions in MS and smoking and found that the T2-weighted lesion volume increased faster in smokers,[10] and a recently published study reported no association between MRI activity and serum cotinine levels.[17] The result of our study supports this latter report. Other hypothesized mechanisms of adverse effects of smoking in MS include chronic cyanide intoxication leading to demyelination, the direct effect of cigarette-smoke components on the blood–brain barrier and smoking-mediated increased frequency and persistence of infections.[11] There are also several other possible reasons for the increased impairment and disability in patients with MS who smoke and it is not clear whether it is a direct effect of tobacco use or a consequence of comorbidities associated with smoking.[38]

Our results do not support a short-term (2 years) influence of tobacco use on MRI and clinical disease activity in MS. Long-term effects and the importance of comorbidities influenced by smoking in MS need to be explored.

AUTHOR CONTRIBUTIONS

Silje Kvistad: study concept and design, acquisition of data. Kjell-Morten Myhr: study concept and design, analysis and interpretation, acquisition of data, critical revision of the manuscript for important intellectual content, study supervision. Trygve Holmøy: analysis and interpretation, critical revision of the manuscript for important intellectual content. Jūratė Šaltytė Benth: analysis and interpretation. Kristin I. Løken-Amsrud, Stig Wergeland: critical revision of the manuscript for important intellectual content. Antonie G. Beiske, Kristian S. Bjerve, Harald Hovdal, Finn Lilleås, Rune Midgard, Tom Pedersen, Søren J. Bakke: acquisition of data, critical revision of the manuscript for important intellectual content. Øivind Torkildsen: study concept and design, analysis and interpretation, acquisition of data, critical revision of the manuscript for important intellectual content, study supervision.

STUDY FUNDING

No targeted funding.

DISCLOSURE

S. Kvistad received research support from Novartis, Biogen. K.-M. Myhr served on the scientific advisory board for Novartis Norway, Biogen, Genzyme, Roche, received travel funding from Sanofi-Aventis, Novartis, Biogen, received speaker honoraria from Genzyme, Sanofi-Aventis, Novartis, Biogen, Roche, Almirall, received research support from Sanofi-Aventis, Novartis, Biogen, ProNova, Norwegian MS Society. T. Holmøy received speaker honoraria from Merck Serono, Sanofi-Aventis, Biogen Idec, Novartis, Genzyme, is an editorial board member for *Journal of the Norwegian Medical Association*, *The Open Virology Journal*, *Future Neurology*, received research support from Merck Serono, Norway. J.S. Benth and K.I. Løken-Amsrud report no disclosures. S. Wergeland received speaker honoraria from Biogen, Novartis. A.G. Beiske served on the scientific advisory board for Genzyme, received travel funding from Teva, has been an author for *Nyhetsbulletinen*, received research support from Novartis. K.S. Bjerve and H. Hovdal report no disclosures. F. Lilleås reports no disclosures. R. Midgard served on the scientific advisory board for Novartis Norway, received travel funding and/or speaker honoraria from Novartis Norway. T. Pedersen reports no disclosures. S.J. Bakke served on the scientific advisory board for Norvasc, received travel funding from ASNR, was a scientific advisor for the Norwegian Medical Association, receives royalties for a textbook. Ø. Torkildsen served on the scientific advisory board for Biogen, Sanofi-Aventis, Merck. Go to Neurology.org/nn for full disclosure forms.

Received February 29, 2016. Accepted in final form June 2, 2016.

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Journal of Neuroimmunology 288 (2015) 92–97

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Body mass index influence interferon-beta treatment response in multiple sclerosis

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ARTICLE INFO

Article history:
Received 4 August 2015
Received in revised form 18 September 2015
Accepted 22 September 2015

Keywords:
Multiple sclerosis
Body mass index (BMI)
MRI disease activity
Interferon-beta treatment
No evidence of disease activity
Treatment response

ABSTRACT

Obesity is a possible risk factor of multiple sclerosis (MS), but the association between obesity and MS disease activity has not been explored. In a cohort of 86 MS patients, 80% of overweight or obese patients (BMI ≥ 25 kg/m^2) had MRI activity compared to 48% of the normal-weight patients (BMI < 25 kg/m^2) ($p = 0.001$) during interferon-beta treatment. NEDA-status (no evidence of disease activity) was defined as a composite that consisted of absence of any relapses, sustained disability-progression and MRI-activity. Among normal-weight patients 26% obtained NEDA-status compared to only 13% of patients with BMI >25 ($p = 0.05$). This may indicate that BMI affects interferon-beta treatment response.

1. Introduction

Multiple sclerosis is an autoimmune disease of the central nervous system, caused by an interaction of genetic and environmental risk factors. Some HLA-alleles, low serum vitamin D levels, Epstein–Barr virus infection and smoking are all factors associated with increased MS risk (Sawcer et al., 2014; Ascherio and Munger, 2007). Obesity has also been suggested as a possible risk factor for MS, and during the last decade, several publications have reported an association between higher BMI in youth and early life and MS (Munger et al., 2009, 2013; Hedstrom et al., 2012; Wesnes et al., 2014; Langer-Gould et al., 2013).

Obesity induces a state of low-grade chronic systemic inflammation (Overs et al., 2012). It is a well-known risk factor for multiple conditions including cardiovascular and related metabolic disorders and some

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http://dx.doi.org/10.1016/j.jneuroim.2015.09.008
0165-5728/

forms of malignancies, and has also been associated with an unfavorable course of several autoimmune diseases (Versini et al., 2014). Obesity in female MS patients at the time of diagnosis is associated with a relapsing course at disease onset (Marrie et al., 2011). Obesity is also associated with a greater risk of depression, lower functional capacity and worse self-rated health status among MS patients (Taylor et al., 2014; Cambil-Martin et al., 2014). Recently, a positive correlation between BMI and disability evaluated by Expanded Disability Status Scale (EDSS) was reported (Oliveira et al., 2014). So far no study has explored the association between BMI and disease activity, or whether BMI influences the response to interferon-beta (IFNβ)-treatment in RRMS.

The main aim of this study was to explore if BMI has an impact on clinical and MRI disease activity in untreated patients with RRMS and during IFNβ -therapy. Based on the association of BMI with systemic inflammation as well as the role of inflammation and vitamin D status in MS disease progression, we also explored possible associations between BMI and selected inflammatory markers and serum vitamin D.

