Environmental risk factors for multiple sclerosis

Results from animal and human studies on diet, vitamin D and Epstein-Barr virus

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Dissertation for the degree philosophiae doctor (PhD) at the University of Bergen, Norway
Scientific environment

The present work was carried out at the Multiple Sclerosis National Competence centre, Department of Neurology, Haukeland University Hospital and at the Institute of Clinical Medicine, Section for Neurology, University of Bergen.
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My wife Nina has been a great inspiration and has always shown a deep interest in my research. She has read through numerous drafts for this thesis and this work would not have been possible without her patience, support and unconditional love. Nina, Thomas and Anna continuously remind me of what really matters in life.

“Without deeper reflection one knows from daily life that one exists for other people - first of all for those upon whose smiles and well-being our own happiness is wholly dependent, and then for the many, unknown to us, to whose destinies we are bound by the ties of sympathy.”

Albert Einstein, “The world as I see it” (1931).
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>25-(OH)D₃</td>
<td>25-hydroxyvitamin D</td>
</tr>
<tr>
<td>1,25-(OH)₂D₃</td>
<td>1,25-dihydroxyvitamin D</td>
</tr>
<tr>
<td>AB</td>
<td>Applied biosystems</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-brain barrier</td>
</tr>
<tr>
<td>BSA</td>
<td>Body surface area</td>
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<tr>
<td>cDNA</td>
<td>Complementary deoxy ribonucleic acid</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CYP27A1</td>
<td>Cytochrome P450 27A1</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>Cytochrome P450 27B1</td>
</tr>
<tr>
<td>DIG</td>
<td>Digoxigenin</td>
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<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
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<tr>
<td>EA</td>
<td>Early antigen</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<tr>
<td>EBERs</td>
<td>Epstein-Barr virus encoded RNA</td>
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<td>EBNA</td>
<td>Epstein Barr virus nuclear antigen</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>EDSS</td>
<td>Expanded disability status scale</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated plus maze test</td>
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<tr>
<td>FcR</td>
<td>Fc-receptor</td>
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<tr>
<td>g</td>
<td>Gram</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
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<td>GM</td>
<td>Grey matter</td>
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<td>GML</td>
<td>Grey matter lesion</td>
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<tr>
<td>IFNβ</td>
<td>Interferon-β</td>
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<tr>
<td>IFNγ</td>
<td>Interferon-γ</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IU</td>
<td>International unit</td>
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</table>
LFB   Luxol fast blue
LMP   Latent membrane protein
MAG   myelin-associated glycoprotein
mRNA  Messenger ribonucleic acid
mg    Milligram
MP    Methylprednisolone
MRI   Magnetic resonance imaging
MS    Multiple sclerosis
MTR   Magnetisation transfer imaging
NAGM  Normal appearing grey matter
NAWM  Normal appearing white matter
NA    Not applicable
ND    Not determined
ng    Nanogram
NK cells  Natural killer cells
OCB   Oligoclonal bands
PCA   Principal component analysis
PCR   Polymerase chain reaction
PLP   Proteolipid-protein
PPMS  Primary progressive multiple sclerosis
PRMS  Progressive relapsing multiple sclerosis
PTLD  Post transplant lymphoproliferative disease
PUFA  Polyunsaturated fatty acid
qPCR  Quantitative real time polymerase chain reaction
RNA   Ribonucleic acid
RRMS  Relapsing remitting multiple sclerosis
RT-PCR Reverse transcriptase polymerase chain reaction
SAM   Significance analysis of microarrays
SPMS  Secondary progressive multiple sclerosis
TBARS Thiobarbituric acid reactive substances
TMEV  Theiler’s murine encephalomyelitis virus
<table>
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<tr>
<th>Acronym</th>
<th>Description</th>
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<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>VCA</td>
<td>Viral capsid antigen</td>
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<tr>
<td>VDDR1</td>
<td>Vitamin D dependent rickets type 1</td>
</tr>
<tr>
<td>VEP</td>
<td>Visually evoked potential</td>
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<tr>
<td>WM</td>
<td>White matter</td>
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Abstract

Multiple Sclerosis (MS) is an immune mediated disease of the central nervous system (CNS). The aetiology is unknown, but environmental factors as well as multiple separate genetic loci contribute to the disease susceptibility. Much research in the last decades has focused on the genetic basis of MS, but increasing evidence is emerging for major impact of environmental risk factors. Research on environmental risk factors is important since they offer a potential for disease prevention. The present study was designed to evaluate and add new knowledge to the role of environmental risk factors associated with the disease. The specific objectives were: A) Explore whether diet intervention with n-3 polyunsaturated fatty acids (PUFAs) could influence MRI disease activity, microglia activation, de- and remyelination in the cuprizone model for demyelination (Paper I). B) Assess if diet intervention with n-3 PUFAs from two different sources could prevent behavioural changes in the cuprizone model for demyelination (Paper II). C) Describe and characterise a case series of patients with co-occurrence of vitamin D dependent rickets (VDDR1) and MS (Paper III), D) Study if relapses in MS were associated with reactivation of latent Epstein-Barr virus (EBV) (Paper IV). E) Examine changes in gene expression in cortical lesions, normal appearing grey matter and meninges of MS patients. Perform an immunohistochemical characterisation of these brain sections and determine if active replication or latent infection with EBV was present in the sections examined (Paper V).

The prevalence of MS is lower in the northern than in the southern parts of Norway. Since the prevalence of MS is known to normally increase with the distance from equator, this is an anomalous distribution of the disease compared to most other parts of the world. One widely accepted hypothesis for this distribution is that the inhabitants in the northern parts of Norway have a higher consumption of fatty fish than in the south, and that some substances in the fish (vitamin D or n-3 fatty acids) offer protection against MS. Two of the papers in this thesis investigate the role of nutrition and diet modification for the susceptibility to demyelinating diseases. We used an animal model for MS (the cuprizone model) in order to study how diet
modification with fatty fish or fish oils could influence the disease course, MRI activity, de- and remyelination in this animal model. Sixty-three mice were fed either a diet enriched with 1) salmon filets, rich in n-3 fatty acids, 2) cod liver oil rich in n-3 fatty acids or 3) soybean oil rich in n-6 fatty acids. Cuprizone was added to the different diets. In the first study (Paper I), we found that the mice fed a salmon-enriched diet had less MRI disease activity and demyelination than the two other groups. In the second study behavioural changes to cuprizone treatment were assessed with the elevated-plus-maze test (EPM). There were striking differences in weight loss, anxiety behaviour and activity levels, with a more favourable clinical outcome for the mice given a diet enriched with salmon filets (Paper II). The findings from these two studies indicated that diets rich in fatty fish could have protective effects against demyelination. There were no effects of giving n-3 supplements in the form of cod liver oil, indicating that other substances than the fatty acids could have accounted for the effect of a salmon based diet. Future studies should aim at isolating the active component in the fish diet, as this could offer a valuable treatment supplement for demyelinating diseases.

Three of the papers deal with the most studied and recognised environmental triggers of MS, Vitamin D and Epstein-Barr virus (EBV). The third study (Paper III) identified three patients with a rare hereditary form of rickets (VDDR1). All of these patients later developed MS. These findings indicated rickets as a possible risk factor for MS and supported the theory that early childhood or the intrauterine periods are the main susceptibility periods for low levels of vitamin D.

The next study (Paper IV) was designed to analyse whether there was an association between exacerbations in MS and reactivation of latent EBV. Sixty-one patients were followed for one year and blood samples were taken in the case of exacerbations or if magnetic resonance imaging (MRI) indicated evidence of new enhancing lesions. All the 61 patients (100%) were anti-viral capsid antigen (VCA) IgG positive, one (2%) was anti-VCA IgM positive and 60 (98%) were anti-Epstein Barr virus nuclear antigen (EBNA) positive. Mean anti-early antigen (EA)-D IgG at baseline was 0.57
(range 0.12-2.70) and at the time of exacerbations 0.61 (range 0.11-2.70). As demonstrated in earlier studies, all the patients in this sample had evidence of previous EBV infections, indicating that EBV could have a role in the pathogenesis of MS. There were, however, no signs of EBV-reactivation at the time of relapse. Thus, this study concluded that EBV reactivations do not play a significant role in MS exacerbations.

The last study (Paper V) was designed to examine changes in gene expression in cortical lesions and in normal appearing grey matter from patients with MS. We observed a strong activation of immunoglobulin (Ig)-related genes in cortical sections of MS patients. The Ig-genes with the strongest activation were mostly variable and constant regions of the kappa and lambda light chains, but also included genes encoding heavy chains for IgM and IgG. No upregulation of Fc-receptor (FcR) genes was found. The cortical sections were immunohistochemically stained for plasma cells, Igs, T- and B-lymphocytes. The stainings revealed Ig-deposition in the meninges of the MS-patients compared to the controls. There were few B-lymphocytes and no B-lymphocyte follicles. Plasma cells were present in the meninges of all the MS patients but not in any of the controls. The activation of Ig-genes observed in the present study was highly interesting, as the synthesis of oligoclonal IgGs have been hypothesised to be caused by activation of EBV infected B-lymphocytes. The samples were screened for the presence of EBV by quantitative real time polymerase chain reaction (qPCR) and immunohistochemistry, but no evidence of active or latent EBV infection was detected. This study demonstrated that genes involved in the synthesis of Igs are upregulated in MS patients, and that this upregulation seems to be caused by a small number of plasma cells located in the meninges. Further, it concluded that EBV infected cells were not necessary for this Ig-upregulation. The findings indicated that the oligoclonal band (OCB) producing B-lymphocytes found in the cerebrospinal fluid (CSF) of MS-patients could have meningeal origin.
**List of publications**


1. Introduction

Multiple sclerosis (MS) is an immune mediated disease of the central nervous system (CNS). It is one of the most common causes of non-traumatic neurological disability among young adults in the western world. The cause of the disease is unknown, but it is widely accepted that environmental factors act in concert with a genetic susceptibility (Dyment et al. 2004; Ebers, 2008).

Epidemiology

MS is a disease affecting about 2.5 million people world-wide (Compston and Coles, 2002) with a large variation in the geographical distribution. Northern and central Europe, USA, Canada, Australia and New Zealand are considered high risk areas, whereas most parts of Africa, Asia, Mexico and northern parts of southern America are considered low risk areas (Kurtzke, 1975; Compston and Confavreux, 2006). The disease affects women more often than men (Pugliatti et al., 2005; Grytten et al. 2006) and the risk of MS seems to be increasing for women (Orton et al., 2006). This increase is not caused by genetic factors and indicates gender specific changes in environmental risk factors.

Norway is considered a high-risk area for the disease and the prevalence seems to be increasing (Grytten et al., 2006). Increasing prevalence rates of MS have been reported in all the Norwegian counties that have been subject to repeated surveys. The prevalence of MS in Norway today is about 150/100000 (Grytten et al., 2006), indicating that about 7000 people are affected, with approximately 300 new cases per year (Torkildsen et al., 2007). The first studies on the distribution of MS in Norway found an east-west and a south-north gradient (Swank, 1950; Westlund, 1970). Two recent updates on the prevalence of MS (Grytten et al., 2006; Smestad et al. 2006) found that the prevalence of MS now is the same in the western and eastern part of Norway. The prevalence of MS seems, however, still to be lower in the northern parts of the country (Gronlie et al., 2000; Alstadhaug et al., 2005). Data from the most
recent epidemiological studies from Norway is given in Figure 1. Since the population in Norway is genetically homogenous, the differences in MS-prevalence seem to reflect that the exposition to environmental risk factors differs between the counties. The prevalence of MS is generally known to increase with the distance from equator, and the distribution in Norway is often refereed to as an anomalous distribution (Ebers, 2008). It has been postulated that the coastal areas of Norway have a lower prevalence of MS due to higher fish consumption than the inland areas (Swank, 1950). Other studies from Norway have also indicated an inverse association between fish consumption and MS-susceptibility (Kampman et al., 2007). It has been demonstrated that the Sami population has a genetic profile that is less susceptible to MS than people with Caucasian heritage (Torkildsen et al., 2005; Harbo et al., 2007). Thus, the lower frequency of MS in the northernmost parts of Norway could also partly have been caused by a high number of people with Sami ancestry in the northernmost counties.
Figure 1. MS distribution in Norway. The east-west gradient for MS postulated by Swank (1950) seems to have disappeared, but there is still a lower prevalence of MS in the two northernmost counties than in the rest of the country. The map is based on prevalence studies from the following counties of Norway: Hordaland county (Grytten et al., 2006), Oslo County (Smestad et al., 2006), Nord-Trøndelag County (Dahl et al., 2004), Nordland County (Alstadhaug et al., 2005) and Troms- and Finnmark Counties (Gronlie et al., 2000).
Symptoms and clinical subtypes

The disease manifests itself with only one symptom (monosymptomatic) in 80% of the patients and with multiple symptoms (polysymptomatic) in 20% (Myhr et al., 2001). Optical neuritis is the initial symptom in about 15-20%. Lesions can occur in the cerebrum, brainstem or cerebellum and the clinical symptoms reflect the part of CNS involved. Lesions in the pyramidal tract, including the spinal cord, typically present as weakness in upper or lower extremities (McDonald and Compston, 2006) while brainstem and cerebellar lesions typically produce symptoms of diplopia, vertigo and ataxia. In addition, sensory symptoms, pain, fatigue and cognitive problems are common (McIntosh-Michaelis et al., 1991; Rao et al., 1991; Beiske et al., 2004). The course and prognosis of the disease is unpredictable. Based on the initial disease course, MS has traditionally been classified into two main clinical patterns (Figure 2):

1. Relapsing-remitting MS (RRMS) is the most common form of the disease, estimated to affect about 80-85% of the patients. It is characterised by unpredictable relapses with subsequent remissions. Deficits may either resolve completely or partially. With time, the recovery from each relapse is often incomplete. When disability progression occurs between relapses, the disease enters a secondary progressive (SPMS) phase. Secondary progression is found in 40-50% of patients with RRMS after 15 years and in 80% after 25 years with the disease (Noseworthy et al., 2006).

