CHAPTER 1

General introduction
Multiple sclerosis – a historical perspective

For centuries there have been anecdotal reports of people who have presented with periods of neurological deficits from which they initially recover, yet with successive episodes their clinical condition gradually worsens over time. The first report in, the 14th century, describes the 16 year old Dutch nun, St. Lidwina from Schiedam, that fell during ice skating after which she developed a clinical picture resembling what we now call multiple sclerosis (MS)\(^1\). However, it was only in the mid-nineteenth century that MS was differentiated as a disease with an individual character, based on its clinical course and pathological features. It was the French clinician Jean-Martin Charcot who combined the clinical and pathological knowledge of people presenting with similar neurological symptoms and gave for the first time, in 1868, the contemporary description of the disease and its name \textit{sclérose en plaques} i.e. multiple sclerosis\(^1\). In his lectures Charcot gave an extensive description of the pathology observed, viz. myelin damage in the central nervous system (CNS) accompanied by inflammation, axonal damage and gliosis\(^2,3\). Although Charcot only saw 34 people with MS during his lifetime, the characterisation of MS as a single disease promoted investigations into the pathology, treatment and crucially the cause of the disease\(^4\). The enormous expansion of research into MS resulted in an increased understanding of its pathogenesis, methods for early diagnosis and search for therapeutic agents. Although more and more pieces of the MS puzzle have been discovered, it is not yet complete and the most relevant questions as what causes the disease and how to cure it still remain to be answered.

Clinical aspects

Multiple sclerosis is a neurological disorder affecting the CNS i.e. the brain, spinal cord and optic nerve, whereas the peripheral nerves are spared. Recurrent focal immune responses in the CNS lead to damage of the myelin and axons, which subsequently result in neurological dysfunction. Depending on the anatomical location of the damage (lesions), patients show various clinical symptoms involving the motor, sensory, visual and autonomic systems (Figure 1). The clinical symptoms of MS are very heterogeneous and most of them are not disease-specific. However, several clinical signs are pathognomic i.e. Lehermitte’s sign (an electrical signal that runs down the back into the limbs upon flexion of the neck) and Uthoff’s phenomenon (a worsening of the neurological symptoms when the body gets overheated, like during hot weather or exercise). Initially, the CNS is able to repair the tissue damage and therefore the early phase of MS is often characterised by periods of neurological symptoms followed by episodes of (partially) recovery. Approximately 85% of people with MS present with this relapsing-remitting neurological course of the disease (RR-MS). However, over time the damage within the CNS becomes more widespread due to the inability of the CNS to repair. The gradual accumulation of damage within the CNS during this phase of the disease is characterised by progressive neurological deficits in which people experience few, if any remissions. Patients need assistance to walk (cane, crutch or brace) and may eventually become wheelchair-bound or in severe cases
bed-bound. In general half of the RR-MS patients enter this secondary-progressive (SP-MS) stage 10 years after the first symptoms. At 25 years after diagnosis, this figures rises to 90%. In contrast to the RR form of MS, 10% of people with MS develop a gradual progression of disability immediately from disease onset. This type of disease is referred to as primary progressive MS (PP-MS)\(^6\).

Typically, the onset of MS occurs between 20 and 40 years, making it the leading cause of neurological disability among young adults. However, MS is also observed in children as young as 3 years old and older people far into the seventh decade\(^6,7\).

The clinical history and presentation is often sufficient to establish the diagnosis. Critical clinical criteria are dissemination of neurological episodes in time (DIT) and space (DIS), implying that a patient presents with 2 or more separate attacks involving at least 2 different areas in the CNS. There is no specific laboratory diagnostic test for MS, although increased immunoglobulins and oligoclonal bands in the cerebrospinal fluid (CSF), and delayed electrical responses after stimulation of specific sensory pathways (evoked potential tests) are used to examine changes associated with MS. MS is more firmly diagnosed using magnetic resonance imaging (MRI) that, while not specific to MS, is highly suggestive of disease when gadolinium enhancing lesions are observed\(^6,8\). The use of MRI allows the criteria of DIT and DIS crucial for diagnosis to be made earlier meaning that treatment regimens can thus be initiated earlier in the disease. For these reasons MRI criteria have been incorporated in the diagnostic criteria for MS; the McDonald criteria\(^9\) (Table 1).

Worldwide more than 2 million people are diagnosed with MS\(^10\). Women are at higher risk compared to men with a female-to-male ratio of 2:1, however this ratio equalises when numbers of people with progressive disease are examined\(^11,12\).