2. Material and methods

2.1. Study population and design

This was a prospective study of 86 patients with RRMS followed for a total of 24 months with repeated serum analyses, MRI scans and EDSS assessments (Table 1). The patients were included in the original OFAMS-study, a randomized double-blind, placebo-controlled multicenter trial of omega-3 fatty acids including 92 Norwegian patients with RRMS according to the McDonald criteria. The patients were aged 18–55 years with an EDSS score ≤5, and ≥1 relapse or new T1-weighted gadolinium enhancing (T1Gd +) or T2-weighted (T2) lesion on MRI in the year prior to inclusion. They did not receive any immunomodulatory therapy at inclusion and the first 6 months, but thereafter all patients received 44 μg IFNβ subcutaneous injections three times weekly. No effects from ω-3 fatty acids supplementation on MS disease activity were detected, and all patients were therefore pooled in the current analysis (Torkildsen et al., 2012).

2.2. Measurement of inflammation markers and 25-hydroxyvitamin D

Serum samples were collected at baseline and after 1, 3, 6, 7, 9, 12, 18 and 24 months and stored at −80 °C until analysis. 25-hydroxyvitamin D was measured with a radioimmunoassay kit (ImmunoDiagnostic Systems, Boldon, UK). The concentrations of osteoprotegerin (OPG), soluble tumor necrosis factor receptor 1 (sTNFR1), the chemokines CXCL16 and CCL21, transforming growth factor (TGF)β1, pentraxin 3 (PTX3), matrix metalloproteinase 9 (MMP-9), interleukin-1 receptor antagonist (IL-1Ra), osteopontin (OPN), and activated leukocyte cell adhesion molecule (ALCAM) were measured by enzyme immunoassay (EIA) obtained from R&D systems (Stillwaters, MN) as described (Holmoy et al., 2013). The analyses were performed simultaneously for all samples from each patient. For each marker consecutive samples from each patient were analyzed in neighboring wells on the same plate. The lab technicians were blinded for treatment as well as MRI activity. The intra- and inter-assay coefficient of variation were <10% for all EIAs.

Table 1
Clinical, MRI and laboratory measurements during study period.

	Month												
	Baseline	1	2	3	4	5	6	7	8	9	12	18	24
	No treatment							Interferon β1a treatment					
MRI examination	•	•	•	•	•	•	•	•	•	•	•		•
EDSS evaluation	•						•				•	•	•
Serum-inflammation markers and 25-hydroxyvitamin D	•	•		•			•	•		•	•	•	•

MRI = magnetic resonance imaging; EDSS = Expanded Disability Status Scale. Relapses were recorded throughout the study period.

2.3. HLA-DRB1 typing

The HLA-DRB1 status was determined by DNA sequencing using SeCoreLoc DRB1 SEQ kit (Invitrogen, Carlsbad, CA, USA) at the Department of Immunology, Oslo University Hospital, Rikshospitalet. Patients carrying at least one DRB1*15 allele were considered HLA-DRB1*15 positive.

2.4. Body mass index (BMI)

Height and weight were registered for all patients at inclusion, and BMI was calculated as weight/height^2. The patients were categorized based on the WHO classification of obesity into three groups; Normal weight patients, (BMI < 25 kg/m^2); Overweight patients (BMI 25–30 kg/m^2); Obese patients (BMI > 30 kg/m^2). For some analyses, overweight and obese patients were merged into one group.

2.5. MRI

MRI was performed at baseline, monthly for 9 months and after 12 and 24 months according to a standardized protocol comprising T2-weighted and T1-weighted gadolinium enhancing (T1Gd +) scan using a standard head coil with a 1,5 Tesla MRI unit. Blinded assessments of new T1Gd + lesions and new or enlarging T2 lesions were conducted by 2 experienced neuroradiologists. The sum of T1Gd + lesions and new or enlarging T2 lesions was denoted as combined unique activity (CUA).

2.6. Composite score of no evidence of disease activity (NEDA)

NEDA was defined as a composite that consisted of absence of any relapses, no evidence of sustained disability progression and no MRI activity (new T1Gd + or new/enlarging T2-lesions) on MRI examinations for the given period (Rotstein et al., 2015). A relapse was defined as the appearance of new symptoms or signs that lasted more than 24 h without concurrent fever or illness. Progression was defined as an EDSS score increase of 1 or more recorded at a clinical visit that was sustained at the subsequent clinical visit 6 months later.

2.7. Statistics

MRI activity was dichotomized to 0 (no activity) and 1 (activity) before the analyses. As the distribution of the inflammation markers was skewed, all markers were LN-transformed for the statistical analyses. Data were described as means and standard deviations (SD) or frequencies and percentages, as appropriate.

The association between BMI and MRI activity before and during IFNβ-treatment was assessed by logistic regression model for hierarchical data. The model contained random effects for patients and fixed effects for BMI-categories, dummy identifying before-during IFNβ period and the interaction between the two. The results were presented as odds ratios (ORs) with corresponding 95% confidence intervals (CI) within each BMI category (normal weight group as reference) before and during IFNβ-treatment as well as ORs for decrease in MRI activity from before to during IFNβ-treatment period. The ORs were also adjusted for age and HLA-DRB1*15 status.

The association between continuous BMI and inflammation markers was estimated by a linear mixed model with random effects for patients and fixed effects for BMI. The results were reported as regression coefficients with the corresponding 95% CI. The change in inflammation markers from before to during IFNβ-treatment was compared between two BMI categories by estimating a linear mixed model with random

effects for patients and fixed effects for BMI categories, dummy identifying before-during IFNβ period and the interaction between the two.

The association between seasonally adjusted vitamin D status and continuous BMI was assessed by estimating a linear mixed model with random effects for patients and fixed effects for vitamin D.

Explorative analyses of MRI activity within different categories of BMI status, (<25 kg/m2 and ≥25 kg/m2) as well as NEDA-status were assessed by z-test for proportions and independent samples t-test. For multiple comparisons, one-way analysis of variance (ANOVA), followed by Fishers' least significant difference method was used as post hoc analysis where applicable. All analyses were performed in SPSS 22 and SAS 9.3. The results with p-values below 0.05 were considered statistically significant. All tests were two-sided.