2. Primary-progressive MS (PPMS) affects about 15-20% of the patients. It is characterised by a continuous increase in neurological deficits without clear relapses or periods of remission. A subgroup of this type is Progressive-relapsing (PRMS), characterised by increases in neurological deficits as well as superimposed relapses. This is the least common form of MS.
Figure 2. Clinical subtypes of MS. The figures show the two main clinical onsets of MS, RRMS and PPMS, and the subgroups, SPMS and PRMS, in each category. (Adapted from Compston and Confavreux, 2006).
Clinical exacerbations

Clinical exacerbations, or relapses, are defined as significant worsening of pre-existing symptoms or appearance of new neurological deficits in the absence of fever and lasting for more than 24 hours (Poser et al., 1983; McDonald et al., 2001; Polman et al., 2005). Stressful life events seem to correlate with exacerbations (Buljevac et al., 2003) and there is also a significant association between systemic infections and the risk of relapses (Correale et al., 2006). Persistent parasitic infections may, on the other hand, protect against disease activity (Correale and Farez, 2007). Pregnancy has been shown to decrease the relapse rate, while it increases in the puerperium (Vukusic et al., 2004). Despite these findings, the disease course is rather unpredictable and it has not been possible to predict relapse-risk in individual patients.
Pathology and pathological subtypes

MS is usually viewed as an inflammatory demyelinating disease (Lassmann et al., 2007). The histopathological characterisation is typically focal areas of myelin loss and partial axonal loss. The hallmark of the disease is formation of sclerotic plaques, which represent the end stage of a process with inflammation, de- and remyelination, astrocytosis, oligodendrocyte death and neuronal and axonal degeneration (Compston and Coles, 2008). The plaques can be located everywhere in the brain or spinal cord, but are most frequently seen periventricular, in the optical nerve, cervical spine and in the subpial cerebral cortex.

The immunological mechanisms leading to the disease are not completely understood, but many immunopathological processes are present. There is usually a marked inflammation in the early lesions, dominated by T-lymphocytes and activated macrophages and microglia, accompanied by a disrupted blood-brain barrier (BBB) (Kirk et al., 2003). Demyelination is accompanied by acute axonal injury and axonal loss (Trapp et al., 1998). The disease progression in MS is caused by accumulated axonal degeneration, and it has been debated whether inflammation is the primary event in MS or whether the inflammation is a secondary reaction to neurodegeneration (Compston and Coles 2008). Remyelination also occurs during the different disease stages, but it is most pronounced in the early phases of the disease course. In the progressive stages of MS, both in PPMS and SPMS, the pathological picture is different. In these forms, there are few active demyelinating plaques, but the pre-existing plaques show evidence of gradual expansion (Prineas et al., 2001). As in RRMS, there is T-lymphocyte and microglia-infiltration present, but only few of the microglia contain myelin degradation products, indicating a slow rate of demyelination (Lassmann et al., 2007). Axonal loss seems to be the pathological substrate for the progressive disability seen in both these forms of MS (Tallantyre et al., 2009).

Although it was previously regarded as a solely white matter disorder, newer studies have reported extensive grey matter involvement (Bo et al., 2003a; Bo et al., 2007). The cytoarchitecture of cortical lesions is usually well preserved and in the purely
cortical lesions, there is no significant T- or B-lymphocyte infiltration compared to control areas (Bo et al., 2003b). Secondary inflammatory changes are thus less pronounced in grey matter lesions. Plasma cells, T- and B-lymphocytes are, however, present in the meninges (Kutzelnigg et al., 2005). The grey matter plaques are generally less visible on MRI.

Four distinctive pathological patterns have been suggested in early MS-lesions (Lucchinetti et al., 2000):

Pattern I: Active demyelinated plaques associated with T-lymphocyte and macrophage dominated inflammation without antibody or complement deposition, but with relative preservation of oligodendrocytes. Loss of all myelin proteins appears to occur simultaneously.

Pattern II: Active demyelination associated with T-lymphocyte and macrophage dominated inflammation. The pattern resembles pattern I, but is distinguished from other lesional patterns by pronounced immunoglobulin (Ig) reactivity. IgG and activated complement (C9neo) is found at sites of active myelin destruction.

Pattern III: Active demyelination with preferential loss of myelin-associated glycoprotein (MAG) compared to proteolipid protein (PLP). The lesions contain infiltrates with T-lymphocytes, macrophages and activated microglia. There is evidence of oligodendrogliopathy and microglial activation as well as partial remyelination and oligodendrocyte apoptosis. The pattern is suggestive of a primary oligodendrocyte dystrophy.

Pattern IV: Sharply demarcated plaques of demyelination. The oligodendrocytes do not show the morphological features of apoptosis, but oligodendrocyte death is revealed by DNA fragmentation. There is no complement
activation or preferential MAG loss and the inflammatory infiltrates are dominated by T-lymphocytes and macrophages.

In the study that first identified these patterns, heterogeneity of lesion pattern was observed between patients, but not in the same individual, thus indicating MS as a heterogeneous disease. The pattern I and II resembled what is seen in T-lymphocyte-or T-lymphocyte and antibody-mediated autoimmune encephalomyelitis (EAE). Pattern III and IV were more consistent with a toxin-induced demyelination and seemed to be more in accordance with the cuprizone model (Torkildsen et al., 2008a). The heterogeneity of MS lesions seems to disappear over time. In a recent study consisting of 93 tissue blocks from 39 MS patients, the authors did not find any signs of heterogeneity (Breij et al., 2008). Thus, the pattern with pathology-heterogeneity is probably an early event in the disease course.
Diagnosis

The principle of diagnosing MS is to establish dissemination in time and space of lesions (Polman et al., 2005). This diagnosis can be based upon clinical evaluation alone or with the assistance of paraclinical features. According to the Poser criteria (Poser et al., 1983), clinical definite MS is defined as two or more relapses affecting two or more separate sites of the CNS and clinical evidence of two or more lesions. The last decade, these criteria have been replaced by the McDonald (McDonald et al., 2001) and revised McDonald criteria (Table 1) (Polman et al., 2005). The principle is still to establish dissemination in time and space, but magnetic resonance imaging (MRI) and CSF-examination can substitute for one of the clinical episodes. The inclusion of the new criteria offers the potential for shorter time delay from the first symptoms to the diagnosis. This is of importance, as early therapy might be important for the long time prognosis of the disease (Compston and Coles, 2008).
Table 1. The 2005 Revisions to the McDonald Diagnostic Criteria for Multiple Sclerosis. (Polman et al., 2005).

<table>
<thead>
<tr>
<th>Clinical Presentation</th>
<th>Additional Data Needed for MS Diagnosis</th>
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<tr>
<td>Two or more relapses; objective clinical evidence of two or more lesions</td>
<td>None, but paraclinical testing (MRI, CSF) should be done to exclude other diagnoses.</td>
</tr>
<tr>
<td>Two or more relapses; objective clinical evidence of one lesion</td>
<td>Dissemination in space, demonstrated by: MRI or Two or more MRI-detected lesions consistent with MS plus positive CSF or Await further clinical relapse implicating a different site.</td>
</tr>
<tr>
<td>One relapse; objective clinical evidence of two or more lesions</td>
<td>Dissemination in time, demonstrated by MRI or Second clinical relapse.</td>
</tr>
<tr>
<td>One relapse; objective clinical evidence of one lesion</td>
<td>Dissemination in space, demonstrated by MRI or Two or more MRI-detected lesions consistent with MS plus positive CSF and Dissemination in time, demonstrated by MRI or Second clinical relapse.</td>
</tr>
<tr>
<td>(monosymptomatic presentation; clinically isolated syndrome)</td>
<td></td>
</tr>
<tr>
<td>Insidious neurological progression, suggestive of MS</td>
<td>One year of disease progression (retrospectively or prospectively determined) and Two of the following: a) Positive brain MRI (nine T2 lesions or four or more T2 lesions with positive VEP), b) Positive spinal cord MRI (two focal T2 lesions), c) Positive CSF</td>
</tr>
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</table>
Treatment

There exists no curative treatment for MS. A number of symptomatic and immunomodulatory treatments are however, available. Clinical exacerbations can be treated with high doses of methylprednisolone (MP), and the medication has been shown to improve symptoms and shorten time of disability after an acute exacerbation of the disease (Miller et al., 2000). Recent evidence also suggests that MP given in pulses every 4 weeks as add on to immunomodulatory treatment could lead to significant reduction in relapse-rate (Sorensen et al., 2009). Immunomodulatory treatment has been available as disease modifying treatment for RRMS since the mid 1990’s. The treatment options are interferon-beta (IFNB) (Betaferon® (IFNB-1b), Avonex® (IFNB-1a), Rebif® (IFNB-1a)), and glatiramer acetate (Copaxone®). These medications have been shown to reduce the relapse-rate and MRI disease activity in MS (Paty et al., 1993; Johnson et al., 1995; Jacobs et al., 1996; PRISMS study group, 1998). The main goals of the treatment are to inhibit disease activity, limit disability and delay disease progression (Miller et al., 2006). Natalizumab (Polman et al., 2006) and thereafter mitoxantrone (Hartung et al., 2002) are treatment options when relapse activity is still present, despite other immunomodulatory treatment. Mitoxantrone is also the only drug with indications for SPMS. There are a number of clinical trials underway, including many phase II and III trials, with promising new therapies for RRMS. Currently there are, however, no proven treatments for PPMS, indicating an urgent need for more research in this area (Compston and Coles, 2008).
2. Risk factors for MS

MS is a complex disease where both genetic and environmental factors act in concert. It may be considered a heterogeneous disease with several clinical and pathological subtypes (Lucchinetti et al., 2000). This could also imply that there are subtypes of the disease, which differ in risk factors. Possible pathological and clinical subgroups are difficult to establish in epidemiological studies and have largely been ignored (Ascherio and Munger, 2008). When discussing risk factors for MS in this context, the disease is therefore referred to as a single entity. It is, however, possible that future studies will reveal subgroups of the disease with unique genetic and environmental risk factors.

2.1 Genetic factors

Although it is not considered a genetic disease, a genetic susceptibility is important for the development of MS. Familial aggregation has been accepted for a long time, and the strongest risk factor for this disease is a positive family history (Ascherio and Munger, 2008). The risk of MS is about 30 times higher among first-degree relatives than in the general population (Compston and Coles, 2002). It has been shown that both first, second and third degree relatives of patients with MS have an increased risk of the disease and the recurrence varies with relatedness (Dyment et al., 2004). If one person in a family has MS, the first-degree relatives have 1-3% risk of developing the disease (compared to 0.1% in the general population). For dizygotic twins, the risk is 4% and for monozygotic, the risk is about 30% (Ebers, 2008). Adoption studies have shown that first-degree non-biological relatives of MS patients had the same risk of MS as the general population (Ebers et al., 1995), thus emphasising the importance of genetic factors. Similarly, in studies of half siblings, the intrafamilial environment had no effect (Ebers et al., 2004) and the risk of MS in stepchildren brought into a family with MS was also demonstrated to be the same as in the general population (Dyment et al., 2006). Thus, the aggregations of MS within families seem to be largely explained
by shared genes and not environmental changes in the microenvironment (Dyment et al., 2004). Environmental risk factors must therefore operate at a larger population level in genetically susceptible individuals. The association between genetic sharing and risk of MS is given in Figure 3.

**Figure 3. Prevalence rates of MS in the general population and in family members of MS patients.** The error bars indicate the estimated 95% confidence intervals. (Adapted from Compston and Coles, 2008).
The susceptibility of MS has been linked to the humane leukocyte antigen (HLA) (Jersild et al. 1972). The markers have been refined as DR15 and DQ6 and the genotypes DRB1*1501, DRB*0101, DQA1*0102 and DQB2*0602 (Dyment et al., 2004; Compston and Coles, 2008). This association is strongest in northern Europeans (Compston et al., 2008). The HLA-C5 (Yeo et al., 2007) and HLA-DRB1*11 (Ramagopalan et al., 2007; Dean et al., 2008) loci seem, on the other hand, to offer a protective effect against MS-susceptibility. Recently, alleles within the interleukin-2-receptor alpha gene (IL2RA) and the interleukin-7 receptor alpha gene (IL7RA) were also shown to be heritable risk factors for the disease (Gregory et al., 2007; Hafler et al., 2007; Lundmark et al., 2007). Development of MS is not likely to be attributed to a single gene, but to an interaction between many genes, where each gene has only a small effect. The fact that the rate for identical twins is less than 100% indicates that environmental factors must act in concert with the genetic susceptibility. Thus, genetic factors can not fully explain the geographic distribution or risk of developing MS in a population. Evidence from epidemiological and adoption studies indicate that the environmental factors act at a broad population level, rather than within the shared familial environment (Orton et al., 2008).

2.2 Environmental factors

The risk of MS differs between populations. In Norway, the Sami has had a much lower prevalence of the disease than the Norwegian population (Torkildsen et al., 2005). It has also been demonstrated that Middle-East immigrants in Oslo have a higher prevalence of MS than other non-western immigrants (Smestad et al., 2006). This could indicate a genetic influence on the susceptibility for the disease in some populations, but it could also reflect that different populations are exposed to different environmental risk factors, or that the gene-environment interaction is important.

The geographical distribution of the disease and change in risk among emigrants has given support to a role of environmental risk factors for the disease susceptibility. It has been demonstrated that migrants who move from an area where the disease is
common to an area where it is rarer have a decreased risk of disease, whereas people who move in the opposite direction retain their low risk of the disease. Their children are, however, at higher risk of MS (Gale and Martyn, 1995). These migrant studies indicate that environmental factors play a role in the susceptibility to MS and that they are particularly important during childhood.

The prevalence of MS seems to be increasing in genetically homogenous populations (Grytten et al., 2006) and the female to male ratio is also growing (Orton et al., 2006). Similarly, there have also been reports of MS in populations who have earlier been regarded as genetically resistant to the disease, like the Sami population in Norway (Gronlie et al., 2000; Harbo et al., 2007). These changes in prevalence and sex ratio are difficult to explain by changes in genetic risk factors, as genetic changes occur over much larger time periods. Thus, this is regarded as implications for changes in the environmental risk factors. Since the prevalence is increasing in females, this could be attributed to changes in lifestyle particularly affecting women (Orton et al., 2006; Compston and Coles, 2008).