The geographical distribution of MS shows an explicit pattern with the highest prevalence in North-America and Europe (140 and 108 per 100.000 respectively) while the disease is almost absent around the equator (2.2 per 100.000).\(^10\)
Table 1 2010 McDonald diagnostic criteria for MS

<table>
<thead>
<tr>
<th>Clinical presentation</th>
<th>Additional Data needed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two or more attacks;</td>
<td>None</td>
</tr>
<tr>
<td>Objective clinical</td>
<td></td>
</tr>
<tr>
<td>evidence of two</td>
<td></td>
</tr>
<tr>
<td>different lesions</td>
<td></td>
</tr>
<tr>
<td>Two or more attacks;</td>
<td>Dissemination in space,</td>
</tr>
<tr>
<td>objective clinical</td>
<td>demonstrated by MRI or</td>
</tr>
<tr>
<td>evidence for one</td>
<td>wait for further</td>
</tr>
<tr>
<td>lesion</td>
<td>clinical attack</td>
</tr>
<tr>
<td>One attack with</td>
<td>Dissemination in time</td>
</tr>
<tr>
<td>objective evidence of</td>
<td>demonstrated by MRI or</td>
</tr>
<tr>
<td>at least two lesions</td>
<td>further clinical</td>
</tr>
<tr>
<td>One attack with</td>
<td>Dissemination in space</td>
</tr>
<tr>
<td>objective evidence of</td>
<td>and time by MRI or</td>
</tr>
<tr>
<td>one lesion</td>
<td>further clinical</td>
</tr>
<tr>
<td>Progressive neurological disability from onset</td>
<td>Progressive disease for more than and dissemination in space and time by MRI, or positive CSF</td>
</tr>
</tbody>
</table>

CNS – central nervous system; CSF – cerebrospinal fluid; MRI – magnetic resonance imaging

**Aetiology**

While the cause of MS is still unknown, it is believed that multifarious factors contribute to the onset and progression of the disease. The uneven geographical distribution could imply a role for genetic predisposition. Approximately 15-20% of MS patients have one or more affected relatives. Twin studies show a concordance rate of 30% between monozygotic twins, which drops to 5% in dizygotic twins, supporting the idea of genetic involvement. Genome wide association studies show strong associations with variations in the human leukocyte antigen (HLA) genes and the risk of MS, especially the HLA-DR*1501 allele. Approximately 65% of Caucasian MS patients carry the HLA-DRB1*1501 haplotype, compared to 30% of healthy controls. Remarkably, HLA-DRB1*1501 has a low frequency in Africa, where MS is almost absent. However, genetic studies do not explain the increased risk to develop MS among people who migrate from low-risk areas to high-risk areas or vice versa. These remarkable changes in MS risk after migration suggest an important contribution of environmental factors. Strongly associated with its geographical distribution, sunlight exposure seems to be unconquerably correlated with MS risk. Sunlight plays an important role in generating active vitamin D, which fulfils important functions in various organ systems, e.g. endocrine, skeletal and immune system. Vitamin D deficiency worsens the development of experimental autoimmune encephalomyelitis (EAE), an animal model of MS, while treatment with bioactive vitamin D suppresses clinical signs of EAE. Supporting the idea that vitamin D also plays a role in MS. Moreover, low serum levels of vitamin D in humans are strongly associated with an increased susceptibility to develop MS. However, the effect of vitamin D supplementation in MS patients is not known, and is currently being investigated in several clinical trials. Another plausible explanation for the epidemiological differences, is the so-called ‘hygiene hypothesis’ first put forward by Rook whereby the lack of early childhood exposure to infectious agents, due to a clean environment, results in excessive responses when challenging infections during young adulthood. For example, infection with Epstein-Barr virus (EBV) during childhood usually occurs without overt clinical symptoms. However, infection
during (young) adulthood often results in the disease infectious mononucleosis (IM; kissing disease). The relevance to MS is overviewed in a meta-analysis of 18 papers in which 19390 MS patients and 16007 controls were examined revealing that IM doubled the risk to develop MS in comparison to an asymptomatic EBV infection, suggesting a link between EBV infection and MS risk. Although EBV seropositivity is very common among the general population (90-95%), the majority of MS patients are seropositive while EBV seronegative individuals rarely develop MS. Moreover, the risk to develop MS increases linearly with the increase of virus specific antibodies in the serum. However, despite convincing epidemiological evidence for a role of EBV in the aetiology of MS, the precise contribution remains to be elucidated.