3. Results

3.1. BMI and disease activity before IFNβ-treatment

The mean BMI in our cohort was 25.7 kg/m^2 (±SD 0.47). There were 46 normal weight patients, 23 overweight patients and 17 obese patients. The mean EDSS scores at inclusion were not correlated with BMI (1.94 ± 0.90 for normal weight patients, 1.83 ± 0.78 for overweight patients and 1.82 ± 0.71 for obese patients, p = 0.57). During the 6 treatment-free months, 10 patients progressed ≥1 EDSS score, comprising 6 normal weight patients and 4 overweight or obese patients. There were a total of 14 clinical relapses in 11 patients, which included 4 normal weight patients and 7 overweight or obese patients. There was no significant association between BMI and MRI activity (CUA) during the six months prior to IFNβ-treatment (Table 2). Adjusting for gender and HLA-DRB1*15 status did not influence our results (data not shown). To increase the power to detect significant differences, we pooled the groups of overweight and obese patients (n = 40), and found a similar pattern with no association between BMI and MRI disease activity (Suppl. Table 1). The mean proportion of positive MRI scans in the period before treatment was 47% for the normal weight patients, 57% for the overweight patients and 43% for the obese patients (Suppl. Figure 1). At baseline 22 (55%) of the overweight and obese patients had MRI activity compared to 22 (48%) of the normal weight patients (p = 0.72). Most patients had active disease in the treatment-free period, for the number of patients without MRI activity, clinical relapses or EDSS progression during the 6 months there were 6 (15%) overweight or obese patients and 12 (26%) normal weight patients (p = 0.43).

3.2. BMI and disease activity during IFNβ-treatment

A total of 28 relapses were recorded in 19 patients during IFNβ-treatment, which included 9 normal weight patients and 10 overweight and obese patients. Twenty-five patients progressed ≥1 EDSS points during the whole study period. All patients had a significant reduction in MRI disease activity (CUA) after initiation of IFNβ-treatment (p ≤ 0.001) (Suppl. Table 2). There was no significant difference between the groups and this was consistent after adjusting for gender and HLA-DRB1*15 status, and after merging the groups of overweight and obese patients (Suppl.Table 1). There were, however, significant differences in the number of patients without MRI activity, clinical relapses or EDSS progression during treatment, in relation to BMI. Thus, for patients with normal weight, 24 patients (52%) had no sign of MRI activity (CUA) during the entire treatment period compared to only 8 patients (20%) with overweight or obesity (p = 0.001) (Fig. 1A). The proportion of patients with no MRI activity and no clinical relapses combined was 41% in the normal weight group compared to 18% in those with overweight or obesity (p = 0.01) (Fig. 1B). Including

Table 2
Odds ratio for MRI disease activity (New T1Gd + lesions or new or enlarging T2 lesions) in different BMI groups before and during IFNβ-treatment.

BMI	Before treatment (M 1-6)		During IFNβ-treatment (M 7-24)	
	OR (95% CI)	p-Value	OR (95% CI)	p-Value
<25 kg/m^2 (n = 46)	1		1	
25–30 kg/m^2 (n = 23)	1.49 (0.63–3.51)	0.36	2.44 (0.96–6.15)	0.06
>30 kg/m^2 (n = 17)	0.78 (0.30–2.04)	0.62	1.18 (0.40–3.47)	0.77

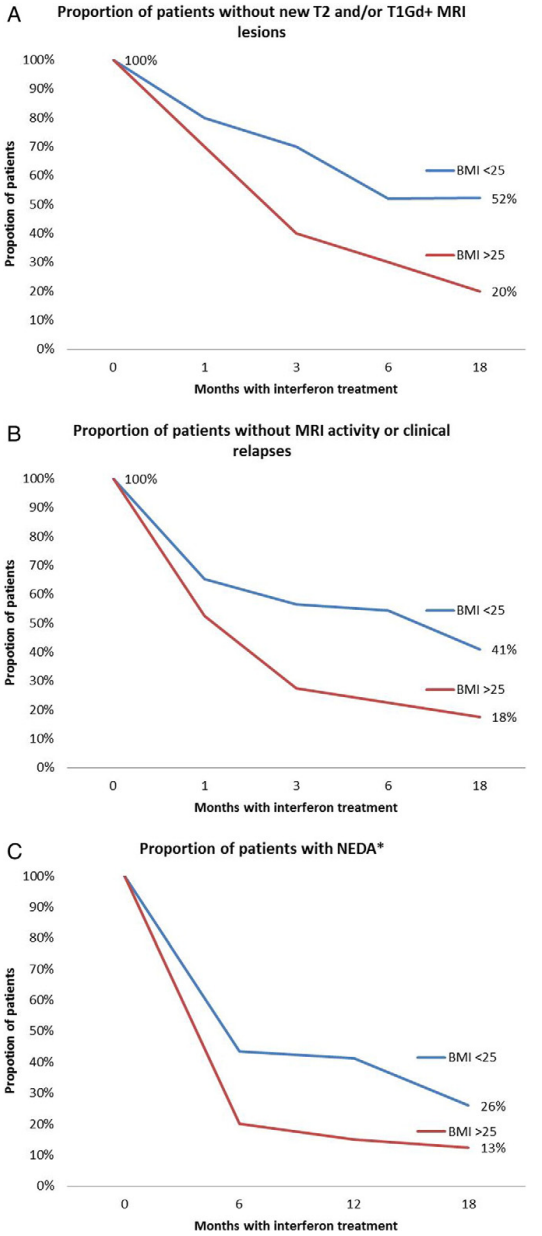

Fig. 1. A. Proportion of patients without new T2 and/or T1Gd + MRI lesions. B. Proportion of patients without MRI activity or clinical relapses. C. Proportion of patients with NEDA*.

also EDSS progression, showed that 26% in the normal weight group obtained NEDA-status, compared to only 13% in the group of overweight and obese patients ($p = 0.05$) (Fig. 1C).

3.3. BMI and the level of inflammation markers and vitamin D

We have previously reported an association between MRI disease activity and different inflammation markers in this cohort (Holmoy et al., 2013). Based on these data, we explored the correlation between inflammation markers and BMI. There was a significant inverse correlation between BMI and serum level of PTX3($r = -0.4$, $p = 0.003$) and a positive correlation with IL-1Ra ($r = 0.08$, $p = 0.005$), but not with any of the other inflammatory markers. (Table 3). We further investigated how IFNβ–treatment affected the inflammation markers and found an overall equal response for normal weight, overweight and obese patients, including a parallel increase in the serum levels of IL-1Ra and PTX3 during treatment (Table 4). There was no correlation between BMI and seasonally adjusted vitamin D levels in serum, ($r = 0.04$; $p = 0.9$).