Several candidate environmental triggers for MS, such as meat consumption, climate (Lauer, 1995), radon exposure (Bolviken et al., 2003), smoking (Riise et al., 2003), vaccination (Hernan et al., 2004) and psychological stress (Li et al., 2004), have been suggested. Many infectious agents have also been associated with the disease, and increased antibody titers have been reported for EBV (Bray et al., 1983; Sumaya et al., 1985) measles virus (Panelius et al., 1971, Shirodaria et al., 1987), rubella virus (Shirodaria et al., 1987) and chlamydia pneumoniae (Sriram et al., 1999). Tetanus vaccination (Hernan et al., 2006) and increased levels of uric acid (Drulovic et al., 2001; Rentzos et al., 2006) have on the other hand been proposed to reduce the disease risk. Most of the suggested environmental risk factors have not been replicated in follow-up studies or there is not enough evidence to give support or reject the hypotheses. Four environmental risk factors have, however, consistently been associated with the disease: Dietary factors, vitamin D, smoking and EBV-infection. The evidence for each of these factors is discussed briefly.
2.2.1 Diet

Early epidemiological studies from Norway indicated that the prevalence of MS was lower in coastal villages with higher fish consumption than in the inland areas (Swank, 1950; Westlund, 1970). These results have later been replicated in other countries (Esparza et al., 1995). Similarly, most population-based epidemiological studies have found an association between the incidence of MS and consumption of saturated fatty acids (Swank, 1950; Agranoff and Goldberg, 1974; Alter et al., 1974; Knox, 1977; Esparza et al., 1995). This led to the hypothesis that a diet rich in fish could have a protective role against MS. The results from case-control studies have, however, been conflicting. While one large cohort study found no effects of a high intake of PUFAs (Zhang et al., 2000), a recent case-control study from Norway (Kampman et al., 2007) indicated an inverse association between fish consumption and the risk of MS. It has been speculated if vitamin D or n-3 fatty acids in the fish could account for the protective effect.

The results from intervention studies have been inconsistent. Some studies have indicated that supplementation with n-3 fatty acids could prevent relapses and slow disease progression (Ghadirian et al., 1998; Nordvik et al., 2000; Weinstock-Guttman et al., 2005) while others have not been able to replicate these findings (Zhang et al., 2000). Studies on n-6 fatty acids have also found conflicting results (Bates et al., 1978; Paty et al., 1978). In general high dropout levels and the use of different types and doses of PUFAs have prevented these studies from giving conclusive results (Farinotti et al., 2007). Another problem is that the follow-up time has been short and that sensitive measures, like MRI, have not been applied to the studies. This could have made it difficult to detect differences between the treatment groups. Other studies have relied on a mixture of n-3 and n-6 PUFAs (Swank and Dugan, 1990). A main problem in these studies has been that different types and sources of PUFAs have been grouped together. Another problem is that the patients who persisted with diet modification could have had a milder disease course than the others (Esparza et al., 1995; Mehta et
There is need for more studies on this area, as 50-75 % of MS patients make use of special diets, especially diets with PUFAs (Farinotti et al., 2007).

### 2.2.2 Sunlight and vitamin D

Ecological studies have shown a strong correlation between low sunlight exposure and the risk of developing MS, and low sunlight exposure seems to be the strongest environmental factor associated with MS (Lauer, 1997). Several studies have found that outdoor activity is inversely correlated to the risk of developing the disease (Kampman et al., 2007). In another study skin cancer was found to be about 50% lower among patients with MS than expected (Goldacre et al., 2004). Skin cancer is directly linked to sun exposure, thus providing an indirect evidence for a possibly protective effect of sun exposure for the susceptibility to MS. There has also been one study on childhood sun exposure and risk of MS for monozygotic twins (Islam et al., 2007). This study demonstrated that childhood sun exposure seemed to have a protective effect against MS in monozygotic twins, where the genetic susceptibility was the same. Sunlight is the major source of vitamin D, and this led to the hypothesis that the protective effect of sun exposure was caused by vitamin D synthesis and that vitamin D deficiencies could increase the risk of the disease (Ascherio and Munger, 2007b).

Studies on the animal model experimental autoimmune encephalitis (EAE) have demonstrated that supplementation of vitamin D can prevent both clinical and pathological evidence of disease activity (Lemire and Archer, 1991; Cantorna et al., 1996), thus giving support to a protective role of vitamin D in MS. In a longitudinal cohort of 200 000 women (the Nurses health study I and II cohorts, http://www.channing.harvard.edu/nhs/), it was demonstrated that the relative risk of MS was 0.59 for women with a vitamin D intake >400 IU/day compared to women with a lower vitamin D intake (Munger et al., 2004). Similarly, in a nested case-control study of 257 individuals with MS, it was demonstrated that the risk of MS declined with increasing serum levels of 25-(OH)D$_3$ (Munger et al., 2006). The risk of MS was 62% lower among individuals within the highest quintile compared to the
lowest. In another study, high serum levels of 25-(OH)D$_3$ were also associated with a lower incidence of MS, but in this study the association was only found among women (Kragt et al., 2008).

The active form of vitamin D, mediates many of its effect on the immune system by binding to the vitamin D receptor (VDR). A number of studies have looked at associations between polymorphisms in the VDR and the susceptibility to MS. Although some studies have indicated an association (Fukazawa et al., 1999; Niino et al., 2000; Tajouri et al., 2005), others have not been able to replicate these findings (Steckley et al., 2000; Yeo et al., 2004; Partridge et al., 2004; Smolders et al., 2009). Some polymorphisms in the VDR seem, however, to be associated with reduced disease disability (Mamutse et al., 2008). A significant genetic influence determines the serum 25-(OH)D$_3$ concentrations and it has also been demonstrated that polymorphisms in the CYP27B1 influences the 25-(OH)D$_3$ status (Orton et al., 2008).

In our study, we have described three patients with co-occurrence of mutations in the CYP27B1 and MS (Torkildsen et al., 2008c). Whether there is an association between these mutations or between polymorphisms in this enzyme and MS remains to be determined.

There have been few intervention studies with vitamin D substitution and all of them have been uncontrolled, comparing the baseline or previous situation after intervention (Goldberg et al., 1986; Achiron et al., 2003; Wingerchuk et al., 2005; Kimball et al., 2007; Burton et al., 2008). Since MS is a disease with an unpredictable disease course, it is not possible to draw affirmative conclusions from any of these studies, indicating an urgent need for well-designed clinical trials of high dose vitamin D substitution for MS.

Vitamin D supplementation could be an effective way to reduce the prevalence of MS in a population. Human breast milk has a low content of vitamin D and infants that are not given supplementation with this vitamin are likely to develop low serum concentrations. There is an increased frequency of MS among people born in May, and
this could be caused by maternal deficiencies in vitamin D during the winter months (Willer et al., 2005; Ebers, 2008). The official recommendations for vitamin D intake are about 400 IU/day. These recommendations are, however, probably too low to give an optimal serum concentration of this vitamin (Bischoff-Ferrari et al., 2006). It has been estimated that an intake of 1000-4000 IU would be required to increase the serum levels of vitamin D to levels associated with MS protection (Vieth, 1999; Ascherio and Munger, 2008). As there has not been documented any adverse effects of vitamin D substitution, increasing the vitamin D intake in the population could offer a good strategy for primary disease prevention.

### 2.2.3 Smoking

Some epidemiological studies have indicated that smoking increases the risk of MS. A study from Norway (Riise et al., 2003) found that smokers had an almost doubled risk of MS. Childhood exposure to passive smoking has also been shown to double the risk of this disease (Mikaeloff et al., 2007). Based on such epidemiological studies, it has been estimated that up to 6% of all cases of MS could have been prevented if smoking was eliminated (Ascherio and Munger, 2008).

Smoking has also been linked to an increased risk of early conversion to clinical definite MS (Di Pauli et al., 2008) and for transition to the secondary progressive form of the disease (Hernan et al., 2005). There have been many attempts to explain the association between smoking and MS, but at present it is not known why it exists. Smoking seems also to be associated with other autoimmune- and immune mediated diseases, like rheumatoid arthritis and systemic lupus erythematosus (Costenbader and Karlson, 2006) thus indicating that smoking could have a general effect on the immune system. Future studies on the mechanisms relating smoking to MS could lead to discoveries of new forms of disease prevention (Ascherio and Munger, 2008).
2.2.4 Epstein-Barr virus (EBV)

The EBV is considered the most compelling infectious candidate for MS. EBV belongs to the herpes virus family and infection with the virus causes a lifelong carrier state. This leads to a continuous stimulation of the immune system and constant elevated antibody titers to EBV. Sero-epidemiological studies have consistently found that the EBV seropositive rate is higher in patients with MS than in controls (Bray et al., 1983; Sumaya et al., 1985; Myhr et al., 1998; Wandinger et al., 2000; Torkildsen et al., 2008b). In children with MS, this association is even stronger (Pohl et al., 2006). This high seroprevalence rate is not found for other viruses (Wandinger et al., 2000; Haahr and Hollsberg, 2006).

There are many different immunological evidences linking EBV to MS. High titers of anti-EBV nuclear antigen (EBNA) antibodies have been shown to predict a higher risk of the disease (Levin et al., 2005). It has also been demonstrated that MS patients with high EBNA titers have more Gd-enhancing lesions on MRI than those with lower titers, as a measure of increased disease activity (Farrell et al., 2009). An increased humoral immune response to EBV has been found in the CSF of patients with MS (Bray et al., 1992). In addition increased CD4+ and CD8+ T-lymphocyte responses for EBV has been detected in the CSF (Holmoy et al., 2004) and in the blood of patients with MS (Hollsberg et al., 2003; Lunemann et al., 2006; Jilek et al., 2008). Further EBNA-1 specific T-lymphocytes have been shown to cross-react with myelin antigens (Lunemann et al., 2008). This supports the theory that clonally expanded EBNA1 specific T cells could contribute to MS by cross-reaction with myelin antigens.

A recent review (Ascherio and Munger, 2007a) concluded that the risk of MS is about 20 times higher in people with a history of mononucleosis compared to EBV negative individuals. It has been suggested that the reason for this association is molecular mimicry between EBV and myelin components (Lang et al., 2002; Holmoy and Vartdal, 2004). There is, however, still no conclusive evidence that this mechanism is relevant for MS. The nature of the EBV makes it a plausible trigger of chronic inflammatory diseases. One study concluded that exacerbations in MS were associated
with evidence for serological reactivation of the virus. Three other studies have not been able to replicate these findings (Buljevac et al., 2005; Torkildsen et al., 2008b; Farrell et al., 2009), and whether this finding was just an occasional association in a small sample set remains to be determined.

A recent study, (Serafini et al., 2007) found EBV infected B-lymphocytes in the brains and meningeal tissue of the majority (21/22) of the MS patients examined. If these results were replicated, this would be compelling evidence for a role of EBV in the pathogenesis of this disease and it would have been a breakthrough in defining the pathogenesis of the disease. Unfortunately, these findings have not been replicated in five other studies (Hilton et al., 1994; Morre et al., 2001; Opsahl and Kennedy, 2007; Willis et al., 2009; Torkildsen et al., 2009c). Further, Willis and colleagues (2009) were not able to detect EBV in 12 MS specimens representing adjacent tissue blocks from the study by Serafini and colleagues (2007). This indicates that EBV seems not to be directly involved in MS pathology in the majority of MS patients. At present, the reason for these discrepant results is unknown. It appears that infiltration of EBV-positive B-lymphocytes could be involved in a subgroup of patients, but this subgroup appears not to have distinct clinical characteristics.

Many features of MS seem to be consistent with a pathogenic role of EBV and the similarities between EBV-infection and MS have been noticed for more than twenty years (Warner et al., 1981). The reduction of MS risk in emigrants from high to low risk areas, is, however, not easily explained by differences in age of EBV infection and it seems likely that other environmental factors, like vitamin D status, modify the susceptibility to EBV (Holmoy, 2008). It is also possible that some EBV-strains are more likely to increase the risk of MS than others (Munch et al., 1998), or that the association is caused by host factors which make people vulnerable both to EBV-infections and the disease (Niller et al., 2008). There exist no effective vaccine against EBV and the role of EBV in MS is only partially understood. Thus, at present it seems not possible to give a good protection against the possibly viral causes of the disease.
3. Aims of the study

The main aim of this study was to investigate whether different environmental factors could influence the risk of developing MS, both in an animal model for the disease and in a patient population. The study consisted of five sub-studies:

1. Explore if differences in diet fatty acid composition can influence MRI activity and the degree of de- and remyelination in the cuprizone model for demyelination.

2. Assess whether differences in diet fatty acid composition can influence behavioural changes in the cuprizone model for demyelination.

3. Describe and characterise patients with co-occurrence of VDDR1 and MS.

4. Study whether EBV reactivation is associated with relapses in patients with MS.

5. Examine changes in gene expression in cortical lesions, normal appearing grey matter and meninges of MS patients. Perform immunohistochemical characterisation of the brain sections examined and evaluate if EBV-infected cells are present.
4. Methodological considerations

4.1 The cuprizone model for demyelination

Although MS is exclusively affecting humans, there exist a number of animal models for the disease. The most widely used models are EAE (Rivers and Schwentker, 1933), Theiler’s virus induced encephalitis (Miller et al., 1997), and the cuprizone model (Blakemore, 1973). In EAE, immunisation with myelin antigens or passive transfer of myelin specific T-lymphocytes induces an inflammatory demyelination in the CNS of the animal. In Theiler’s virus induced encephalitis, infections of mice with the neurotropic picornavirus Theiler's murine encephalomyelitis virus (TMEV) leads to a progressive CD4 (+) T-lymphocyte-mediated demyelinating disease. The cuprizone model (Blakemore, 1974) is a model of toxic demyelination. In this model, young adult mice are fed with the copper chelator cuprizone (bis-cyclohexanone oxaldihydrazone). This leads to a consistent demyelination. Spontaneous remyelination can be observed as early as 4 days after withdrawal of the neurotoxin (Lindner et al., 2008), thus making the cuprizone model excellent for studying factors which can prevent demyelination and stimulate remyelination.

Mice strain and cuprizone administration
The cuprizone model was first established in Swiss mice (Carlton, 1967; Blakemore 1972), but most recent publications have relied on the C57BL/6 strain (Matsushima and Morell, 2001; Hoffmann et al., 2008). The use of C57BL/6 mice offers a good potential of using the model in knockout gene studies. The dosage necessary to give demyelination is strain and age dependent (Irvine and Blakemore, 2006). The standard protocol applied the recent years has been feeding 8-weeks-old C57BL/6 mice with 0.2 % cuprizone (w/w) for 6 weeks. It has been demonstrated that a higher degree of demyelination can be achieved by increasing the dosage to 0.3 % cuprizone (w/w) (Lindner et al., 2008). The major drawback, is that the mortality rate in the cuprizone
mice rises from <5% with 0.2 % cuprizone to 10-15% with 0.3 % concentration (Maren Lindner, personal communication.)