Disease mechanisms

The name multiple sclerosis originates from the many sclerotic plaques found in the CNS in response to the damage. These plaques represent an end stage of a damaging process involving inflammation, demyelination, oligodendrocyte damage and death, astrogliosis, blood-brain barrier (BBB) breakdown, axonal loss and neurodegeneration. The initial trigger and the connection between these different pathology elements is currently unknown. For many years MS was widely considered to have an autoimmune-mediated aetiology. The presence of T-lymphocytes in demyelinating lesions supported the hypothesis that autoreactive T-cells, triggered in the periphery, migrate across the BBB and cause, together with B-lymphocytes and macrophages, damage to myelin, oligodendrocytes and axons. While T- and B-cells are clearly involved in MS lesions, researchers over the past years have questioned whether an autoimmune response really initiates the disease. Although many candidate autoantigens have been proposed, so far the target of these autoimmune reactions in MS patients is not clear. Moreover, T-cell responses to myelin or neuronal proteins do not differ between MS patients and control subjects adding to the idea that induction of peripheral autoimmune T responses is not the trigger of the disease. In addition, autologous haematopoietic stem cell transplantation in patients with MS, with the aim of resetting the immune system, reduces relapses and inflammation but fails to stop neurodegenerative processes and disease progression. This has led to the idea that the driving force of MS may originate within the CNS and that inflammation is a secondary event. Advanced techniques in neuroimaging indicate that abnormalities in the CNS appear before the fulminant inflammation associated with active lesion formation. Axonal pathology, impaired myelin integrity, changes in oligodendrocytes, and microglia activation can already be observed in the normal appearing brain tissue of people with MS. However, the underlying mechanisms leading to these abnormalities are unclear. A variety of factors could induce these noticeable changes, including oxidative stress, mitochondrial deficits and ion channel dysfunction, but also pathogenic antibodies or viral infections.

In summary, while inflammation, myelin damage and neurodegeneration are the key pathological features of MS, the exact role of inflammation and the mechanism leading to such damage remain to be clarified.
Experimental models of multiple sclerosis

Experimental models are essential in MS research to gain more insight in fundamental disease mechanisms as well as testing the efficacy of new therapeutic approaches.

Single cell culture systems or co-cultures of different cell types allow investigation of cellular processes, responses and interactions. Preferably primary human neural cells are key to understanding human specific mechanisms and developing human specific therapies. Isolation protocols for primary human microglia and astrocytes are successful, although isolation material is sparse and the yield cells limited\textsuperscript{42-49-51}. Of particular relevance is that obtaining primary human neurons and oligodendrocytes is more challenging and often impossible. Primary cultures of rodent embryonic stages are able to provide all CNS cells, but still the yield is relatively low\textsuperscript{52-54}. For this reason various transformed cells and cell lines of neural cells are used (reviewed by van der Star et al.\textsuperscript{55}). Yet, care must be taken to ensure they preserve features associated with their cell type \textit{in vivo}.

A disadvantage of single cell culture systems is that the cellular behaviour observed may not fully reflect what happens in the context of intact tissue. An intimate contact between CNS cells can be maintained in co-culture systems of neural cells or in brain slice cultures, that represent even more the interactions within brain tissue\textsuperscript{56,57}.

Although \textit{in vitro} models provide information on cellular pathways and possible targets for treatment, \textit{in vivo} models are necessary to investigate the broader effect on other organ systems and, in the case of therapeutic compounds, efficacy and safety. Over the past several decades, various animal models have been developed to study the manifold aspects of MS\textsuperscript{55,58,59}. The most commonly used animal model in MS is experimental autoimmune encephalomyelitis (EAE) in which an autoimmune response to CNS antigens results in a spectrum of neuropathological features, including demyelination, inflammation and neurodegeneration\textsuperscript{55}. To induce EAE, laboratory animals are immunised with myelin, neural or glia antigens emulsified in an adjuvant that augments the immune response to the selected antigens\textsuperscript{55}. The clinical course of EAE varies from a monophasic acute course to a secondary progressive one, depending on the immunising antigen and immunisation protocol used as well as the species, age, gender and strain of the laboratory animals\textsuperscript{55,60}. Thus, it is a matter of selecting the most relevant model depending on the research question. A model that does not fit, will not provide the answers translatable to MS\textsuperscript{60}. Over a thousand treatments have been tested in EAE, but only a few have eventually been licensed for MS. This apparent low gain of usable therapies has resulted in a growing criticism of the EAE model\textsuperscript{61}. However when using a different study design, the outcomes could have been more promising\textsuperscript{60}. EAE provides a good platform to investigate (auto)immune related questions. Mechanisms like focal demyelination, remyelination and factors that can induce demyelination other than autoimmunity, however, are more appropriate to study in different models such as viral models or toxic models as explained below.

Viral infections of the CNS can induce demyelination in mammals. The best studied is the Theiler’s murine encephalomyelitis virus, a murine specific single stranded RNA virus belonging to the picornaviruses. The virus induces a transient meningo-encephalo-myelitis followed by a chronic neurological disease with
demyelination in susceptible mice. This virus-induced pathology has clear similarities to chronic progressive MS, however no persistent viral infection has been demonstrated in MS. Another way to induce demyelination is by administration of toxins such as the phospholipase A2 activator lysolecithin, and the copper chelator cuprizone, - the most extensively used toxins to induce demyelination. Although precise mechanisms of actions are unknown, injection of lysolecithin and feeding of cuprizone result in focal demyelination by damaging the myelin membrane and oligodendrocytes, respectively. While inflammation is not very prominent in these models, remyelination is evident in both. Thus these models are ideal to study the mechanisms involved in remyelination crucial for recovery in MS. Recently genetic approaches for selective oligodendrocyte apoptosis have been used to study de- and remyelination. The availability of transgenic, mutant and parabiotic experimental animals has helped the understanding of multifarious mechanisms important for MS, including inflammation, neurodegeneration as well as neuroprotection.