4. Discussion

In this study, we found that overweight and obese patients had higher disease activity, as evaluated by NEDA-status, during IFNβ-treatment compared to normal-weight patients. There was no association between BMI and MRI disease activity before IFNβ -treatment and we did not find any difference in disability (EDSS) progression between the groups although this has been reported in another study (Oliveira et al., 2014). The mean BMI in our cohort was 25.7 kg/m^2. This is lower than the mean BMI in the Norwegian population, but in coherence with the findings of BMI for MS patients in Norway (Nortvedt et al., 2005).

Obesity is associated with a low-grade inflammatory state and release of cytokines that influence immune responses (Cao, 2014). It has been shown to worsen the disease course in several autoimmune diseases (Versini et al., 2014) and studies have reported a positive association between BMI and disability among MS patients (Oliveira et al., 2014). Adiposity induces a pro-inflammatory macrophage profile in the fatty tissue (Lumeng et al., 2007) and a study of experimental autoimmune encephalomyelitis (EAE) in mice showed more relapses in mice with this macrophage profile (Mikita et al., 2011).

There was a significant reduction in MRI disease activity in all three BMI-groups during IFNβ-treatment (Suppl. Table 2). However, there was a significant difference between normal weight and overweight- and obese patients regarding the number of patients obtaining NEDA-status. Our results indicate that overweight and obesity may have an impact on IFNβ-treatment response. It is uncertain whether this may be due to a suboptimal treatment response among overweight and obese patients or a generally more active disease in these groups. However, the IFNβ-treatment had a similar response on the levels of inflammation markers for all patients. This may indicate that the difference in treatment response was not due to a lesser effect on the inflammation. Different BMI between patients is not accounted for in routine IFNβ-treatment regimens since all patients receive the same dose. Most studies on IFNβ-treatment have not addressed the issue of BMI (Clanet et al., 2002; Kalincik et al., 2013; Kappos et al., 2004) and others have found conflicting results regarding the effect of different doses of IFNβ, however not adjusting for BMI (Li and Paty, 1999; O'Connor et al., 2009). Only one study has explored the effect of BMI regarding IFNβ-treatment, but this was a study on treatment of secondary progressive MS (SPMS) reporting no effect of IFNβ-treatment overall (Panitch et al., 2004). We did not find an association between BMI and MRI activity for the 6 months without treatment, possibly due to low numbers of patients in each group and large variation in MRI disease activity.

Obesity may influence systemic inflammation, and in the present study we found an inverse correlation between serum PTX3 levels, an acute-phase protein produced at the site of inflammation, and BMI. Our results are in accordance with a recent study reporting an inverse association between serum PTX3 levels and BMI in the general population (Witasp et al., 2014). However, increased PTX3 levels are observed in several autoimmune disorders (Shimada et al., 2014). A previous study found that plasma levels of PTX3 in MS patients were significantly increased during MS relapses (Wang et al., 2013), suggesting that a link

Table 3
Correlation between inflammation markers and BMI during the study period.

Inflammation marker	Regression coefficient (95% CI)	p-Value
PTX3	−0.4 (−0.06; −0.01)	**0.003**
sTNFR1	0.006 (−0.007; 0.019)	0.35
CXCL16	0.008 (−0.01; 0.03)	0.38
MMP-9	0.01 (−0.03; 0.05)	0.49
CCL21	0.008 (−0.01; 0.03)	0.46
IL-1RA	0.08 (0.03; 0.14)	**0.005**
OPN	−0.004 (−0.03; 0.02)	0.79
OPG	0.0006 (−0.01; 0.01)	0.92
sFRP3	0.004 (−0.03; 0.04)	0.84
TGFβ1	0.01 (−0.003; 0.03)	0.11
ALCAM	0.005 (−0.01; 0.02)	0.59

PTX3, pentraxin 3; sTNFR1, soluble tumor necrosis factor type 1; CXCL16, chemokine (C-X-C motif) ligand 16; MMP-9, matrix metalloproteinase 9; CCL21, chemokine (C-C motif) ligand 21; IL-1Ra, interleukin-1 receptor antagonist; OPN, osteopontin; OPG, osteoprotegrin; sFRP3, secreted frizzled-related protein 3; TGFβ1, transforming growth factor β1; ALCAM, activated leukocyte cell adhesion molecule. Bold figures indicate p ≤ 0,005.

Table 4
Effect of IFNβ-treatment on serum inflammation markers in patients categorized into two groups, normal weight (BMI < 25 kg/m^2) and overweight or obese (BMI ≥ 25 kg/m^2).

Inflammation markers	BMI		p-Values for difference in change
	<25 kg/m^2 (n = 46)	> = 25 kg/m^2 (n = 40)	BMI < 25 kg/m^2 vs. BMI ≥ 25 kg/m^2
LN_PTX3			
Before	7.0 (6.9; 7.2)	6.7 (6.6; 6.9)	0.18
During	7.1 (6.9; 7.2)	6.8 (6.7; 7.0)	
LN_sTNFR1			
Before	6.8 (6.7; 6.9)	6.8 (6.7; 6.9)	0.22
During	6.9 (6.8; 7.0)	6.9 (6.8; 7.0)	
LN_CXCL16			
Before	7.0 (6.9; 7.1)	7.0 (6.9; 7.2)	0.05
During	7.1 (7.0; 7.3)	7.2 (7.1; 7.4)	
LN_MMP9			
Before	5.9 (5.7; 6.2)	6.2 (6.0; 6.5)	0.13
During	5.6 (5.3; 5.8)	5.7 (5.4; 5.9)	
LN_CCL21			
Before	5.6 (5.5; 5.8)	5.6 (5.5; 5.8)	0.14
During	5.8 (5.6; 5.9)	5.8 (5.6; 5.9)	
LN_IL1RA			
Before	3.5 (3.1; 3.8)	4.1 (3.7; 4.4)	0.46
During	4.1 (3.7; 4.4)	4.6 (4.2; 4.9)	
LN_OPN			
Before	1.8 (1.7; 2.0)	1.9 (1.7; 2.1)	0.46
During	2.2 (2.1; 2.4)	2.3 (2.1; 2.5)	
LN_OPG			
Before	6.9 (6.9; 7.0)	6.9 (6.8; 7.0)	0.16
During	7.1 (7.0; 7.2)	7.1 (7.0; 7.1)	
LN_sFRP3			
Before	8.3 (8.0; 8.5)	8.2 (8.0; 8.5)	0.48
During	8.3 (8.1; 8.6)	8.3 (8.0; 8.5)	
LN_TGFβ1			
Before	2.9 (2.8; 3.0)	3.0 (2.9; 3.1)	0.47
During	2.8 (2.7; 2.9)	2.9 (2.8; 3.1)	
LN_ALCAM			
Before	5.0 (4.9; 5.1)	5.1 (4.9; 5.2)	0.69
During	5.0 (4.9; 5.1)	5.1 (5.0; 5.2)	