**Effects of cuprizone administration**
The administration of cuprizone causes cell death of the oligodendrocytes, which leads to a subsequent demyelination (Morell *et al.*, 1998; Mason *et al.*, 2004). It is not known exactly why administration of cuprizone only leads to a specific cell death in the oligodendrocytes, but cuprizone is a copper chelator, which in turn leads to inhibition of the copper dependent mitochondrial enzymes cytochrom oxidase and monoamine oxidase (Venturini, 1973; Matsushima and Morell, 2001). Hence, a plausible hypothesis is that disturbance in energy metabolism leads to apoptosis in the oligodendrocytes, which causes demyelination. If mice are exposed to a higher dose of cuprizone, this will lead to formation of megamitochondria in the liver, thus emphasising the role of mitochondrial dysfunction in this model. Although cuprizone is a copper chelator, the effect of cuprizone is not antidoted by administration of copper (Carlton, 1967). Another feature in this animal model for demyelination is that the mice develop hydrocephalus. Early experiments in the cuprizone model indicate that this development is caused by aqueductal stenosis (Kesterson and Carlton, 1970).

**Areas of demyelination and correlation to MS**
The demyelination was previously believed to predominantly affect the corpus callosum and superior cerebellar peduncles (Suzuki and Kikkawa, 1969; Matsushima and Morell, 2001). Newer studies, which have used immunohistochemical techniques, have however demonstrated extensive cortical demyelination in mice given cuprizone (Skripuletz *et al.*, 2008). Thus, the pathological pattern may resemble what is found in MS (Bo *et al.*, 2007). Since the cuprizone model is a model of toxic demyelination, leading to a primary loss of oligodendrocytes, the pathological pattern may resemble the type 3 or type 4 lesions in MS, as described by Luchinetti (Lucchinetti *et al.*, 2000). Cuprizone-induced demyelination is characterised by a microglia/macrophage response. However, the cuprizone model differs from MS and EAE in that the BBB remains intact (McMahon *et al.*, 2002). Both MS and EAE involve complex
immunological processes and T-lymphocyte activation is frequently seen in both conditions. T-lymphocytes are, however, almost completely absent during cuprizone-induced demyelination (Matsushima and Morell, 2001). The intact BBB could perhaps account for these differences. Recent evidence has challenged the hypothesis that MS is a result of primary autoimmune processes (Barnett and Prineas, 2004). Studies have indicated that, for at least some MS patients, apoptosis of oligodendrocytes seem to be the earliest change in newly forming lesions (Lucchinetti et al., 2000; Barnett and Prineas, 2004). Thus, the cuprizone model seems to offer a valuable supplement to the EAE model for studies of de- and remyelination.

**Behavioural responses**

Mice fed cuprizone exhibit weight loss, which is reversed when the cuprizone diet is discontinued (Franco-Pons et al., 2007; Xiao et al., 2008). The mice develop both motor and behavioural deficiencies. Morell and colleagues (1998) were the first to report behavioural deficiencies in cuprizone-treated animals. From visual observations, they reported that the cuprizone-treated animals appeared to have a lower activity level compared to the control animals. These observations have been replicated with standardised tests (Liebetanz and Merkler, 2006; Franco-Pons et al., 2007; Xiao et al., 2008), and it appears that the C57Bl/6 mice given 0.2% cuprizone first go through a period with hyperactivity and decreased anxiety behaviour (after 3 weeks of cuprizone treatment) and then develop motor dysfunction (after 5 weeks). The motor dysfunctions seem to persist even 6 weeks after withdrawal of the neurotoxin (Franco-Pons et al., 2007).

**Effects of chronic exposure**

Although the demyelination observed in the cuprizone model is usually reversible, chronic cuprizone exposure (≥12 weeks) leads to impairment of oligodendrocyte regeneration and insufficient remyelination (Armstrong et al., 2006). Mice that are chronically exposed to cuprizone (≥12 weeks) also experience tonic-clonic seizures upon stress-inducing stimuli (Kesterson and Carlton, 1972; Hoffmann et al., 2008).
These results also suggested that the seizures are a consequence of neuronal degeneration in the hippocampal formation (Hoffmann et al., 2008).

**Quantification of demyelination**

*Histology*

Both luxol fast blue (LFB) staining, immunohistochemical studies and electron microscopy (EM) have been used to evaluate the degree of de- and remyelination in the cuprizone model (Matsushima and Morell, 2001; Lindner et al., 2008). It has been demonstrated that EM data correlated well with LFB myelin staining and immunohistochemical myelin protein staining (Lindner et al., 2008). In this study, immunohistochemical staining for myelin proteins was detected before significant remyelination is observed in EM. It seems therefore that immunohistochemical studies (Figure 4) may be sensitive for the detection of remyelination in the cuprizone model.
Figure 4. Stainings with luxol fast blue (LFB) and anti-proteolipid protein (PLP). The panel presents staining for myelin with LFB and anti-PLP from the corpus callosum of female C57Bl/6 mice. Normal myelin is shown in the controls; whereas the mice fed cuprizone for 6 weeks have a marked loss of myelin fibres. After one week of cuprizone withdrawal, it is possible to see some myelin-regeneration. (Adapted from Torkildsen et al., 2009a).

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<th>Controls</th>
<th>6 weeks of cuprizone</th>
<th>1 week of cuprizone withdrawal</th>
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<td><strong>LFB</strong></td>
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<tr>
<td><strong>PLP</strong></td>
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Magnetic resonance imaging (MRI)
Animal models have proven to give valuable insight to confirm and validate the correlation between MRI findings and histopathology (Wu et al., 2008). Further, the use of MRI in the cuprizone model offers the potential of using MRI to follow the disease progression and monitor the effect of interventions (Figure 5). This could lead to a reduction in the number of animals necessary to study de- and remyelination. It has been demonstrated that MRI can be used as a reliable and valid surrogate marker of the extent of pathology in this model (Wu et al., 2008). Imaging may even be used for evaluating the dynamic process of de- and remyelination (Chen et al., 2007). However, more studies confirming the close relationship between MRI and histology are necessary before it can be used without histological verification of the results.

Figure 5. **T2 weighted MRI from C57Bl/6 controls and mice fed cuprizone for 5 weeks.** The picture shows hyperintense lesions (arrows) in the mice fed cuprizone for 5 weeks. (Adapted from Torkildsen et al., 2009a)
**Behavioural testing**

MS leads to cognitive impairment in about 50% of the patients (McIntosh-Michaelis et al., 1991; Rao et al., 1991). Only few studies have evaluated behavioural changes in the cuprizone model (Franco-Pons et al., 2007; Xiao et al., 2008). It has been demonstrated that the behavioural deficits induced by cuprizone treatment, correlate well with the extent of white matter demyelination in the animals (Franco-Pons et al., 2007). Thus, behavioural testing may serve as a valuable surrogate marker for demyelination.

**Gene expression**

The cuprizone model has been used to identify temporal changes in levels of mRNAs during remyelination and demyelination (Jurevics et al., 2002; Bedard et al., 2007). Using these techniques, genes coding for myelin structural components have been found to be greatly down-regulated during demyelination and upregulated during remyelination (Jurevics et al., 2002). The cuprizone model has also been used to identify selective genetic markers for microglia activation (Bedard et al., 2007). The use of these methods offer the potential for a better understanding of both genetic mechanisms involved in microglia activation, de- and remyelination. Ultimately, this could lead to new therapeutic strategies for MS.
4.2 Study design of the diet intervention studies (article I and II)

Mouse strain
The present studies (article I and II) made use of the C57Bl/6 strain. It was chosen because it is the most common used strain in the cuprizone model and dose-response relations are well established (Lindner et al., 2008; Torkildsen et al., 2008a). The mice are genetically homogenous, thus reducing the intra-individual variability and the number of mice required for significant results. Our study was performed with female mice, while most researchers occupied with the cuprizone model have used male mice. Sex differences have been noted for SJL-mice exposed to cuprizone, but not for the C57Bl/6 strain, indicating that sex differences should not interfere with our results (Taylor et al., 2009). Since MS is a disease that affects women more often than men, one could argue that the use of female mice is even more transferable to human studies. Another reason for choosing female mice was that male mice have a more aggressive behaviour. Female mice were therefore chosen to avoid aggressive behaviour and subsequent injuries to the mice.

Nutritional components
The three diets used in the animal experiments were composed of 53.9 % dextrin, 18.0 % protein, 9.0 % sucrose, 5.0 % α-cellulose, 5.0 % starch, 4.0 % lipids, 3.5 % mineral mix, 1.0 % vitamin mix, 0.3 % L-cystein, and 0.3 % choline. The diets differed mainly in the lipid source. The salmon based diet was made of salmon filets that were grinded and freeze dried and then grinded again to powder consistency before they were added. Thus, salmon was the main protein and lipid source for the mice in this group. The energy balance and balance between the different components were made to be as identical as possible (Table 2, 4).
Table 2. Energy balance in the different diets

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<tr>
<th>Energy balance</th>
<th>Salmon (J/g)</th>
<th>Cod liver oil (J/g)</th>
<th>Soybean oil (J/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>17832</td>
<td>17694</td>
<td>17771</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>5.6</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>21.2</td>
<td>17.7</td>
<td>18.1</td>
</tr>
</tbody>
</table>

The soybean based diet used in this study was made to contain a composition of nutrients comparable to commercially available standard rodent diets (for example the Rat and mouse No.I maintenance diet, Scanbur, Special Diets Services, Karlslunde, Denmark). The standard n-6/n-3 ratios in these diets are about 10-13 (compared to 9.5 in our diet). It could be argued that the studies should ideally include one group given standard rodent chow in addition to the soybean-oil group. By making the rodent diet ourselves we were, however, able to make sure that there were no main differences in the diets except for the lipid source.

Lipid peroxidation

Double bounds in PUFAs are unstable and easily oxidated by free radicals. It is therefore important to control that no significant peroxidation has occurred in the special diets. In our study, Thiobarbituric Acid Reactive Substances (TBARS) were measured to make sure there were no large differences in lipid peroxidation between the different diets (Table 3). The measurement of TBARS is one of the most commonly used methods for detection of peroxidation (Valenzuela, 1990). This is an in vitro indirect method of measurement. This could lead to an over- or underestimation of lipid peroxidation. Measurements of peroxide- and anisidine number in addition to TBARS could have given an even more sensitive and accurate measure (Brunborg, 2006).
Table 3. Results from analysis of TBARS, as a measure of peroxidation in the different diets.

<table>
<thead>
<tr>
<th></th>
<th>Salmon</th>
<th>Cod liver oil</th>
<th>Soybean oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS</td>
<td>5.7 ng/g</td>
<td>3.7 ng/g</td>
<td>2.1 ng/g</td>
</tr>
</tbody>
</table>

Table 4. The fatty acid content of the different diets. The results of the measurements of fatty acid contents are given in the following table. (Fatty acid content is given as g fatty acid/100 g feed, wet weight.)

<table>
<thead>
<tr>
<th></th>
<th>Salmon</th>
<th>Cod liver oil</th>
<th>Soybean oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Σ saturated</td>
<td>7.6</td>
<td>8.1</td>
<td>7.9</td>
</tr>
<tr>
<td>Σ monoenes</td>
<td>24.2</td>
<td>20.3</td>
<td>15.0</td>
</tr>
<tr>
<td>16:1n-7</td>
<td>1.5</td>
<td>3.0</td>
<td>0.2</td>
</tr>
<tr>
<td>18:1n-9</td>
<td>15.3</td>
<td>6.4</td>
<td>14.0</td>
</tr>
<tr>
<td>20:1n-9</td>
<td>2.5</td>
<td>3.9</td>
<td>0.1</td>
</tr>
<tr>
<td>Σ n-6</td>
<td>5.9</td>
<td>1.4</td>
<td>22.9</td>
</tr>
<tr>
<td>18:2n-6</td>
<td>5.2</td>
<td>1.1</td>
<td>22.9</td>
</tr>
<tr>
<td>20:4n-6</td>
<td>0.2</td>
<td>0.3</td>
<td>N.D.</td>
</tr>
<tr>
<td>Σ n-3</td>
<td>9.8</td>
<td>13.3</td>
<td>2.4</td>
</tr>
<tr>
<td>18:3n-3</td>
<td>2.1</td>
<td>0.4</td>
<td>2.4</td>
</tr>
<tr>
<td>20:5n-3</td>
<td>1.9</td>
<td>4.7</td>
<td>N.D.</td>
</tr>
<tr>
<td>22:5n-3</td>
<td>1.0</td>
<td>0.8</td>
<td>N.D.</td>
</tr>
<tr>
<td>22:6n-3</td>
<td>3.5</td>
<td>5.6</td>
<td>N.D.</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>0.6</td>
<td>0.1</td>
<td>9.5</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>1.5 mg/100 g</td>
<td>1.6 mg/100 g</td>
<td>1.2 mg/100 g</td>
</tr>
<tr>
<td>Vitamin D₃</td>
<td>4.3 μg/100 g</td>
<td>13.7 μg/100 g</td>
<td>2.6 μg/100 g</td>
</tr>
</tbody>
</table>
Administration
In the cuprizone model for demyelination, the different diets are available ad libitum and cuprizone is added to the diets. It is therefore possible that cuprizone could have interacted with substances in the salmon-based diet or that a gastric interaction neutralising the neurotoxin could have occurred. This is a problem with all dietary intervention studies performed in this animal model. It is not possible to measure the amount of cuprizone in serum. It has, however, not been possible to antidote the effect of cuprizone in earlier studies in the cuprizone model, where the mice were given high doses of copper (Carlton, 1967; Kesterson and Carlton, 1972). These experiments were designed to give copper, which would interact with cuprizone in the stomach of the animals. The fact that these studies reported no effects of their interventions indicates that a gastric interaction is less likely.

Study design
The mice were grouped into 6 groups (three control groups given salmon, cod liver oil or soybean oil without cuprizone and three groups given cuprizone). MRI and behavioural testing was performed after 5 weeks of cuprizone administration (indicated with grey boxes). After 6 weeks of administration, half of the mice were sacrificed and after one week of remyelination, the remaining mice were sacrificed (Figure 6).

Figure 6. Study design of the cuprizone experiments. The grey boxes indicate the week when the MRI and behavioural testing were performed. The asterisks indicate the time points when the mice were sacrificed.