In conclusion, although MS is a unique human disease there are many experimental models, both in vivo and in vitro, that contribute to the notion of important pathways in MS and even more important that lead to the development of new treatments, however if used wisely (Table 2 summarises the models used in this thesis).

**Therapies**

Current therapeutic approaches in MS focus on disease modulation and management of symptoms, with the goal of amending or preserving function and maintaining the patient’s quality of life.

Since the 1950s corticosteroids, such as methylprednisolone, have been used in the treatment of acute exacerbations, that have an enormous impact on the physical and mental health of MS patients. Additionally, incomplete resolvement of the relapses is one of the main causes for early disability in the disease course. The use of short-term high-dose methylprednisolone regimes has been proven to be effective in decreasing the duration and severity of symptoms during a relapse. Yet, there is no evidence that they prevent new exacerbations, nor that such approaches have beneficial effects on long-term disability. Thus administration of corticosteroids has been restricted to relapse-management and for many years was the only therapeutic option for MS patients. However, in the mid-1990s the US Food and Drug Administration (FDA) approved interferon β (IFNβ) and glatiramer acetate (GA) for the treatment of MS. These two immunomodulatory medications were the first disease-modifying therapies (DMTs) used in MS and are still considered first-line treatment for RR-MS, due to their relatively benign safety profile. Despite their efficacy not all patients respond to these therapies, for example up to 49% IFNβ treated patients fail to respond after 2 years of treatment. As part of the body’s normal defence mechanism, the immune system responds effectively to foreign agents by producing antibodies that bind these agents. The production of these so-called neutralising antibodies is one of the main reasons, that long-term efficacy cannot be conserved in some MS regimens especially for IFNβ.
Table 2. Experimental models used in this thesis

<table>
<thead>
<tr>
<th>Model</th>
<th>Characteristics</th>
<th>Chapter</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell culture systems</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary human microglia</td>
<td>Human microglia isolated from fresh post mortem brain material</td>
<td>5,6,8,9</td>
</tr>
<tr>
<td>Monocyte derived macrophages</td>
<td>Immunomagnetically selected peripheral blood monocytes are differentiated into macrophages in vitro</td>
<td>5,8</td>
</tr>
<tr>
<td>Oligodendrocyte (MO3.13) cell line</td>
<td>A fusion of a human mutant rhabdomyosarcoma cell line with primary human oligodendrocytes</td>
<td>9</td>
</tr>
<tr>
<td><strong>Animal models</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C57BL/6 mice immunised with MOG35-55</td>
<td>Mice develop a chronic progressive form of EAE</td>
<td>6</td>
</tr>
<tr>
<td>Biozzi ABH mice immunised with SCH</td>
<td>A relapsing-remitting EAE form that becomes secondary progressive with disease duration</td>
<td>2,6</td>
</tr>
<tr>
<td>12 month old Biozzi ABH mice immunised with SCH</td>
<td>EAE is normally induced in young mice (6-8 weeks), immunising 12 month mice result in a more severe secondary progressive form of EAE (data described in chapter 2)</td>
<td>2</td>
</tr>
<tr>
<td>Rhesus monkeys (Macaca mulatta) immunised with MOG1-125 dissolved in CFA</td>
<td>A severe acute disease, most animals die within 48h after disease onset</td>
<td>7</td>
</tr>
<tr>
<td>Rhesus monkeys (Macaca mulatta) immunised with MOG1-125 dissolved in IFA</td>
<td>Acute disease, due to the lack of mycobacteria in IFA that are present in CFA, less side effects are observed and thus the model is thought to be more animal friendly</td>
<td>7</td>
</tr>
</tbody>
</table>

ABH, antibody high; CFA, complete Freund’s adjuvant; EAE, experimental autoimmune encephalomyelitis; IFA, incomplete Freund’s adjuvant; MOG, myelin oligodendrocyte glycoprotein; SCH, spinal cord homogenate * Immunomagnetic separation is a laboratory tool making use of paramagnetic beads coated with selective antibodies to separate cells magnetically