PTX3, pentraxin 3; sTNFR1, soluble tumor necrosis factor type 1; CXCL16, chemokine C-X-C motif ligand 16; MMP-9, matrix metalloproteinase 9; CCL21, chemokine C-C motif ligand 21; IL-1Ra, interleukin-1 receptor antagonist; OPN, osteopontin; OPG, osteoprotegrin; sFRP3, secreted frizzled-related protein 3; TGFβ1, transforming growth factor β1; ALCAM, activated leukocyte cell adhesion molecule.

between high BMI and MS activity did not involve PTX3 related mechanisms. We also detected a correlation between serum level of IL-1Ra and BMI. IL-1Ra is a cytokine antagonist secreted by immune cells and adipocytes that inhibits the binding of IL-1 to its receptor. In MS circulating levels have been shown to correlate with disease activity and IFNβ-treatment and it has been suggested involved in the counter-regulation of inflammatory activity (Voltz et al., 1997; Nicoletti et al., 1996). The serum level of IL-1Ra is known to be higher among overweight and obese patients (Meier et al., 2002), which we also found in our cohort. Again, our findings suggest that any link between obesity and enhanced MS activity seem not to involve IL-1Ra related mechanism.

The strengths of this study were that the analyses were done in a well characterized cohort of patients with RRMS. The study had a prospective study design with repeated and paired MRI scans and measurements of serum levels before and during IFNβ-treatment. All MRI, biochemical and clinical assessments were performed with strict and standardized procedures. The main limitation of our study is that the numbers of patients in each group were small, implying limited power to detect small differences between the groups. Further we only measured BMI at inclusion, and there is a possibility that patients may have changed BMI status during the study period of two years. Moreover, a number of parameters were investigated in this cohort, and the possibility that some of the significant findings are by chance should be taken into account. Several reports have found a correlation between circulating levels of vitamin D and obesity (Konradsen et al., 2008; Wortsman et al., 2000). There was however no evidence for this in our cohort. This may be explained by different study populations. Most of the studies reporting an association are based on subjects with morbid obesity and BMI > 35 kg/m^2. The number of patients with BMI > 30 kg/m^2 was also small and there might be an association that we did not find because of these low numbers.

The low number of morbidly obese patients may also explain why we did not find any correlation between most of the inflammation markers and BMI. Obesity and metabolic syndrome are known to effect serum inflammation markers (Cao, 2014; Park et al., 2005). Our cohort included only one patient with BMI > 35 kg/m^2 and we do not know if any patients had metabolic syndrome. Further, the choice of inflammation markers for analyzes was made in a previous study on this cohort emphasizing markers that are likely to reflect different steps in the pathogenesis of MS, and may not necessarily reflect systemic inflammation associated with it. Increased levels of inflammation markers like tumor necrosis factor α (TG1α), interleukin-6 (IL-6) and adipokines have been reported in obese patients (Cao, 2014), but were not examined in our study. There is a possibility that we would have found a correlation with BMI and a difference in serum level during treatment had other more obesity-related markers been tested.

5. Conclusion

Obesity has a major effect on several diseases and effect the risk of MS. This study indicates that patients with overweight and obesity has a lesser chance of obtaining complete remission and NEDA-status during IFNβ-treatment. Whether this is due to a suboptimal treatment response or a generally more active disease remains to be determined. Our findings suggest that BMI should be taken into account when evaluating the effect of IFNβ-treatment in MS patients and should also encourage weight reducing measures as part of the therapy in overweight and obese patients. It also emphasizes a need for further studies on this issue.

Conflict of interest

SSK have received unrestricted grants from Novartis and Biogen Idec.

KMM has participated on scientific advisory boards for Novartis Norway, Biogen Idec, and Genzyme; received funding for travel from Bayer, Novartis, Merck-Serono and Biogen Idec; received speaker honoraria from Bayer, Genzyme, Sanofi-Aventis, Novartis, Merck-Serono and Biogen Idec; and received unrestricted research support from Bayer, Sanofi-Aventis, Novartis, Merck-Serono, Biogen Idec, Pronova Biocare and Norwegian MS Society.

SW has received unrestricted grants and honoraria as a speaker from Alexion Pharmaceuticals and Novartis.

ØT has participated on scientific advisory boards for Biogen Idec, Genzyme and Merck-Serono and received speaker honoraria and travel grants from Genzyme, Merck-Serono, Novartis and Biogen-Idec.

TH has participated on scientific advisory boards from Biogen Idec and Genzyme; received funding for travel from Sanofi-Aventis, Novartis, Merck-Serono and Biogen Idec; received speaker honoraria from Bayer, Genzyme, Sanofi-Aventis, Novartis, Merck-Serono and Biogen Idec; and received unrestricted research support from Bayer, Sanofi-Aventis, Novartis, Merck-Serono and Biogen Idec.

Acknowledgments

This study was supported with unconditional research grant from Novartis and Biogen Idec.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jneuroim.2015.09.008.

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III

Journal of Neuroimmunology 323 (2018) 73–77

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

Serum levels of leptin and adiponectin are not associated with disease activity or treatment response in multiple sclerosis

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ARTICLE INFO

Keywords:
Multiple sclerosis
Adipokines
MRI disease activity
Interferon-beta treatment
No evidence of disease activity
Treatment response

ABSTRACT

Adipokines secreted by fatty tissue have inflammatory properties and are suggested biomarkers of MS disease activity. To assess this, 88 MS patients were followed with nine repeated measurements of leptin and adiponectin and 12 magnetic resonance imaging (MRI) scans for two years; six months without any immunomodulatory treatment followed by 18 months during interferon-beta (IFNB) treatment. Serum levels of leptin dropped and adiponectin increased upon initiation of IFNB-therapy, but were not associated with clinical or MRI disease activity or with treatment response. Our findings indicate that leptin and adiponectin are not useful as biomarkers of MS disease activity.

1. Introduction

Multiple sclerosis is a neuro-inflammatory disease caused by an interaction of genetic and environmental factors (Thompson et al., 2018; Huynh and Casaccia, 2013). Obesity increases the risk of MS and has been reported to affect disability and relapse rate (Munger et al., 2009; Munger et al., 2013; Hedstrom et al., 2012; Langer-Gould et al., 2013; Wesnes et al., 2015; Marrie et al., 2011; Oliveira et al., 2014; Cortese et al., 2018). In an earlier study, we showed that body weight influenced interferon-beta (IFNB) treatment response, as fewer of the overweight MS patients achieved NEDA-status compared to patients with normal weight (Kvistad et al., 2015). The mechanism for this remains unclear.