<table>
<thead>
<tr>
<th>Control groups</th>
<th>0 % Cuprizone</th>
<th>0 % Cuprizone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (weeks)</td>
<td>1 2 3 4 5 6 7</td>
<td>8 9 10 11</td>
</tr>
<tr>
<td>Group 1, Salmon (N=9)</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td>Group 2, Cod liver oil (N=9)</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Group 3, Soybean oil (N=9)</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>0 % Cuprizone</th>
<th>0,2% Cuprizone</th>
<th>0 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (weeks)</td>
<td>1 2 3 4 5 6 7</td>
<td>8 9 10 11</td>
<td></td>
</tr>
<tr>
<td>Group 1, Salmon (N=12)</td>
<td>**</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Group 2, Cod liver oil (N=12)</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>Group 3, Soybean oil (N=12)</td>
<td>*</td>
<td>*</td>
<td></td>
</tr>
</tbody>
</table>
As there was no intraindividual variability in the control groups for weight gain, behaviour, MRI activity, demyelination, inflammation or astrocytosis, future studies can probably reduce the size of the control groups. The sizes of the intervention groups were large enough to detect significant changes in de- and remyelination and we lost only one mouse during the experimental procedure. It would, depending on outcome measure and expected effect sizes, be advisable to increase the size of the cuprizone-intervention groups in future studies in order to increase the likelihood of reaching significant differences.

**Behavioural testing and MRI**
The elevated plus maze (EPM) test was administered for behavioural testing and T2 weighed MRI sequences were used as MRI measures of disease activity.

**Histochemistry and immunohistochemistry**
For quantification of de- and remyelination, the tissue sections were stained with LFB. In addition immunohistochemistry was used for detection of demyelination (anti-PLP), BBB-damage (anti-fibrinogen), astrocytosis (anti-glial fibrillary acidic protein; GFAP) and microglia activation (anti-MAC-3) (Table 5).

**Table 5. Primary antibodies used in the diet intervention study.**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Detection</th>
<th>Species/isotype</th>
<th>Dilution</th>
<th>Primary antibody</th>
<th>Source</th>
<th>Pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP</td>
<td>Myelin</td>
<td>Mouse IgG2a</td>
<td>1:1000</td>
<td>24 h, 4°C</td>
<td>Serotec</td>
<td>Citrate buffer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Serotec Citrate</td>
<td></td>
<td></td>
<td></td>
<td>15 min.</td>
</tr>
<tr>
<td>GFAP</td>
<td>Astrocytes</td>
<td>Mouse IgG1</td>
<td>1:2000</td>
<td>½ h, 22°C</td>
<td>Dako</td>
<td>EDTA buffer</td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>BBB-damage</td>
<td>Rabbit polyclonal</td>
<td>1:20000</td>
<td>½ h, 22°C</td>
<td>Dako</td>
<td>EDTA buffer</td>
</tr>
<tr>
<td>MAC-3</td>
<td>Microglia/macrophage</td>
<td>Rat IgG1,K</td>
<td>1:200</td>
<td>24 h, 4°C</td>
<td>BD Biosciences</td>
<td>Citrate buffer</td>
</tr>
</tbody>
</table>

*Abbreviations: GFAP=Glial fibrillary acidic protein, PLP=Proteolipid protein*
**Dose translation to human studies**

It has been common to extrapolate the dosage used in animal studies to humans by simply multiplying with body weight. A more correct dose translation is probably the use of normalisation of body surface area (BSA). This method has been demonstrated to correlate across several species with oxygen utilisation, basal metabolism, blood volume, renal function, plasma proteins and caloric expenditure (Reagan-Shaw *et al.*, 2008). It has also been applied to antineoplastic drugs and has been shown to correlate with maximum tolerated doses in rodents and humans (Freireich *et al.*, 1966).

The formula for dose translation based on BSA is given with the following formula (Reagan-Shaw *et al.*, 2008):

\[
\frac{\text{Human estimated dose (mg/kg)}}{\text{Animal dose (mg/kg)}} = \frac{\text{Animal Km}}{\text{Human Km}}
\]

where Km is equal to body weight (kg) divided by BSA (m\(^2\)). For humans the weight is approximately 60 kg with a BSA of 1.6 m\(^2\), giving a Km factor of 37 and for mice the weight is 0.02 kg with a BSA of 0.007 m\(^2\), giving a Km factor of 3.

The major drawback with this formula is that BSA is estimated with a formula incorporating measures of body weight and height, the Du Bois height-weight formula (Du Bois and Du Bois, 1915), from which the constants were derived from only 9 patients:

\[
\text{BSA (m}^2\text{)} = \text{body weight (kg)}^{0.425} \times \text{height (cm)}^{0.725} \times 0.007184
\]

It is likely that more reliable conversion-methods will be developed in the future, but currently the BSA-calculations are far superior to body-weight extrapolations (Reagan-Shaw *et al.*, 2008).
4.3 Vitamin D

Vitamin D is a fat-soluble vitamin. It can be considered a hormone rather than a vitamin, as it can be synthesised in the body, is released in the circulation and has distinct target organs (Gillham et al., 2001). It is included in the vitamin group, as it becomes an essential dietary factor when endogenous synthesis is inadequate to meet the physiological requirements. It is usually synthesised in the skin from 7-dehydrocholesterol upon exposure to ultraviolet-B radiation. Ultraviolet light causes scission of the B-ring of 7-dehydrocholesterol to form previtamin D$_3$. This is followed by a spontaneous rearrangement to cholecalciferol, or vitamin D$_3$. The vitamin can also be obtained from the diet and dietary sources become increasingly important when there is little ultraviolet radiation. The vitamin D$_3$ is biological inactive and must be metabolised to its biological active form. The active form of vitamin D$_3$, 1,25-dihydroxycholecalciferol (1,25-(OH)$_2$D$_3$), is produced by a 2-step hydroxylation; first at the 25 position in the liver by the mitochondrial cytochrome P450 27 A1 (CYP27A1) isozyme. 25-(OH)D$_3$ is then hydroxylated at the 1$\alpha$ position in the kidney by the cytochrome P450 27B1 (CYP27B1) isozyme. This leads to the formation of 1,25-(OH)$_2$D$_3$. The 1,25-(OH)$_2$D$_3$ form of the vitamin is the most active form and it is 500 times more potent than 25-(OH)D$_3$. Vitamin D in the form of 1,25-(OH)$_2$D$_3$ is a strong immunomodulator. It works on the vitamin D receptor (VDR), expressed on T-lymphocytes, macrophages and dendritic cells (Lin and White, 2004). The serum level of 1,25-(OH)$_2$D$_3$ is normally well regulated and is within the normal range even in people who are vitamin D deficient (Ascherio and Munger, 2008).

**Mechanisms of vitamin D in the immune system**

1,25-(OH)$_2$D$_3$ regulates calcium and phosphate homeostasis. As shown in figure 7, the classical target tissues are bone, kidney and intestine. It has also been reported to have a number of other functions, including inhibition of proliferating malignant cells (Raghuwanshi et al., 2008). It mediates many of its functions by binding to the VDR. It has been demonstrated that the VDR is present on T-lymphocytes and that 1,25-
(OH)\(_2\)D\(_3\) can inhibit T-lymphocyte proliferation and activation (Bhalla et al., 1983). This suppressive effect is followed by a decrease in interleukin-2 (IL-2) and interferon-γ (IFN-γ) mRNA levels (Bhalla et al., 1986). Due to the effect of 1,25-(OH)\(_2\)D\(_3\) on various parts of the immune system, the vitamin has been implicated in various autoimmune and immune-mediated diseases. It has been used with great success in a variety of animal models for diseases, including experimental lupus erythematosus (Lemire et al., 1992), arthritis (Cantorna et al., 1998) and EAE (Cantorna et al., 1996).

**Requirements of vitamin D**

Sunlight exposure is the main source of vitamin D in most parts of the world. It has been estimated that one day of sun exposure is equivalent to a single dose of 10 000 to 25 000 IU of vitamin D (Holick, 2005). Fish oils and egg yolk are natural dietary sources of vitamin D (Figure 7). In the northern parts of the world, there is no ultraviolet radiation between November and April, thus making exogenous supply necessary to avoid vitamin D deficiencies (Gillham et al., 2001). Seasonal variation, time spent outdoors and skin pigmentation will influence the vitamin D serum levels. Babies born in the autumn and breast fed during the winter will also have low levels of vitamin D, if they are not given supplementation, as breast milk contains only small amounts of the vitamin.

Increased exposure to sunlight or increased dietary intake of vitamin D increases serum levels of 25-(OH)D\(_3\), thus making it a useful measure of vitamin D nutritional status. Serum level of 25-(OH)D\(_3\) is considered the best indicator of vitamin D deficiency. It is highly sensitive to vitamin D intake and sun exposure and is a marker of vitamin D availability to tissues (Ascherio and Munger, 2008). The cut-off values for normal 25-(OH)D\(_3\) status are, however, under debate (Zittermann, 2003). It is generally agreed that serum levels of less than 20-25 nmol/L indicate severe deficiency associated with rickets. 50 nmol/L has been suggested as the lower limit of the normal range, but PTH levels seem not be normalised before vitamin D levels reach at least 80 nmol/L (Thomas et al., 1998). A daily dose of 1000 IU of vitamin D seems to be
required to bring the concentration in a population up to 75 nmol/L 25-(OH)D₃ in at least 50% of the population (Bischoff-Ferrari et al., 2006). A very high intake of vitamin D can, on the other hand, lead to vitamin D toxicity. This can result in bone loss, kidney stone formation and calcification of heart and kidneys. Vitamin D toxicity has not been observed as a result of sun exposure and it is very seldom occurring with an intake of less than 10 000 IU/day (Holick, 2007). In general, a daily intake of 4 000 to 10 000 IU per day is safe for young adults (Vieth, 2001; Heaney et al., 2003) and serum concentrations above 600 nmol/L are required to elicit hypercalcemia (Hathcock et al., 2007).

Several factors other than sun exposure and dietary intake also determines the levels of circulating 25-(OH)D₃. Women have normally lower serum levels than men and skin colour, amount of body fat and age have also been shown to determine the requirements of vitamin D (Myhr, 2009). Finally, polymorphisms in the 1-alfa-hydroxylase (CYP27B1) are also significantly associated with the 25-(OH)D₃ concentration (Orton et al., 2008), indicating that the vitamin D regulation is under important genetic influence.
Figure 7. Vitamin D metabolism. The figure demonstrates the major sources of vitamin D, how it is metabolised and its major biological effects (Modified from Crawford et al., 2006).
4.4 Epstein-Barr virus (EBV) and EBV serology

The EBV was first discovered in 1964. It is a virus in the herpes family and infections lead to a lifelong carrier state. It has a 173-kb DNA genome and is capable of infecting B- and T-lymphocytes, squamous epithelial cells in the oro- and nasopharynx, glandular epithelium of the thyroid, stomach and salivary gland, smooth muscle cells and follicular dendritic cells (Gulley, 2001). In Norway, about 15-25% of children below the age of 5 years are infected and by the age of 30, about 90-95% are carriers. When children become infected with the virus, these infections usually produce no symptoms or only mild flu-like symptoms. The virus causes infectious mononucleosis in 35-50% when the infection occurs during adolescence. Most of the symptoms of mononucleosis are attributable to the T-lymphocyte response of the infection (Cohen, 2000).

Mechanism of infection

The virus is transmitted in salvia. Epithelial cells or resting B-lymphocytes in the oropharynx are infected. The cells then either undergo lytic infection with the production of more viruses or express the latent viral proteins (Cohen, 2000). Cytotoxic T-lymphocytes and Natural Killer (NK) cells regulate the latently infected B-lymphocytes. Cell lysis is associated with the release of virions with viral spread to lymphoid tissue. Further replication leads to viremia with infection of the liver, spleen and B-lymphocytes in the peripheral blood. Cytotoxic T-lymphocytes and NK cells eventually reduce the number of infected B-lymphocytes (Figure 8). Since an infection with the virus causes a carrier state, it remains dormant in B-lymphocytes for the rest of a person’s life. EBV infected cells are rare in healthy virus carriers (~1-50 per 10⁶ cells) and cannot be analysed directly (Kuppers, 2003). The virus can reactivate and is then found in salvia of the infected person. These reactivations usually occur without clinical symptoms.
Figure 8. **EBV infection in humans.** The figure shows how the EBV is transmitted by salvia to the epithelium in oropharynx. From here it can infect resting B-lymphocytes who are either transformed to a lytic or resting (latent) phase. The primary infection is eventually controlled by cytotoxic T-lymphocytes and NK cells. (Modified from Cohen, 2000).

![Diagram of EBV infection](image)

**Latent and lytic cycle**

The latent infection is characterised by a limited expression of latent genes, where the virus does not replicate, but persists within the host cells (Bergallo *et al.*, 2007). The EBV encodes nine latency-associated proteins. Six of them are located in the nucleus (EBV nuclear antigens; EBNAs) and three of them are located in the membrane (latent membrane proteins; LMPs) (Thorley-Lawson, 2001). Three different latency forms have been recognised (Table 6) (Thorley-Lawson, 2001; Kuppers, 2003). In the type I
pattern only EBNA1 is expressed, in the type II pattern EBNA1 and LMP1 and/or 2 are expressed and in type the III pattern EBNA1, EBNA2, EBNA3a-c, EBNA-LP, LMP1 and LMP2 are expressed. The type III pattern is also known as the growth program, because EBV-transformed B-lymphocytes proliferating in vitro have this gene expression pattern. In addition, it is recognised a latency pattern 0, in which EBV protein expression is limited to LMP2 (Bergallo et al., 2007). LMP2A is the only gene product that is consistently detected in healthy carriers. The lytic cycle is initiated by the transcription of the EBV immediate early BZLF1 gene. The gene is a hallmark of lytic infection, and is used to distinguish between the latent and lytic stage (Kubota et al., 2008).

**EBV-associated diseases**
The EBV is a cofactor for the development of a number of inflammatory and neoplastic diseases. The virus is known to be able to cause infectious mononucleosis if the disease occurs in adolescence. Infectious mononucleosis is a predisposing factor for the development of Hodgkin’s disease. EBV is present in 40-60% of the cases of the disease and it is predisposing to both the EBV positive and negative Hodgkin’s disease (Thorley-Lawson, 2001). In Hodgkin’s disease, the type II pattern of gene expression is usually found. EBV is also present in almost all endemic cases of Burkitt's lymphoma and 20-30% of cases outside the endemic areas (Rickinson and Kieff, 2001). In Burkitt's lymphoma, only EBNA-1 (type I pattern) is expressed. The virus is also found in almost all undifferentiated forms of nasopharyngeal carcinoma and has then typically the latency II pattern of gene expression (Sudo et al., 1997). Finally, T-lymphocyte immunosuppressive therapy given to patients after transplantation is associated with increased risk of lymphoproliferative diseases (post-transplant lymphoproliferative diseases; PTLD) (Kuppers, 2003). These diseases are almost always of B-lymphocyte origin and positive for EBV (Knowles, 1998). The majority of PTLD cases have the latency III pattern of gene expression.
Table 6. EBV latency forms. (Adapted from Kuppers, 2003). The table illustrates the latent protein expression of the EBV virus in different latency forms.