From 2000 on the choice of treatments has expanded to a total of 6 DMTs with new mechanisms of action that have been approved by the FDA\(^7\) (Table 3). The first to be approved was mitoxantrone, however due to its severe cardiac toxic side effects and the introduction of newer therapies, mitoxantrone is now only reserved for rapidly progressing patients failing other treatments\(^7\). In 2004 natalizumab was approved on the basis of two phase III clinical trials, showing a reduction of 68% of clinical relapses compared to placebo\(^22,71,73,74\). However, shortly afterwards this drug was withdrawn from the market as a consequence of various reports on the development of progressive multifocal leukoencephalopathy (PML) in natalizumab treated patients. Progressive multifocal leukoencephalopathy is a serious, often fatal, progressive white matter disorder of the brain, caused by a reactivation of the John Cunningham (JC) virus in immune deficient patients reduction of clinical relapses balanced the relatively low risk to develop PML\(^72,85\). However, the risk increases with duration of natalizumab treatment, seropositivity for JC virus antibodies and previous immunosuppressive therapies, hence a strict risk evaluation is necessary prior to and during treatment\(^71\). Such evaluation is particularly necessary as reflected by a recent case report describing a 70 year old
woman who developed PML two weeks after testing negative for JC serology\(^{86}\). Natalizumab was the first of several monoclonal antibody therapies to be investigated for MS treatment aimed at binding of different cells or proteins to regulate their function. In the case of MS, all monoclonal antibody therapies target the immune system to modulate the immune response seen in the CNS. Natalizumab prevents lymphocytes and monocytes to enter the brain by binding the protein VLA-4\(^{71,87}\). The recently FDA approved alemtuzumab depletes and thus allows repopulation of circulating lymphocytes by binding to CD52\(^{87}\). In addition, daclizumab targets CD25 on T-cells while ocrelizumab and oftamumab are both directed against the surface maker CD20 on B-cells and a small subset of T-cells\(^{88}\). Although the latter three are still under investigation in phase II and III studies, they show promising results\(^{22,71}\).

All of the above mentioned drugs are injectables, either subcutaneous, intramuscular or intravenous, which patients could experience as tedious and therefore negatively influencing compliance. Since 2010 three oral treatments have been approved by the FDA, fingolimod, teriflunomide and dimethyl fumarate\(^{89}\). Teriflunomide and dimethyl fumarate both have a relatively low side effect risk profile, although long-term safety data are not available yet. Nevertheless, they are suggested to be first line treatment next to IFNβ and GA\(^{87}\). In contrast, due to its potential cardiac effects fingolimod is only used as second line therapy\(^{87}\).

In conclusion, the introduction of DMTs have resulted in an expansion of treatment options for RR-MS patients. However, none of the available drugs are able to stop the progression of the disease and fully stop lesion development. Preliminary results of ocrelizumab in PP-MS show promising effects on the progression of clinical disability, however this study is still ongoing\(^{90}\). In addition, dimethyl fumarate has neuroprotective properties and shows a halt on disease progression in a pilot study\(^{31}\). Yet, limited data is available. Therefore therapies to stop progressive MS should be a major area of research interest and the focus might shift from immunomodulation to neuroprotection.