Obesity induces a chronic state of low-grade inflammation and is known to affect onset and progression of several autoimmune diseases (Versini et al., 2014). Adipokines are produced by adipocytes in the fatty tissue and are involved in the regulation of inflammation and immune responses (Tilg and Moschen, 2006). The role of serum adipokine levels has been explored for several autoimmune diseases with various results (Otero et al., 2006; Lee and Song, 2018; Mei et al., 2016; Li et al., 2015; Cao et al., 2016). Both clinical and experimental studies have described findings supportive of a link between metabolic status and the immune system in the pathogenesis of MS (Matarese et al., 2010). Especially two adipokines have been explored in MS, adiponectin and leptin, which also represent the most abundant adipokines produced by adipocytes (Guerrero-Garcia et al., 2016). Adiponectin is a

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https://doi.org/10.1016/j.jneuroim.2018.07.011
Received 4 June 2018; Received in revised form 29 June 2018; Accepted 23 July 2018

primarily anti-inflammatory acting adipokine, and low levels are associated with worse experimental autoimmune encephalomyelitis (EAE) (Piccio et al., 2013). Leptin has a pronounced part in the regulation of the Th1/Th2-balance, is negatively associated with the number of regulatory T-cells and has a key involvement in the secretion of several pro-inflammatory cytokines (Fantuzzi and Faggioni, 2000). Several reports have described an association between leptin and the induction and progression of EAE (Matarese et al., 2001; Sanna et al., 2003).

Serum leptin levels has also been suggested as a biomarker of treatment-response in relapsing-remitting MS (RRMS), (Batocchi et al., 2003) and a recent study found an association between serum leptin levels and MS severity and number of relapses (Lanzillo et al., 2017). The aim of this study was to examine if adiponectin and leptin were associated with MS disease activity and could be used as biomarkers of MS disease activity and IFNB-treatment response.

2. Material and methods

2.1. Study population and design

The study population comprised MS patients from an omega-3 fatty acids study in MS (OFAMS) described in previous publications (Torkildsen et al., 2012; Kvistad et al., 2014). In brief this was a prospective study of 88 RRMS patients followed for a total of 24 months with repeated serum analyses, magnetic resonance imaging (MRI) scans and clinical (relapse and Expanded Disability Status Scale; EDSS) assessments. The patients were included in the original OFAMS study, that was a randomized double-blind, placebo-controlled multicenter trial of omega-3 fatty acids including 92 Norwegian patients with RRMS according to the McDonald criteria. The patients were 18–55 years of age with an EDSS score ≤ 5, and ≥ 1 relapse or new T1-weighted gadolinium enhancing (T1Gd+) or T2-weighted (T2) lesion on MRI in the year prior to inclusion. They did not receive any immunomodulatory therapy at inclusion and the first six months, but thereafter all patients received 44 μg IFNB -1a subcutaneous injections three times weekly (Torkildsen et al., 2012).

2.2. Serum adipokine analyses

Serum samples were collected at baseline and after 1, 3, 6, 7, 9, 12, 18 and 24 months and stored at −80 °C until analysis. Serum-leptin and adiponectin were measured by radioimmunoassay kit (Merc Millipore Corporation, Hormonlaboratoriet, OUS, Norway).

The analyses were performed simultaneously for all samples from each patient. For each marker consecutive samples from each patient were analyzed in neighboring wells on the same plate. Each sample was analyzed in duplicates. The laboratory technicians were blinded for treatment as well as MRI activity.

2.3. MRI

MRI was performed at baseline, monthly for nine months and after 12 and 24 months according to a standardized protocol comprising T2-weighted and T1-weighted gadolinium enhancing (T1Gd+) scan using a standard head coil with a 1,5 Tesla MRI unit. Blinded assessments of new T1Gd + lesions and new or enlarging T2 lesions were conducted by two experienced neuro-radiologists. The sum of T1Gd + lesions and new or enlarging T2 lesions was denoted as combined unique activity (CUA).

2.4. No evidence of disease activity (NEDA-3)

NEDA-3 was defined as a composite that consisted of absence of any relapses, no evidence of sustained disability progression and no MRI activity (new T1Gd + or new/enlarging T2-lesions) on MRI examinations for the given period (Rotstein et al., 2015). A relapse was defined as the appearance of new symptoms or signs that lasted > 24 h without concurrent fever or illness. Progression was defined as an EDSS score increase of 1 or more recorded at a clinical visit that was sustained at the subsequent clinical visit 6 months later.

2.5. Missing values

A total of nine MRI scans (one during study months 1–6 and eight during study months 7–24) were missing. Six blood samples for serum leptin and adiponectin were missing for months 0–6 and 22 blood samples for months 7–24 (24 for leptin).

2.6. Statistics

Data are described as means and standard deviations (SDs).

Within- and between-patient variance was estimated by a linear mixed model (LMM) with random intercepts for patients. Mean serum levels before and during treatment were compared by including fixed effects for period into LMM. Associations between baseline levels of adipokines and body mass index (BMI), relapses, NEDA and EDSS score at baseline were assessed by linear regression model.

Generalized linear mixed models with random intercepts for patients were used to estimate the association between MRI activity and adipokines. LMM was estimated to assess the associations between time profile in adipokines, NEDA status and EDSS progression at baseline as well as relapses. To compare the periods before and during IFNB-treatment, the relevant models were re-estimated by including the interaction between the period and variable of interest. All models were also adjusted for gender, age and BMI. Intra-center correlations were negligible hence no adjustments for cluster effect on center level were included into the regression models. Results with p values $< .05$ were considered statistically significant. The analyses were performed in SAS version 9.4 (SAS Institute, Cary, NC) and SPSS version 25 (IBM Corp., Armonk, NY).

Table 1
Concentrations of adipokines in multiple sclerosis patients at different time points before and during interferon-beta treatment.