<table>
<thead>
<tr>
<th>Latency form</th>
<th>EBERs</th>
<th>EBNA1</th>
<th>LMP1</th>
<th>LMP2A</th>
<th>EBNA2</th>
<th>EBNA3/EBNA-LP</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+</td>
<td>N.D.</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Memory B-cells in peripheral blood, healthy carriers</td>
</tr>
<tr>
<td>I</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Burkitt's lymphoma</td>
</tr>
<tr>
<td>II</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>Hodgkin's lymphoma and nasopharyngeal carcinoma</td>
</tr>
<tr>
<td>III</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Post-transplant lymphoma</td>
</tr>
<tr>
<td>IV</td>
<td>N.D.</td>
<td>N.D.</td>
<td>-</td>
<td>N.D.</td>
<td>+</td>
<td>N.D.</td>
<td>Infectious mononucleosis</td>
</tr>
</tbody>
</table>

**Abbreviations**: EBERs= Epstein-Barr virus encoded RNA, EBNA= Epstein Barr virus nuclear antigen, LMP=Latent membrane protein, N.D=Not determined
4.4.1 Detection of EBV

Epstein-Barr virus encoded RNA (EBER) in situ hybridisation
This is considered the gold standard for detection of EBV in tissue samples (Ambinder and Mann 1994; Gulley, 2001). EBER transcripts are expressed in latently infected B-lymphocytes at high levels, thus making this a very sensitive detection method. There are two main pitfalls with this technique. First, there is a possibility of false negative hybridisation results as a result of RNA degradation (Gulley, 2001). This can be avoided by running a hybridisation in parallel to ensure that RNA is preserved. Secondly, there is a risk of false positive results if the results are not adequately distinguished from background lymphocytes and artefacts.

Immunohistochemistry
This method can be used to detect viral proteins expressed on EBV-infected cells. These stainings, especially LMP1, have proven to be nearly as effective as in situ hybridisation for identifying EBV in PTLD, Hodgkin’s disease and infectious mononucleosis (Lones et al., 1997). True LMP1 positive stainings are granular in character and are localised to the cytoplasm. False positive signals have been reported from eosinophils, plasma cells, cells of the nervous system and poorly fixated cells (Gulley, 2001).

Reverse-transcriptase polymerase chain reaction (RT-PCR)
The use of this technique offer the potential for identifying and distinguishing between different latency forms of the infection and also to distinguish between latently infected cells and cells in the lytic phase. The most common targets are EBNA1 and 2, LMP2A and BZLF1 (Gulley, 2001). Although this method is not used on a routine basis, it has just been validated with a high degree of sensitivity and specificity (Kubota et al., 2008).
**Serology**

Antibodies to several antigen complexes may be measured in the serum of patients. Immunosuppressed patients can have inconsistent responses to EBV and serology is not a reliable marker of infection in these patients. In healthy individuals, serology is considered the gold standard to detect acute versus remote infections (Gulley, 2001). The following antibodies are usually measured: IgM and IgG to the viral capsid antigen (VCA), IgM to the EBV nuclear antigen (EBNA) and IgG to the early antigen (EA). IgM to the viral capsid antigen (VCA) appears early in the infection and disappears after 4-6 weeks. IgG to the viral capsid antigen (VCA) appears in the acute phase of the infection. It reaches a peak after about 2-4 weeks and persists for the rest of life. The EBV nuclear antigen (EBNA) appears 2-4 months after the acute infection and persists for life. IgG to the early antigen (EA) appears in the acute phase and usually falls to undetectable levels after 3-6 months. With viral reactivations, the anti-EA-IgG titer increases.

**Figure 9. Serological reactions to active (lytic) and latent EBV infection.** The figure illustrates the production of antibodies to different components of the EBV virus during the acute viral infection and in the latent phase.

---

**Abbreviations:** EA=Early antigen, EBNA=EBV nuclear antigen, VCA=Viral capsid antigen,
4.5 Microarray

In a gene expression microarray, thousands of genes can be monitored to study the effect of a disease on gene expression. A number of different microarray platforms have been established. The microarrays are glass microscope slides, silicon chips or nylon membranes where thousands of different genes are attached to different locations. The principle of running a cDNA microarray experiment is simple (Figure 10). First RNA is purified from control and experimental samples. Then reverse transcription is used to convert the extracted RNA into cDNA. This is labelled with fluorescent probes. It is usual to use Cy3 for the controls (which have a fluorescent emission wavelength corresponding to the green part of the light spectrum) and Cy5 for the experimental samples (corresponding to the red part of the light spectrum). The two-labelled samples are mixed and incubated into the microarray. The labelled samples then bind to the sites on the array corresponding to the genes expressed.

Figure 10. The principle of gene expression analysis by DNA microarray technology. (From Khan et al., 1999)
Gene expression studies in MS

Microarray techniques were first applied to MS brain tissue in 1999 (Whitney et al., 1999). Since then, a number of studies have used these techniques to study gene expression in demyelinated lesions (Tajouri et al., 2003), normal appearing white (Zeis et al., 2008) and grey (Dutta et al., 2006; Dutta et al., 2007) matter. The main findings in the published microarray studies on MS brain tissue are given in table 7. The studies differ in the number of genes examined and in characterisation of the brain tissue material, thus making a direct comparison between the studies difficult.
Table 7. Main findings and characteristics of the studies using microarray techniques on MS brain tissue.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Number of patients</th>
<th>Genes examined</th>
<th>Tissue used</th>
<th>Brain area</th>
<th>Main findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whitney et al., 1999</td>
<td>1 MS (PP), 0 controls</td>
<td>5 000</td>
<td>Acute lesions vs. NAWM</td>
<td>NA</td>
<td>Inflammation related genes upregulated</td>
</tr>
<tr>
<td>Whitney et al., 2001</td>
<td>2 MS (PP, RR), 3 controls</td>
<td>2 798</td>
<td>WM lesions</td>
<td>NA</td>
<td>5-lipoxygenase upregulated in WM lesions</td>
</tr>
<tr>
<td>Lock et al., 2002</td>
<td>4 MS (SP), 2 controls</td>
<td>7 000</td>
<td>Lesions vs. Control. Acute vs. Chronic</td>
<td>NA</td>
<td>Granulocyte stimulating factor upregulated in active lesions. FcγR upregulated in chronic lesions.</td>
</tr>
<tr>
<td>Tajouri et al., 2003</td>
<td>5 MS (SP), 0 controls</td>
<td>5 000</td>
<td>WM lesions, chronic and active</td>
<td>WM lesions and NAWM</td>
<td>69 common genes with increased expression in chronic and acute lesions and 70 transcripts uniquely expressed in each.</td>
</tr>
<tr>
<td>Mycko et al., 2003</td>
<td>4 MS (SP), 0 controls</td>
<td>588</td>
<td>Lesions (chronic active vs chronic inactive)</td>
<td>NA</td>
<td>Upregulation of inflammatory and apoptosis related genes</td>
</tr>
<tr>
<td>Graumann et al., 2003</td>
<td>10 MS (SP, PP), 7 controls</td>
<td>3 528</td>
<td>NAWM vs Ctrl WM</td>
<td>Subcortical white matter</td>
<td>Upregulation of genes involved in maintenance of cellular homeostasis and neuroprotection in NAWM</td>
</tr>
<tr>
<td>Mycko et al., 2004</td>
<td>4 MS, 0 controls</td>
<td>588</td>
<td>Chronic active vs-chronic inactive lesions</td>
<td>NA</td>
<td>Upregulation of inflammatory and apoptosis related genes</td>
</tr>
<tr>
<td>Lindberg et al., 2004</td>
<td>6 MS (SP), 12 controls</td>
<td>12 556</td>
<td>Lesions vs Ctrl. WM</td>
<td>NA</td>
<td>Cellular immune response in NAWM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NAWM vs. Ctrl. WM</td>
<td></td>
<td>Humoral immune response in lesions</td>
</tr>
<tr>
<td>Dutta et al., 2006</td>
<td>6 MS (SP, PP), 6 controls</td>
<td>33 000</td>
<td>NAGM vs. Ctrl GM</td>
<td>Motor cortex</td>
<td>Mitochondrial dysfunction in NAGM in MS</td>
</tr>
<tr>
<td>Dutta et al., 2007</td>
<td>6 MS (SP, PP), 6 controls</td>
<td>33 000</td>
<td>NAGM vs. Ctrl GM</td>
<td>Motor cortex</td>
<td>Activation of the ciliary neurotropic factor signalling pathway</td>
</tr>
<tr>
<td>Zeis et al., 2008</td>
<td>11 MS (SP, PP, PR), 8 controls</td>
<td>3 528</td>
<td>NAWM vs Ctrl WM</td>
<td>Subcortical white matter</td>
<td>Upregulation of genes involved in anti-inflammatory mechanisms in NAWM</td>
</tr>
<tr>
<td>Torkildsen et al., 2009c</td>
<td>6 MS (SP, PR), 8 controls</td>
<td>27 868</td>
<td>NAGM vs. GML vs Ctrl GM and meningeal tissue</td>
<td>Cortical and meningeal tissue</td>
<td>Upregulation of Ig-related, but not FcγR-genes.</td>
</tr>
</tbody>
</table>

Abbreviations: NA=Not applicable, SP=Secondary progressive, PP=Primary progressive, PR=Progressive relapsing, GM= Grey matter, GML=Grey matter lesions, NAGM=Normal appearing grey matter, NAWM=Normal appearing white matter, WM=White matter.
The use of the microarray method raises many methodological considerations. The most important are discussed briefly:

**Normalisation and statistical analysis**
A number of different normalisation methods exist, depending on the system used. Global normalisation is often applied to microarray systems covering a large number of genes (Kinter et al., 2008). In our study, inter-array quantile normalisation was performed in order to minimise the effect of external variables introduced into the data. Examination of similarities and differences in global gene expression profiles of the samples was done by principal component analysis (PCA). Identification of probes that differed significantly in expression level (i.e. hybridisation signal intensity) between sample groups was carried out by significance analysis of microarrays (SAM) (Tusher et al., 2001), comparing the expression levels of all probes in one group with those of the other. The SAM analysis threshold was set to a q-value of five (Storey, 2003).

**Standardisation**
There exist many different platforms for RNA profiling. They differ with respect to the number of genes analysed, in the length of cDNA probes and how RNA is processed before labelling (Kinter et al., 2008). This makes it difficult to compare the results from microarray studies directly (Comabella and Martin, 2007). Our study was performed with the Applied Biosystems (AB) Expression Array system, which is based upon chemiluminescence detection (emission of electromagnetic radiation during the course of a chemical reaction). This method does not require excitation and results in low background noise, thus enhancing the sensitivity of the array (Figure 11). The AB human microarray contains 31,700 probes against 27,868 genes, as well as about 1,000 control probes.
**Figure 11. The principle of the chemiluminescence detection method.** First digoxigenin (DIG) labelled cDNA hybridises to the microarray. Then DIG antibody-alkaline phosphatase conjugates and finally an enhancer interacts with the activated chemiluminescent substrate, which in turn produces light. (Adapted from Applied biosystems, http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/gereraldocuments/cms_040420.pdf)
Immunohistochemical characterisation of the material

An important issue in microarray studies is identification of the cellular composition of the samples examined (Kinter et al., 2008). An immunohistochemical characterisation of both the control and MS-samples is therefore important. In our study, the control and MS-samples were screened for demyelination (anti-PLP), B- and T-lymphocyte infiltration (anti-CD20 and anti-CD3), plasma cell accumulation (anti-CD138), Ig-deposition (anti-IgA, -IgG, -IgM, -Kappa, Lambda) and latent EBV infection (anti-EBNA2 and anti-LMP1). The primary antibodies used in this study are given in table 8.

Table 8. Primary antibodies used in the microarray study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Detection</th>
<th>Species/isotype</th>
<th>Dilution</th>
<th>Primary antibody</th>
<th>Source</th>
<th>Pre-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLP</td>
<td>Myelin</td>
<td>Mouse IgG2a</td>
<td>1:2000</td>
<td>24 h</td>
<td>Serotec</td>
<td>95 % ethanol 10 min.</td>
</tr>
<tr>
<td>CD20</td>
<td>B-lymphocytes</td>
<td>mouse IgG</td>
<td>Prediluted</td>
<td>10 min</td>
<td>Dako</td>
<td>95 % ethanol 10 min.</td>
</tr>
<tr>
<td>CD3</td>
<td>T-lymphocytes</td>
<td>rabbit polyclonal</td>
<td>1:800</td>
<td>1 h</td>
<td>Sigma</td>
<td>95 % ethanol 10 min.</td>
</tr>
<tr>
<td>CD138</td>
<td>Plasma cells</td>
<td>mouse IgG</td>
<td>1:400</td>
<td>1 h</td>
<td>Serotec</td>
<td>95 % ethanol 10 min.</td>
</tr>
<tr>
<td>IgA, -IgG, -IgM, -Kappa, Lambda</td>
<td>Immunoglobulins</td>
<td>rabbit polyclonal</td>
<td>1:800</td>
<td>1 h</td>
<td>Dako</td>
<td>95 % ethanol 10 min.</td>
</tr>
<tr>
<td>EBNA2</td>
<td>EBV-EBNA2</td>
<td>mouse IgG</td>
<td>1:50</td>
<td>1 h</td>
<td>Abcam</td>
<td>95 % ethanol 10 min.</td>
</tr>
<tr>
<td>LMP1</td>
<td>EBV-LMP1</td>
<td>mouse IgG</td>
<td>Prediluted</td>
<td>½ h</td>
<td>Abcam</td>
<td>95 % ethanol 10 min.</td>
</tr>
</tbody>
</table>

Abbreviations: EBNA= Epstein Barr virus nuclear antigen, LMP= Latent membrane protein, PLP= Proteolipid-protein
Sample and case selection

It is important to avoid false positive results because of sample and case selection. Gender, age differences and medical treatment could influence the microarray results (Kinter et al., 2008). In our study, brain samples from six patients with MS and eight control patients without any signs of neurological disease were obtained from the Dutch Brain Bank, the Netherlands for microarray examination. The mean age of MS cases was 49.2 years (range 41-58) with a mean post-mortem delay of 8.6 hours (range 8-11). The mean age of the control cases were 81.9 years (range 74-89) with a mean post-mortem delay of 7.3 hours (range 4-15). For qPCR validation, five additional MS patients and four additional controls were included. The mean age of these MS patients were 57.8 years (range 34-75) with a mean post-mortem delay of 10.7 hours (range 6.3-18.0) and the mean age of these controls were 73.7 years (range 57-82) with a post-mortem delay of 6.7 hours (range 5.4-8.2). The possible implications of the age differences between the MS and control samples are discussed in Chapter 5.
5. Summary of results and general discussion

5.1 Summary of results

5.1.1 Effects of diet intervention on MRI activity, de- and remyelination in the cuprizone model for demyelination (article I)

The aim of this study was to analyse if diets with a different composition of PUFAs could influence demyelination, as measured by histopathology and MRI, and stimulate remyelination in cuprizone fed mice, a widely used animal model for de- and remyelination.