**Neuropathological features of multiple sclerosis**

The major challenge for MS therapy is to develop approaches that stop lesion formation and neurodegeneration - the underlying cause of the clinical relapses and progressive neurological deficit - and to promote remyelination and repair. Pathology studies of the CNS from MS patients have thrown light on how lesions may develop, thus providing novel clues how therapies might be initiated to modulate these pathogenic processes\(^{3,37,48,95–97}\). Much of the material used in these studies is derived during autopsy, however in rare cases biopsy material of rapidly expanding active MS lesions is available, taken when there is suspicion of a tumour. Although the majority of the tissue is obtained post-mortem after a long-standing disease, this does not mean that early changes in MS lesion formation are restricted to early stage MS disease. Newly forming lesions can be found throughout the whole course of the disease, even many years after the onset of disease during the secondary progressive phase\(^{97–99}\).
<table>
<thead>
<tr>
<th>Drug</th>
<th>Mechanisms of action</th>
<th>Adverse events</th>
<th>Efficacy - ARR</th>
<th>FDA approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon Beta INF-β 1a</td>
<td>Plays a role in several immunomodulatory pathways, including inhibition of T-cell activation and proliferation, down regulation of MHCII expression and stimulation of anti-inflammatory cytokines</td>
<td>Reactions injection site, flu-like symptoms, leukopenia, anaemia, liver-enzyme elevation and neutralizing antibodies directed to INF-β abolishing the efficacy of the drug</td>
<td>30% reduction versus placebo</td>
<td>1996, 2002</td>
</tr>
<tr>
<td>Avonex ®, Rebif®</td>
<td>Precise MOA is not completely understood. Plays a role in several immunomodulatory pathways, including inhibition of T-cell activation and proliferation, down regulation of MHCII expression and stimulation of anti-inflammatory cytokines</td>
<td>30% reduction versus placebo</td>
<td>1993, 2009</td>
<td></td>
</tr>
<tr>
<td>IFN-β 1b Betaferon® Extavia®</td>
<td>Plays a role in several immunomodulatory pathways, including inhibition of T-cell activation and proliferation, down regulation of MHCII expression and stimulation of anti-inflammatory cytokines</td>
<td>30% reduction versus placebo</td>
<td>1996, 2002</td>
<td></td>
</tr>
<tr>
<td>Glatiramer acetate Copaxone®</td>
<td>Exact MOA not known. GA is thought to compete with MHC binding of various myelin antigens for their presentation to T-cells and therefore limit the activation of myelin reactive T-cells. Additional GA leads to a immunoregulatory response pattern.</td>
<td>Reactions injection site and immediate systemic reaction post-injection</td>
<td>28% reduction versus placebo</td>
<td>1996</td>
</tr>
<tr>
<td>Mitoxantrone Novantrone®</td>
<td>Reduces B-cell, T-cell and macrophage proliferation, by interfering with DNA repair through inhibition of topoisomerase II.</td>
<td>Cardio toxicity, therapy-related acute leukaemia, nausea, vomiting, alopecia, infections, amenorrhea and infertility</td>
<td>65% reduction versus placebo</td>
<td>2004, reapproved since 2006</td>
</tr>
<tr>
<td>Nataluzimab Tysabri®</td>
<td>Humanized monoclonal antibody against VLA-4, hindering lymphocyte migration through the BBB and into the CNS</td>
<td>PML, infusion related hypersensitivity, infections and headache</td>
<td>68% reduction versus placebo</td>
<td>2004, reapproved since 2006</td>
</tr>
<tr>
<td>Fingolimod Gilenya®</td>
<td>Binds to S1P receptor In absence of signals from S1P receptors lymphocytes are unable to migrate from secondary lymphoid tissue, resulting in a decrease of these cells in the periphery. Additionally a neuroprotective role can be described by direct interaction with neural cells expressing S1P receptors.</td>
<td>Headache, nasopharyngitis, fatigue, infections, bradycardia, atioventricular conduction block, hypertension, macular oedema, lymphopenia and liver enzyme elevation</td>
<td>55% reduction versus placebo</td>
<td>2010</td>
</tr>
<tr>
<td>Teriflunomide Aubagio®</td>
<td>Activated lymphocytes depend on de novo pyrimidine synthesis. This is prevented by inhibition of dihydro-orotate dehydrogenase by teriflunomide. This results in suppression of immune-cell proliferation.</td>
<td>Gastrointestinal symptoms, alopecia, increased levels of liver enzymes, skin rashes, weight loss, infections and hypertension</td>
<td>31% reduction versus placebo</td>
<td>2012</td>
</tr>
<tr>
<td>Dimethyl fumarate</td>
<td>Active component is dimethyl fumarate. The activation of immune cells leads to the expression of S1P receptors.</td>
<td>Flushing, headaches, gastrointestinal</td>
<td>53% reduction versus placebo</td>
<td>2013</td>
</tr>
</tbody>
</table>
Tecfidera®: precise MOA is not known. Fumarate activates the nuclear factor E2-related factor 2 transcriptional pathway, which plays an important role in the oxidative stress response and immune homeostasis. It affects both neuronal and immune cells by changing their enzyme and cytokine expression.

Alemtuzumab (Campath®): Humanized mAb to CD52, which is expressed on the surface of all mature leukocytes. Binding to CD52 leads to a complement and cell mediated lysis, which causes a rapid depletion of the target cells.

Pathological examination of the MS brain and spinal cord displays macroscopically visible scar-like areas that represent end-stage lesions. Many other lesions are not visible to the naked eye and can be detected using an MRI guided protocol. Although MS lesions develop throughout the whole CNS, there is a preference for certain anatomical locations, such as the optic nerve, spinal cord, brainstem and the periventricular white matter areas. The location, size and number of lesions differ among patients, however no correlation between disease progression and lesion distribution has been observed. The formation of lesions is complex and differences in composition between the early and chronic phases of the disease have been described. Analysis of brain biopsy samples taken from rapidly expanding lesions from early MS cases has revealed a heterogeneous pattern of pathological characteristics. This has led to the suggestion by the authors that there are four different pathological mechanisms leading to demyelination. This classification could have important implications for the diagnosis and treatment of MS if the mechanisms underlying the damage vary between patients. In contrast, examination of lesions from patients with a long disease duration showed a homogenous pattern of the pathology. One explanation for this dichotomy is that the heterogeneity of early MS may represent the initial phase of the disease and in time the lesions could converge into a more uniform type. However, most of the acute MS biopsy samples are obtained from patients with an atypical, often rapidly progressive, clinical course in which a rapidly expanding lesion is observed. Thus whether the lesion heterogeneity is present in patients with a milder, more typical onset is unknown.
Neuropathological features of multiple sclerosis

The major challenge for MS therapy is to develop approaches that stop lesion formation and neurodegeneration - the underlying cause of the clinical relapses and progressive neurological deficit - and to promote remyelination and repair. Pathology studies of the CNS from MS patients have thrown light on how lesions may develop, thus providing novel clues how therapies might be initiated to modulate these pathogenic processes. Much of the material used in these studies is derived during autopsy, however in rare cases biopsy material of rapidly expanding active MS lesions is available, taken when there is suspicion of a tumour. Although the majority of the tissue is obtained post-mortem after a long-standing disease, this does not mean that early changes in MS lesion formation are restricted to early stage MS disease. Newly forming lesions can be found throughout the whole course of the disease, even many years after the onset of disease during the secondary progressive phase.