	Before interferon beta treatment				During interferon-beta treatment				
	Study months								
	0	1	3	6	7	9	12	18	24
Leptin, n	85	85	87	88	80	85	82	82	87
pmol/l (SD)	1038 (1031)	1013 (955)	1101 (984)	1052 (1016)	976 (782)	1054 (977)	979 (856)	926 (812)	1011 (950)
Adiponectin, n	85	86	87	88	80	86	83	82	87
mg/l (SD)	8.8 (4.0)	9.0 (4.2)	9.3 (4.3)	9.4 (4.3)	10.0 (4.8)	9.5 (5.0)	9.2 (4.3)	9.3 (4.3)	9.3 (4.5)

3. Results

3.1. Serum levels of leptin and adiponectin

A total of 764 serum samples were analyzed in 88 patients. Mean levels of leptin and adiponectin at baseline and during different phases of the study are shown in Table 1. The serum leptin levels were significantly higher (mean difference 55.7 pmol/l, 95% CI 14.5; 96.9, $p = .008$) while the serum adiponectin levels were significantly lower (mean difference 0.24 mg/l, 95% CI 0.01–0.46, $p = .037$) before IFNB-treatment as compared to during treatment.

The minimum serum-leptin level was 50 pmol/l and the maximum was 6250 pmol/l. The minimum serum-adiponectin level was 0.8 mg/l and the maximum was 31.8 mg/l. There was a great inter-individual variation in the serum levels of adipokines, but the level for each patient was relatively stable for the nine measurements during the 24 months period. The maximum difference in serum-leptin was 3510 pmol/l within- and 6200 pmol/l between-patients. The maximum difference in serum-adiponectin was 15.8 mg/l within- and 31.0 mg/l between-patients. Close to 90% of the total variation in serum levels could be attributed to between-patient variations. There was a positive association between BMI and serum-leptin measured at baseline ($p < .001$), but no association between BMI and serum-adiponectin ($p = .137$).

3.2. Serum leptin and adiponectin levels and MRI disease activity

There was no association between the serum levels of adipokines and MRI activity, OR for CUA = 0.99 (0.99–1.00) for leptin and 1.02 (0.96–1.08) for adiponectin; $p > .50$ for both). The results were consistent for the six months before and 18 months during IFNB-treatment (Fig. 1). Adjusting for gender, age and BMI did not influence the results. Neither new T1Gd + -lesions or new and enlarging T2 lesions were associated with any of the adipokines (data not shown).

3.3. Serum leptin and adiponectin levels and clinical relapses

A total of 11 of the 88 patients (6%) had clinical relapses during the study period. There was no differences between patients with and without relapses in the serum-leptin ($p = .70$) or serum-adiponectin levels ($p = .20$) at baseline, nor did the patients differ in serum adipokine levels throughout the study-period. This was also consistent after adjusting for gender, age and BMI.

3.4. Serum levels of leptin and adiponectin and EDSS score

The mean EDSS score was 1.9 (0.84) at baseline. Mean serum-leptin and serum-adiponectin at baseline were 1037.0 (1030.6) pmol/l and 8.8 (4.0) mg/l, respectively. There was no association between serum-leptin and EDSS score at baseline ($p = .89$) or between serum-adiponectin level and EDSS score at baseline ($p = .38$).

Twenty-five patients progressed ≥1 EDSS points during the whole study period. There was no association between EDSS progression and baseline serum-leptin ($p = .95$) or adiponectin level ($p = .38$). There was no difference in serum levels between patients with EDSS progression and those without progression throughout the study, and adjusting for age and gender did not change the results.

3.5. Serum levels of leptin and adiponectin and NEDA-3 status

Only 16 of the 88 patients (18%) obtained NEDA-3-status during IFNB -treatment. There was no difference between patients that obtained and those that did not obtain NEDA-3-status in baseline serum-leptin or adiponectin ($p = .21$ and $p = .33$, respectively). The groups did not differ in serum-levels throughout the study-period. This was also consistent after adjusting for gender, age and BMI.

4. Discussion

In this study we explored the potential role of two adipokines, leptin

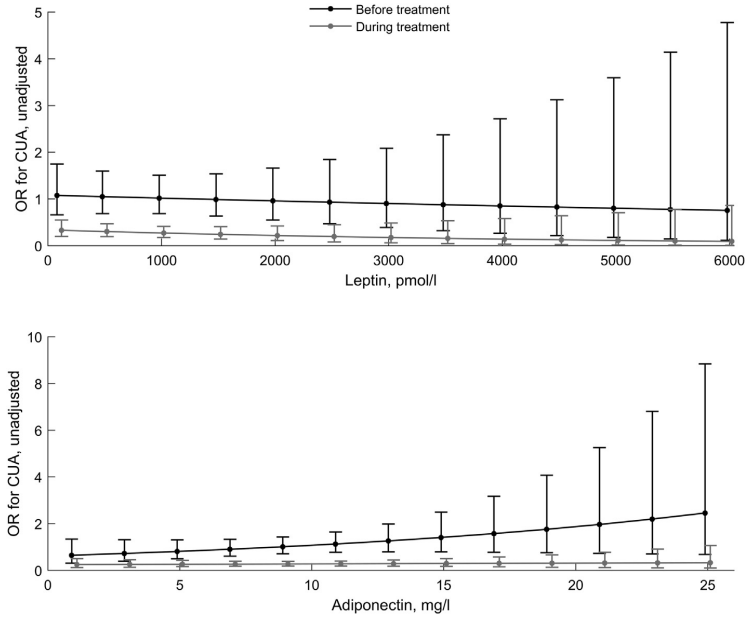

Fig. 1. Odds ratio for MRI disease activity (New T1Gd + lesions or new or enlarging T2 lesions) depending on serum level of leptin and adiponectin before and during IFNB –treatment. There was no significant difference in OR for CUA depending on the serum level of leptin or adiponectin.

and adiponectin, as biomarkers of disease activity and IFNB-treatment response in MS. We found no association between the serum levels of leptin or adiponectin and MRI- and clinical disease activity or NEDA-3-status, indicating that neither of the adipokines is useful as biomarkers of MS disease activity or treatment-response. We did however find a difference in serum levels of the adipokines before and during IFNB-treatment, reflecting the anti-inflammatory effect of the drug.