Sixty-three female C57Bl/6 mice were fed with 0.2% cuprizone on three different diets that consisted of the same ingredients, except the lipid source which came from 1) salmon fillets rich in marine n-3 PUFAs, where the salmon filets served both as a lipid and protein source, 2) cod liver oil rich in marine n-3 PUFAs, or 3) soybean oil rich in n-6 PUFAs. MRI was performed after 5 weeks of continuous cuprizone treatment. After 6 weeks, half of the animals were sacrificed and the corpus callosum was examined using LFB histochemistry and immunohistochemistry for PLP, GFAP, fibrinogen and MAC-3. After one week of remyelination, the rest of the mice were sacrificed and the corpus callosum examined with the same histo- and immunohistochemical techniques.

After 5 weeks of cuprizone-treatment the mice fed salmon-cuprizone had a significantly lower hyperintense lesion volume on T2-weighted MRI than the two other groups (P<0.0005). The mice given salmon-cuprizone had significantly less demyelination after 6 weeks of cuprizone treatment, as measured with LFB (P<0.0005) and PLP (P=0.014). There was less infiltration of mac-3 immunopositive cells in the corpus callosum of mice fed salmon-cuprizone compared to the mice given soybean oil-cuprizone (P=0.041), but not compared to mice receiving cod liver oil-
cuprizone (P=0.073). The salmon-cuprizone group had also enhanced remyelination compared to the cod liver oil-cuprizone group (LFB; P=0.003, PLP; P=0.018). Immunostaining for fibrinogen revealed no sign of BBB damage in the cuprizone-treated mice or in the controls at any of the time points.

5.1.2 A salmon based diet protects mice from behavioural changes in the cuprizone model for demyelination (article II)

The aim of this study was to evaluate whether diets with n-3 PUFAs from two different sources could influence behavioural changes induced by demyelination in cuprizone pre-treated mice, compared to a control diet with an n-3/n-6 ratio more like a western diet. The cuprizone model is well suited for studying dietary factors that may influence neuropsychological dysfunction induced by demyelination (Franco-Pons et al., 2007; Xiao et al., 2008). Our hypothesis was that supplementation with omega-3 PUFAs would have a protective effect on demyelination, leading to less dysfunction in the two n-3 groups than in the control group.

Female mice were fed three different diets enriched with 0.2% cuprizone. The diets consisted of the same ingredients, except the lipid source, which came from 1) salmon fillets rich in marine n-3 PUFAs, where the salmon fillets also served as a protein source, 2) cod liver oil rich in marine n-3 PUFAs, or 3) a control diet containing soybean oil rich in n-6 PUFAs. The mice were weighed twice weekly throughout the experimental period. After 5 weeks of continuous cuprizone treatment, animal activities were assessed with the elevated plus-maze (EPM) test and after 6 weeks the mice were sacrificed and tissue sections from their brains were stained with LFB.

The mice fed salmon-cuprizone had significantly less demyelination than the two other groups (P<0.001). They had also more visits in both open and closed arms of the EPM than the other cuprizone treated groups (P’s<0.0001). In addition they had more entries in the open arms than both the cod liver oil-cuprizone (P<0.02) and the soybean oil-
cuprizone treated mice (P<0.0001). Their weight loss was also smaller than the other cuprizone-treated mice (P<0.0001).

5.1.3 Vitamin D-dependent rickets as a possible risk factor for multiple sclerosis (article III)

This article is a case study of three patients in two families with co-occurrence of MS and VDDR1. VDDR1 is a very uncommon genetic subtype of rickets, caused by a mutation in CYP27B1. The active form of vitamin D, 1,25-(OH)_{2}D_{3}, is produced by a two-step hydroxylation, first at the 25-position in the liver by mitochondrial CYP27A1 and then at the 1α-position in the kidney by CYP27B1. Thus, CYP27B1 is the key enzyme in determining the rate of 1,25-(OH)_{2}D_{3} production. Vitamin D is emerging as an important cofactor for the susceptibility to MS (Ascherio and Munger, 2007b). There have, however, not been any studies on the possible association between hereditary forms of rickets and this disease. The prevalence of MS in Norway is 1.5 per 1000 people (Torkildsen et al., 2007). The prevalence of VDDR1 is not known, but we were only able to identify three patients diagnosed with VDDR1 at Haukeland University Hospital, which serves a population of 460,000 people.

All three patients identified in this study had VDDR1 verified by genetic testing and fulfilled the Poser criteria for MS (Poser et al., 1983). Two of the patients had undergone magnetic resonance imaging, which confirmed the diagnosis of long-lasting MS. The patients in our case series had been given cholecalciferol or ergocalciferol (vitamin D_{3} and D_{2}) supplements since childhood. Blood samples from two of the patients were available and they had normal and above normal levels of 1,25-(OH)_{2}D_{3}. 
5.1.4 Epstein-Barr virus reactivation and multiple sclerosis (article IV)

The aim of this study was to look for evidence of reactivation of latent EBV infection during MS-exacerbations. Several lines of evidence have implicated an association between EBV infection and the risk of MS. All patients have evidence of previous EBV infection (Myhr et al., 1998), the risk of MS is increased after infectious mononucleosis and there seems to be an age-dependent relationship between EBV infection and development of MS (Ascherio and Munger, 2007a). One study has also suggested that exacerbations in MS are associated with reactivation of latent EBV infection (Wandinger et al., 2000). These findings could, however, not be reproduced in another study (Buljevac et al., 2005), and whether EBV plays an underlying role as an activator of MS remains controversial.

Sixty-one patients with definite RRMS according to the Poser criteria were available from a clinical trial on interferon treatment (Myhr et al., 1999). All patients had definite RRMS according to the Poser criteria (Poser et al., 1983). Mean age was 34.9 years (range 21-48) with mean expanded disability scale (EDSS) score of 2.8 (range 0-5.5). The patients were followed for one year and blood samples were drawn at baseline, months 3, 6 and 12 and in case of clinical exacerbations. MRI was performed at baseline, monthly during the first six months and at month 12. Blood samples for analysis of anti-EA-D IgG were drawn at the time of clinical exacerbations or when the MRI examination revealed new T1 gadodiamide-enhancing lesions. All included patients were analysed for evidence of previous EBV infection by measuring IgM and IgG antibodies against VCA and EBNA-1. Twenty-three baseline-paired exacerbation samples were quantitatively analysed to examine whether exacerbations in MS were associated with a change in anti-EA-D IgG ratio.

All the 61 patients in our study (100%) were anti-VCA IgG positive, one (2%) was anti-VCA IgM positive and 60 (98%) were anti-EBNA positive. Mean anti-EA-D IgG at baseline was 0.57 (range 0.12-2.70) and at the time of exacerbations 0.61 (range
Wilcoxon signed rank test revealed no differences between the 23 baseline and paired samples (P=0.58).

5.1.5 Upregulation of immunoglobulin-related genes in cortical sections from multiple sclerosis patients (article V)

In the present study, we have compared global gene expression in normal appearing grey matter and grey matter lesions of the cortex of MS patients as well as comparing grey matter samples in MS- and control patients. The brain sections examined were then immunohistochemically characterised and qPCR and immunohistochemistry was performed for detection of both potential EBV transcripts and proteins. Microarray-based global gene expression profiling is increasingly used to study potential genes involved in the pathogenesis of MS. The method offers the potential for a better understanding of the pathogenesis involved in MS (Kinter et al., 2008). Previous studies have mainly focused on white matter tissue. To date, there have only been two studies on global gene expression in the cortex of MS patients (Dutta et al., 2006, Dutta et al., 2007).

For the microarray examination, brain samples from six MS patients and eight controls were available from the Dutch brain bank, the Netherlands. All microarray experiments were performed using the Applied Biosystems Expression Array system. The results were then validated using Taqman qPCR. qPCR was also used for detection of latent (EBNA1, EBNA2, LMP1, LMP2) and lytic (BZLF1) EBV transcripts in the samples examined. Finally, immunohistochemistry was used for detection of demyelination (anti-PLP), Igs (anti-IgA, IgG, IgM, kappa, lambda), B-lymphocytes (anti-CD20), T-lymphocytes (anti-CD3), plasma cells (anti-CD138), and latent EBV-infected cells (anti-EBNA2 and anti-LMP1).

We observed a strong activation of Ig-related genes in the cortical sections of MS patients. Among these, the genes with the strongest activation were variable and constant regions of the kappa and lambda chains, but also included genes encoding
heavy chains for IgM and IgG. No upregulation of Fc-receptor genes was found. The results were verified with Taqman qPCR. The cortical sections were then immunohistochemically stained for plasma cells, Igs, T- and B-lymphocytes. The stainings revealed Ig-deposition in the meninges of the MS-patients compared to the controls. There were few B-lymphocytes and no B-lymphocyte follicles. Plasma cells were present in the meninges of all the MS patients but not in any of the controls.

The activation of Ig-genes observed in the present study is highly interesting as the synthesis of oligoclonal IgGs have been hypothesised to be caused by activation of EBV infected B-lymphocytes. The sensitivity of the qPCR for EBV-transcripts was tested using the EBV positive cell line B95.8 in dilutions with the EBV-negative cell line CRL-1593.2. It was possible to detect EBV-positive cells at concentrations down to 1:1000-10 000. The sensitivity of the immunohistochemistry was tested using the EBV-positive cell line B95.8 as a positive control. We then screened for the presence of this virus in our MS- samples using both techniques, but were unable to detect signs of active or latent EBV.
5.2 General discussion

5.2.1 Article I and II

Outcome measures

*MRI*

T2 weighted MRI was taken after 5 weeks of cuprizone administration. The examination revealed large group differences in volume of hyperintense lesions. Other sequences could have differentiated if this partly resulted from increased intra- or extracellular fluid volume. It would especially have been interesting to use magnetisation transfer ratio (MTR) to visualise myelin loss and repair (Zaaraoui et al., 2008) and to include more time points for MRI analysis.

*Histology and immunohistochemistry*

LFB and PLP have been proven to be reliable markers for the degree of demyelination in this animal model (Lindner et al., 2008). Thus, inclusions of other methods, like EM would probably have added only limited information. It would have been interesting to add an immunohistochemical staining for oligodendrocytes, such as anti-neuritic outgrowth factor (NOGO). Since cuprizone is thought to affect the oligodendrocytes directly, this could have added more knowledge to the effects of dietary interventions.

*Behavioural responses and weight loss*

The mice were weighed twice weekly and their behavioural responses to cuprizone administration were assessed with the EPM test. The EPM test is primary used to measure anxiety behaviour, but it can also be used to measure activity levels (Lister, 1987). Thus, the present study (article II) was able to draw conclusions on group differences in weight loss, activity levels and anxiety behaviour. This behaviour study was largely explorative and future studies should include a larger battery of behaviour assessment tests. It would especially be interesting to include open-field assessment,
the rota-rod test and functional observation battery since these are tests that have been evaluated in the cuprizone model (Golub et al., 2004).

**Validity and reliability**

One of the main concerns with intervention studies in the cuprizone model is that cuprizone could theoretically interact with the intervention substance in the stomach of the animal (Zhang et al., 2008). Thus, the validity of the experiment for MS would be low. However, two studies have found that it is not possible to antidote the effect of cuprizone by co-administration of dietary copper supplements (Carlton, 1967; Kesterson and Carlton, 1972). In addition mice exposed to dietary interventions with the salmon based diet had less behavioural changes than the other mice (Torkildsen et al., 2009b) and cuprizone is not known to affect other cells in the CNS than oligodendrocytes. The best way to make sure there was no antidote effect would have been to measure the serum-cuprizone level in each animal. Unfortunately, there are currently no methods for this detection.

The diets were available ad libitum and a possible confounder could be that the energy content or fatty acid composition differed between the diets. There was, however, little variation between the diets in this aspect. It could also be argued that the salmon fed mice ate more of the chow than the other groups and thus had less weight loss. Since this would also have led to more ingestion of cuprizone, this should have given them more demyelination than the two other groups. The fact that they had less weight loss and demyelination than the two other groups, seem to confer that the salmon based diet offered a protection against the effects of cuprizone.

Another concern is how valid the cuprizone model is for MS. In the cuprizone model, there is no disruption of the BBB and little inflammation (Torkildsen et al., 2008a), which are prominent features of MS (Lassmann et al., 2007). The processes underlying de- and remyelination are probably well suited for studies in the cuprizone model. Histopathological analysis indicate that the cuprizone model has a pathological pattern which could resemble the type III or IV lesions described by Lucchinetti and
colleagues (2000). Thus, the pathological pattern resembles some aspects of MS, and the model seems to be a valuable supplement to EAE. There have been few intervention studies in the cuprizone model and the validity of the model for therapeutic interventions are yet to be determined. If the results from the diet intervention studies were replicated in another animal model for MS, like the EAE-model, this would significantly strengthen the validity of the conclusions for MS.

The two dietary intervention studies contained a number of measures to increase the reliability of the results. Since the salmon based diet had a positive influence on weight loss, behavioural changes, MRI activity, demyelination and microglia activation, the conclusion that salmon offers a protective effect against the neurotoxicity of cuprizone seems to be consistent independent of the measures used. In order to increase the reliability of the experiment, the results should be confirmed in a new study.
5.2.2 Article III

As this study suggests that rickets and low levels of 1,25-(OH)$_2$D$_3$ are possible risk factor for MS, it would have been interesting to include repeated measures of the vitamin D status in these patients. The patients were however, diagnosed with vitamin D dependent rickets in the 1950s and analysis of the active components of vitamin D (25-(OH)D$_3$ and 1,25-(OH)$_2$D$_3$) were not available at the time. The diagnosis was based on clinical signs of rickets, changes in the meta-epiphysis on x-ray, hypophosphatemia and hypocalcaemia; it was confirmed in 2003 with genetic testing.