Pathological examination of the MS brain and spinal cord displays macroscopically visible scar-like areas that represent end-stage lesions. Many other lesions are not visible to the naked eye and can be detected using an MRI guided protocol. Although MS lesions develop throughout the whole CNS, there is a preference for certain anatomical locations, such as the optic nerve, spinal cord, brainstem and the periventricular white matter areas. The location, size and number of lesions differ among patients, however no correlation between disease progression and lesion distribution has been observed.

The formation of lesions is complex and differences in composition between the early and chronic phases of the disease have been described. Analysis of brain biopsy samples taken from rapidly expanding lesions from early MS cases has revealed a heterogeneous pattern of pathological characteristics. This has led to the suggestion by the authors that there are four different pathological mechanisms leading to demyelination. This classification could have important implications for the diagnosis and treatment of MS if the mechanisms underlying the damage vary between patients. In contrast, examination of lesions from patients with a long disease duration showed a homogenous pattern of the pathology. One explanation for this dichotomy is that the heterogeneity of early MS may represent the initial phase of the disease and in time the lesions could converge into a more uniform type. However, most of the acute MS biopsy samples are obtained from patients with an atypical, often rapidly progressive, clinical course in which a rapidly expanding lesion is observed. Thus whether the lesion heterogeneity is present in patients with a milder, more typical onset is unknown.

Grey matter lesions

A general approach to classify MS lesions is based on the degree of myelin damage and the presence of activated microglia/macrophages in the white matter (WM) and the anatomical location of the demyelinated lesion in the grey matter (GM). For a long time MS was considered to be a disease that mainly affects the WM, a concept that has been challenged in the last decade by advanced imaging and immunohistochemical techniques. Cortical lesions can already be found early in the disease course. Detailed studies of demyelinated areas in the cortex show that GM lesions substantially differ from WM lesions with regard to the degree of visible inflammation; there is no clear evidence of local blood-brain...
barrier disruption, minimal leukocyte infiltration and less microglia activation compared to WM lesions\textsuperscript{96,109}. On the basis of the anatomical location 3 grey matter lesion types have been identified\textsuperscript{96,105}. Lesions that extend over the border between white and grey matter are classified as leukocortical lesions, whereas lesions completely within the cortex are termed intracortical. Demyelination along the surface of the brain is classified as subpial, which is the most common grey matter lesion type (Figure 2). Some cortical lesions can extend to the full width of the cortex, whether these are widespread subpial lesions or an autonomous lesion type is under debate. Some research groups classify this as a fourth lesion type, although histologically it is difficult to distinguish them from the other types\textsuperscript{96,101,102}.

**White matter lesions**

White matter lesions in the brain are categorised according to the presence and distribution of myelin-laden macrophages, foamy macrophages, within the demyelinated area. Active lesions are characterised by a defined area of myelin damage, with relative axonal preservation, filled with foamy macrophages and profound perivascular infiltrates, composed of lymphocytes. Chronic active lesions have foamy macrophages in a rim around the demyelinated area. This suggests that the macrophages move outside the center of the lesion after clearance of the myelin. Inactive lesions are completely demyelinated areas, hypocellular, with hardly any inflammation. The few cells present are mostly hypertrophic astrocytes\textsuperscript{97,100,102,110,111} (Figure 3). In some cases remyelination occurs. Oligodendrocyte progenitors repopulate the damaged area and allow axons to be remyelinated, though the myelin sheaths are thinner than normal. During histopathological examination this is visible as a clearly demarcated region of pale myelin, referred to as shadow plaques\textsuperscript{101,102}. Significantly more shadow plaques are observed in early MS cases in relation to chronic disease\textsuperscript{112}.

**Preactive lesions**

Pathological, longitudinal positron emission tomography (PET) and MRI studies reveal that lesions continually form and regress during disease and disease activity. Yet, it remains unknown how MS lesions really start. The use of the MRI-guided sampling protocol during MS brain autopsies revealed lesion-like signals on the MRI-scan that were not traditional MS lesions upon
In regions without myelin damage, BBB dysfunction or leukocyte influx, clusters of activated microglia are visible\textsuperscript{97,113} (Figure 4). Thus in normal-appearing tissue innate immune cells in the CNS are already in a state of alertness. Sometimes foamy macrophages can be detected in these clusters, although rare, which could be the start of active demyelination\textsuperscript{37,114}. PET-scan techniques labelling TSPO, a mitochondrial protein that is present on activated microglia, revealed focal signals in normal-appearing regions several months before classical lesions were visible\textsuperscript{115}. This strongly suggests that the microglia clusters found are a distinct stage that could be the start of classical inflammatory lesions and therefore referred to as preactive lesions\textsuperscript{37}.