Studies have shown that MS patients have higher serum levels of leptin and lower serum levels of adiponectin than the normal population (Kraszula et al., 2012; Matarese et al., 2005; Musabak et al., 2011; Frisullo et al., 2007). This has been of great interest since these adipokines are regulators of the immune system. Leptin acts as a pro-inflammatory hormone and induces Th1-cytokin production, and adiponectin has anti-inflammatory properties as it suppresses synthesis of several pro-inflammatory cytokines (tumor necrosis factor-α, interleukin-6, interferon-γ) (Tilg and Moschen, 2006). Both adipokines are important for introduction and disease progression in EAE, (Piccio et al., 2013; Matarese et al., 2008) and there are reports supporting a link between metabolic status and MS pathogenesis (Matarese et al., 2010). Hence, the role of serum-leptin and adiponectin in MS has been given much attention, but the results related to MS disease activity are so far conflicting. A report on 59 MS patients followed for 12 months found that serum levels of leptin was increased before clinical relapses and decreased during IFNB-treatment (Batocchi et al., 2003), opposed to others that found higher levels during remission (Chatzantoni et al., 2004; Frisullo et al., 2007). A recent study on 45 MS patients followed for 12 months reported that higher serum leptin levels at baseline was associated with greater risk of clinical relapses and MRI-activity the upcoming year, and it was proposed as a tool for prediction of disease course during IFNB-treatment (Lanzillo et al., 2017). All these studies included a limited number of patients and had a short follow-up time. A prospective study of 80 patients followed for two years did however not find any association between serum-leptin or adiponectin levels and disease activity (Natarajan et al., 2015). This is in accordance with our findings.

In our study, the levels of leptin were lower and the levels of adiponectin higher during IFNB- treatment as compared to the treatment-naïve period, indicating a more favorable inflammatory profile during treatment. This was expected considering the anti-inflammatory effect of the drug. It is also in accordance with a previous report on inflammation markers in this cohort, showing favorable inflammatory changes during treatment (Holmoy et al., 2013). Changes in serum levels were, however, not associated with disease activity during IFNB-treatment and the adipokines were concluded as not useful biomarkers for treatment response.

We have previously shown that fewer overweight and obese patients achieved NEDA-3 status during IFNB-treatment compared to patients with normal weight. We found that 26% of normal weight patients obtained NEDA-status during IFNB-treatment compared to only 13% of patients with BMI > 25 (Kvistad et al., 2015). The cause of this is unknown, but overweight is associated with a chronic low-grade inflammation, and variable levels of adipokines have been suggested to contribute to a pro-inflammatory status associated with fewer regulatory T-cells and cytokine-release (Guerrero-Garcia et al., 2016; Cao, 2014). We aimed therefore to explore whether the differences in treatment response was associated to different serum levels of adipokines. We found no association between serum levels of leptin or adiponectin and treatment response.

In our cohort there was no association between serum levels of leptin and adiponectin and baseline EDSS score, and the serum levels did not differ between patients regarding their disease course assessed by clinical relapses or achievement of NEDA-status. One study of 110 MS patients described an association between obesity in MS and EDSS score in the setting of insulin-resistance (Oliveira et al., 2014) and one study of 45 MS patients described a non-significant correlation between serum-leptin and EDSS score (Lanzillo et al., 2017). However, most studies are coherent with our findings and report no correlation between EDSS score and serum leptin or adiponectin (Chatzantoni et al., 2004; Batocchi et al., 2003; Rotondi et al., 2013; Natarajan et al., 2015; Evangelopoulos et al., 2014).

The OFAMS study consisted of a cohort that was very well characterized over a two year-period with nine serum measurements and 12 MRI examinations for each patient. All MRI, biochemical and clinical assessments were performed with strict and standardized procedures. The cohort has been explored in great extent with many published reports where factors proven to be associated with disease activity, like vitamin-D level and Epstein-Barr virus antigen levels have been confirmed (Loken-Amsrud et al., 2012; Kvistad et al., 2014). There are however some limitations. The number of overweight patients was rather small, and only 18% of these obtained NEDA-status. Hence there is a possibility that the number of patients were too low to find an association between serum-leptin levels and treatment-response after adjusting for BMI. However the vast majority of other reports on MS and adipokines have included fewer patients. Another weakness with our study was that only one patient had BMI > 35 kg/m^2, and the group of overweight patients were not characterized regarding insulin-resistance and metabolic syndrome. It is likely that the grade of inflammation and level of adipokines would have been different in a group of morbidly obese patients with comorbidities, and we cannot be sure that our results can be applied under such circumstances. However, in Europe RRMS patients with BMI > 35 are uncommon. The patients in this study were randomized to omega 3 fatty acids or corn oil. There have been reports on a relation between the serum levels of leptin and adiponectin and omega-3 fatty acids, where omega 3 fatty intake can decrease circulating levels of leptin in non-obese subjects and increase the levels in obese patients (Gray et al., 2013). This is a possible confounder in our study. However, considering that we wanted to explore if higher leptin levels could possibly explain why less obese patients obtained NEDA-status in our previous study compared to normal weight patients, the possible changes induced by the omega-3 fatty acid would be likely to influence our results so that we would have found a greater difference. Since we did not find any effects of serum leptin levels, we assume that this is not a major confounder in our study. Several factors may affect serum leptin-levels included fasting, emotional stress, physical exercise and diurnal variation with higher levels at early morning and during night. The serum-leptin levels vary during the day and are higher at night and early morning. This may have affected our results, but most probably only to a minor degree. The blood samples were collected mainly at equal time points during daytime. The intra-individual variance was also low in this material.

5. Conclusion

Our findings indicate that serum-leptin and adiponectin do not have a profound role in the immune mechanisms of RRMS-disease activity, and are therefore not suitable as potential biomarkers of disease activity or IFNB-treatment response.

Conflict of interest

SSK have received unrestricted grants from Novartis and Biogen Idec.

KMM has served on advisory board, received speakers honoraria, travel funding and/or unrestricted research grants from Novartis, Biogen, Genzyme, Merck, Almirall, Roche, Teva and/or the Norwegian MS Society.

TH has received speaker honoraria, and/or served on advisory board, and/or received unrestricted research grants from Biogen, Roche, Merck, Novartis, and Genzyme.

JSB has no conflict of interest.

SW has received honoraria as speaker from Novartis, Sanofi-Aventis and Biogen.

AGB has received speaker honoraria from Merck Serono, Teva, Biogen Idec and Norwegian MS Society and travel funding from Teva.

KSB has no conflict of interest.

HH has no conflict of interest.

RM has served on scientific advisory boards for Novartis Norway and Merck Norway and has received travel funding and/or speaker honoraria from Biogen, Novartis Norway, and Sanofi Genzyme.

JVS has no conflict of interest.

ØT has participated on scientific advisory boards for Biogen Idec, Genzyme and Merck-Serono and received speaker honoraria and travel grants from Genzyme, Merck-Serono, Novartis and Biogen-Idec.

Acknowledgements

This study was supported with unconditional research grant from Novartis and Biogen Idec.

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Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

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ISBN: 9788230847374 (print)
9788230852705 (PDF)