All the patients have been treated with supplementation of vitamin D since the time of diagnosis. We have performed a detailed analysis of vitamin D status in the patients (vitamin 25-(OH)D$_3$ and 1,25-(OH)$_2$D$_3$). Their vitamin D levels were close to normal or above the reference values at the time of measurement due to their substitution-therapy. Blood samples were available for two of the patients, one receiving substitution with Rocaltrol (Roche) and one receiving AFI-D$_2$ forte (Nycomed Pharma). Rocaltrol contains 1,25-(OH)$_2$D$_3$ and AFI-D$_2$ forte contains 25-(OH)D$_2$. For the patient receiving substitution therapy with Rocaltrol, the 25-(OH)D$_3$ level was 6.5 nmol/L and the 1,25-(OH)$_2$D$_3$ level was 42 nmol/L. For the patient receiving AFI-D$_2$ forte, the 25-(OH)D$_3$ level was 672 nmol/L and the 1,25-(OH)$_2$D$_3$ level was 24 nmol/L. The normal reference value for 25-(OH)D$_2$ and 1,25-(OH)$_2$D$_3$ taken together is 30-150 nmol/L.

Since all the patients in this case series had a mutation in the CYP27B1, responsible for the final hydroxylation to 1,25-(OH)$_2$D$_3$, supplementation with 25-(OH)D$_2$ in the form of AFI-D$_2$ forte would not increase the level of the most potent form of vitamin D$_3$, 1,25-(OH)$_2$D$_3$. Further AFI-D$_2$ forte contains ergocalciferol (vitamin D$_2$) and not cholecalciferol (vitamin D$_3$) and it has been suggested that vitamin D$_3$ has a greater bioefficacy than vitamin D$_2$ (Houghton and Vieth, 2006). It is therefore possible that the patients have had a sub-optimal treatment of their rickets and that this has given an increased risk of MS. Since they have received substitution therapy, the findings may
also indicate that low level of vitamin D in early childhood, or maybe even in the intrauterine period, could account for increased susceptibility to MS observed in individuals with low levels of circulating vitamin D.

### 5.2.3 Article IV

A crucial issue regarding this study is the value of performing isolated EA-D IgG as a measurement of EBV-reactivation. The study by Wandinger and colleagues (2000) and Buljevac and colleagues (2005) included both measurements of anti-EA-D IgG and PCR-samples in selected patients. Inclusion of both these parameters could have increased the sensitivity of our study. In relation to reactivations and exacerbations in MS, the immunological response is however probably of greater importance than the viral load in serum. Buljevac and colleagues (2005) were in their study able to find viraemia on PCR in only 3 samples, despite an elevated immune response against anti-EA-D IgG in many more samples. It is well known that chronic T-lymphocyte responses against EBV proteins of the early replication phase can prevent viral replication (Cohen et al., 2000, Buljevac et al., 2005). Thus, including PCR analysis would not necessarily provide more information regarding the relationship between exacerbations and reactivation.

One of the strengths of our study compared to the two earlier papers on this subject (Wandinger et al., 2000, Buljevac et al., 2005) was that we were able to include systematic MRI-scans. Although Buljevac and colleagues (2005) were able to perform three MRI-scans from a selected sample of 37 patients, their study did not include systematic MRI-scans. In our study, we have followed all the 61 patients included with MRI-scans at 8 time-points (month 0, 1, 2, 3, 4, 5, 6, 12). This could potentially have detected subclinical lesion activity and increased the likelihood of detecting a correlation between rises in anti-EA-D IgG titers and exacerbations.

In the study by Buljevac and colleagues (2005) there was a tendency towards a higher number of gadolinium enhancing lesions in the EA+ than the EA- group. Only three
samples in our study showed, however, signs of elevated anti-EA-D IgG titers and there was no sign of increased disease activity or a higher number of gadolinium enhancing lesions in this group. This further emphasises the fact that there seems to be no correlation between rises in anti-EA-D IgG titers and disease activity in our patient sample.

5.2.4 Article V

Sample and case selection
The availability of affected MS tissue is limited. Brain biopsies from MS patients are very rare and most studies rely on post-mortem material. It is therefore possible that other genes are up- or down regulated in earlier phases of the disease course. RNA degrades shortly and it is important to use cases with a short post-mortem interval. The control cases should be at the same age as the samples (Lu et al., 2004). It is also important to use control samples from the same brain areas as the MS lesions to avoid false results due to brain region differences (Stansberg et al., 2007). Differences in gene expression could be due to an up- or down regulation in the cells in the region, but it could also be caused by differences in cellularity (Kinter et al., 2008). Immunohistochemical characterisation of the material with markers for gliosis, demyelination and inflammation is therefore important for the interpretation of the data (Kinter et al., 2008). In addition, some of the genes found differently expressed between the samples should be validated using qPCR.

Eight Ig-related genes were selected for validation by TaqMan qPCR. This validation confirmed significant upregulation of the IGKC, IGHG1 and IGKV41 genes in the MS samples. Thus, the upregulation of Ig-related genes seems to be consistent.

In our study, it was difficult to obtain age-matched MS and control samples. In general it is difficult to obtain brain tissue from MS patients and very few centres have frozen tissue available, as we used in our study. Age differences between the MS- and control samples are known to affect the expression of some genes, like anti-oxidative stress
genes and genes involved in mitochondrial function (Lu et al., 2004). On the other hand, there are no reports of changes in Ig-expression with increasing age. In order to exclude an age effect, qPCR was performed on additional samples not included in the microarray assay. In these samples, the age was overlapping between the MS- and control groups and we did not observe any age-effect on Ig upregulation in these qPCR analyses. Thus, the upregulation of Ig-gene related probes seemed to be attributable to MS and not age-related changes in gene expression in our study.

On the global level we were unable to detect systematic differences between GML and NAGM. The findings may be influenced by heterogeneity of the samples and batch effects interfering with our results. Since there is little inflammation in cortical MS-lesions (Bo et al., 2003b), one would especially expect genes involved in myelin production to be substantially downregulated in GML compared to NAGM. It has, however, been demonstrated that there is substantial remyelination at the lesion border of cortical lesions (Albert et al., 2007; Stadelmann et al., 2008), and this could have made it more difficult to detect downregulated genes in the lesions. Microarray studies with laser dissected material could maybe increase the likelihood of detecting these changes in gene expression.

Immunohistochemistry

All the MS and control cases were screened for degree of demyelination (anti-PLP), immunoglobulin deposition (anti-IgA, IgG, IgM, Kappa, Lambda), plasma cells (anti-CD138), B (anti-CD20)- and T-lymphocytes (anti-CD3) with immunohistochemistry. This was done to detect cells that could account for the differences in gene expression observed in the MS and control samples.

Our immunohistochemical staining revealed no B-lymphocyte follicles in any of the MS-samples examined. There have been three articles reporting findings of these follicles in MS-patients (Serafini et al., 2004; Magliozzi et al.; 2007, Serafini et al., 2007). The reason for this discrepant result could have been caused by the small number of tissue blocks examined in our study, or that ectopic follicles have only
developed transiently and disappeared at the time of death (Serafini et al., 2007). Since the immunohistochemical sampling in our study was limited, the most plausible hypothesis seems to be that there could have been B-lymphocyte follicles located in other blocks not examined. Since B-cell follicles have been demonstrated to be the main focus of EBV-persistence (Serafini et al., 2007), it could also be argued that the chance of identifying EBV-infected cells is very small when B-lymphocyte follicles are not present. A recent study questions the findings by Serafini and colleagues (2007). Despite looking for evidence of EBV-infections in some of the same tissue blocks, they were not able to find evidence for EBV-infection (Willis et al., 2009). Our study was not designed to give a detailed investigation of the possible presence of B-lymphocyte follicles or EBV-infection in the CNS of MS-patients. It seems, however, that persistent EBV infection did not cause the Ig-upregulation found in our sections, as neither B-cell follicles nor EBV-infected cells were found in close proximity to the microarray sections examined.
5.3 Future perspectives

Paper I and II
These studies indicate that dietary intervention could offer a therapeutic strategy in demyelinating diseases. The results need to be replicated in the cuprizone model and preferably in other animal models for MS, like the EAE model. Since the cuprizone model seems to be a model with primary oligodendrocyte death, the effects in a primary inflammatory model, like EAE would be very interesting. It would also be interesting to study if diet intervention could improve the disease course if it is introduced after cuprizone administration is started. In our study, the mice were already fed the three different diets at the time of cuprizone administration. If the results are consistent in different animal models for MS, human intervention studies should be considered.

Although we were able to perform a detailed analysis of energy balance, vitamin- and fatty acid content in the different diets, no blood samples were collected from the mice in this experiment. Thus, we were not able to measure if n-3 fatty acids from the salmon diet had a better uptake than the n-3s from cod liver oil. Since some human studies have indicated that the source of n-3s could influence their uptake (Visoli et al., 2003; Elvevoll et al., 2006; Harris et al., 2007), this would be an interesting analysis for future studies.

At present it is not known which substance in the salmon is responsible for the treatment effect. These studies could provide the basis for more specific diet interventions, with isolated compounds from the salmon-based diet, like vitamin D-supplementation. Analyses of vitamin D levels in the different diets used in our study show, however, that the cod liver oil diet actually had a higher level of vitamin D than the salmon-based diet. This could indicate that vitamin D is not as important in the cuprizone model as in EAE. Since the 25-hydroxylation of vitamin D is performed by the mitochondrial CYP27A1 isozyme and cuprizone is known to inhibit a number of
mitochondrial enzymes, the effects of vitamin D could have been inhibited by cuprizone. More studies of vitamin D supplementation in the cuprizone model, preferably with measures of 25-(OH)D$_3$ serum levels, should be performed.

In our study, salmon was both the lipid and protein source in the salmon-cuprizone group. It would also be interesting to feed mice only the lipid fraction in salmon in order to exclude an interaction with the proteins. It has also been speculated if antioxidants could have a protective effect in the cuprizone model (Zhang et al., 2008) as well as in EAE (Hendriks et al., 2004). This indicates that another interesting candidate could be the antioxidant astaxanthin. Salmon is known to contain large amounts of astaxanthin and this substance could be a possible candidate for future pharmaceutical interventions (Jackson et al., 2008).

**Paper III**

This case study supports the theory that early childhood or the intrauterine periods are the main susceptibility periods for low levels of vitamin D. To date, no association study on VDDR1, or other forms of rickets, and MS has been conducted. Since VDDR1 is a very rare disease, such association studies would be difficult to perform in most parts of the world. It has, however, been estimated to affect 1 in 2916 children in the Saguenay-Lac-St-Jean region of Quebec, Canada (De Braekeleer and Larochelle, 1991) and this region would have been particularly interesting for this kind of studies. Finally, it would have been interesting to perform studies on the association between other forms of childhood rickets and the risk of MS.

**Paper IV**

EBV remains one of the key candidates in the pathogenesis of MS. Our study is the second to conclude that EBV reactivation is not associated with MS exacerbations and our results are also in accordance with the findings from a recent study (Farrell et al., 2009). The association found by Wandinger and colleagues (Wandinger et al., 2000) may be an occasional association in a small sample set. Larger study samples that
include measures of both PCR and serology may be necessary to fully determine this possible association.

**Paper V**

Microarray-based studies offer the potential to discover disease specific alterations in gene expression and give a better understanding of the molecular pathomechanisms in MS. More studies are needed to replicate the findings from these studies and to hopefully generate new targets for disease intervention. Since we were unable to identify genes differentially expressed between grey matter lesions and normal appearing grey matter in MS samples, there is also need for more microarray studies comparing grey matter and grey matter lesions, preferably with laser dissected material.

In our study, very few plasma cells seem to be responsible for the Ig upregulation. Since recent findings have indicated that EBV-persistence and reactivation in the CNS could be important for development and disease progression in MS, more studies on this subject are warranted. The few plasma- and B-lymphocytes we found present in the cortical sections, indicate that a very sensitive method is probably necessary to detect the presence of EBV infected cells in MS patients. Thus, the reason for the discrepant findings could be caused by a low sensitivity of the methods used in the negative studies. Another possible reason for the discrepant findings could be that EBV is only present in a subgroup of MS-patients. In other diseases where EBV infections are known to be an important co-factor, like Burkitt’s lymphoma, EBV is only found in about 30-40% of the cases (Kuppers, 2003). Our findings indicate that cortical and meningeal EBV infections are not necessary for the Ig-upregulation detected in our study. More studies are needed to confirm our results and to elucidate if persistent EBV-infections in the CNS are important for subgroups of patients.
6. Conclusions

I-II. The extent of demyelination, macrophage/microglia-infiltration and MRI lesion volume was significantly lower in the animals receiving a salmon-based diet, compared to the two other experimental groups. These mice had also less behavioural changes, with less weight loss, anxiety behaviour and preserved total activity levels compared to the other diet groups. Thus, diet modification with a salmon based diet as the source of n-3 PUFAs seems to protect against demyelination in the cuprizone model. These results were not found in diets with purified n-3 or n-6 PUFAs. Our findings indicate that discrepant results in previous dietary intervention studies for MS could have been influenced by differences in type and source of fatty acids. Thus, this study provides a basis for further studies exploring the potential role of diet intervention in this disease.

III. We proposed that VDDR1, and possibly other hereditary rickets mutations that influence vitamin D metabolism, could be risk factors for MS. All our patients have received vitamin D supplementation since their childhood, and this could indicate that insufficient vitamin D substitution could be a risk factor for the disease. Since the patients have received substitution therapy, our data could also be interpreted to be in accordance with other studies, indicating early childhood or the intrauterine period as the main susceptibility periods for low levels of vitamin D.
IV. All the patients in our study showed evidence of previous EBV-infection. Thus, our findings support the hypothesis that previous infections with EBV may have a role in the pathogenesis of MS. We found however, no evidence for an association between EBV reactivation and exacerbations in MS. Our study suggests that reactivation of latent EBV infection do not play a significant role for exacerbations in RRMS.

V. This study demonstrates that genes involved in the synthesis of Igs are upregulated in cortical grey matter lesions in MS patients and that this activation seems to be caused by a small number of meningeal plasma cells. Latently EBV-infected B-lymphocytes seem not to be necessary for this Ig-upregulation. Further, our findings indicate that the OCB-producing cells found in the CSF of MS patients could have meningeal origin.
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