Preactive lesions are defined as round or oval clusters of activated microglia in an area that lacks detectable demyelination, hypertrophic astrocytes, gliosis and leukocyte infiltration. In addition, viral or bacterial antigens, neurofibrillary aggregates or insoluble protein inclusions should not be present\textsuperscript{37}. While the term preactive lesions is relatively new, the phenomenon of microglia cluster formation in normal-appearing regions in MS has been described by others, yet different terms to describe them were used. Already in the early nineties Sanders et al.\textsuperscript{111} identified ‘earliest lesions’ and classified them as type I lesions, Barnett et al.\textsuperscript{116} termed them “new forming lesions”, and Marik et al.\textsuperscript{117} called them pattern III lesions, while more recently they were referred to as microglia nodules\textsuperscript{118}.

A detailed pathological study of 21 MS brains revealed that preactive lesions were present in 67% of the cases\textsuperscript{113}. In this study there was no correlation between the occurrence of preactive lesions and gender, MS type or disease duration of the donor. Even in patients with long standing disease (>20 years) preactive lesions could be observed, indicating that their presence is not restricted to the early phase of the disease\textsuperscript{113}. Although the term preactive lesion implies that they would develop into full-blown active lesions, the relatively high numbers of preactive lesions compared to active lesions, suggests that the majority of preactive lesions resolve. This indicates a level of intrinsic regulation that is able to restore the abnormalities in this early stage. Moreover, failure of this repair mechanism has been suggested to underlie the transformation from the preactive into the damaging active lesions\textsuperscript{42}. Thus understanding the formation, progression and resolution of preactive lesions could provide novel clues how to stop lesion formation in MS.

**Microglia key players in preactive lesions**

A fundamental event in preactive lesion formation is the attraction and activation of microglia. Microglia are the innate immune cells of the CNS and represent 10-20% of all glia cells. Unlike oligodendrocytes and astrocytes, microglia have a mesodermal origin and during early embryogenesis move from the yolk sac into the CNS\textsuperscript{119,120}. Although their unique ramified morphology, under normal conditions, would not immediately associate microglia with macrophages, they fulfill similar functions as other tissue macrophages, including antigen presentation, cytokine production and phagocytosis\textsuperscript{121}. Microglia are very dynamic and continuously screen the environment for possible pathological conditions.
Figure 3. White matter MS lesions In normal brain tissue oligodendrocytes produce the myelin that enwraps the axons, microglia and astrocytes are in a resting state (A). Immunohistochemical studies that visualise the myelin protein proteolipid protein (PLP) show no abnormalities (B) and activated microglia (visualised by an anti-HLA-DR antibody) are sparse (C). In MS white matter lesions demyelination and inflammation are prominent features. In active lesions the demyelinated area is filled with activated microglia, macrophages and lymphocytes (D-F). Chronic active lesions are characterised by a hypocellular center with a rim of activated microglia and macrophages (G-I). This rim disappears in inactive lesions and an hypocellular area remains, the few cells present are mostly hypertrophic astrocytes forming a gliotic scar (J-L).

Figure 4. Pathological features of preactive lesions In an area where immunohistochemical studies reveal no clear deviations of the myelin (A, C; PLP stain) clusters of activated microglia are already detectable (B, D; HLA-DR stain). C and D represent a magnification of the outlined square in B. Pictures are adapted from van Horssen et al.
Upon triggering they take on an amoeboid morphology, like macrophages, and become functionally activated. In order to kill invading pathogens microglia produce various pro-inflammatory cytokines, chemokines and free radicals. These mediators may also induce bystander damage to the surrounding environment, which might contribute to axonal injury, neuronal and oligodendrocyte cell death and demyelination. Many studies, however, have also reported beneficial roles for microglia, including clearance of myelin debris and death cells, promoting remyelination, releasing neurotrophic factors and producing anti-inflammatory chemokines and cytokines. The functional duality of macrophages has led to a polarised view of macrophages as well as microglia: the damaging pro-inflammatory microglia phenotype is commonly referred to as M1 or ‘classically activated’ and the repairing anti-inflammatory as M2 or ‘alternatively-activated’.

As mentioned above, most preactive lesions are thought not to develop into destructive lesions. This strongly suggests that an internal regulation mechanism exists to manage the situation and avoid damage. Microglia in an alternative (M2) activation state may very well fulfil this role, by producing factors that can control the inflammation. Van Horssen et al. described that microglia in preactive lesions express the anti-inflammatory interleukin (IL) IL-10 and tumour necrosis factor-α (TNF). Although TNF is widely considered as a pro-inflammatory mediator, several lines of evidence indicate that is also exhibits immunosuppressive and cell survival properties. While Singh et al. reported the presence of the M2 marker CD163 on clustered microglia in MS, they also observed strong expression of CD40 and iNOS, two classical M1 markers. However the study by Singh et al. focused on microglia nodules in biopsy material taken from brain regions close to rapidly expanding lesions. A switch from the repairing (M2) microglia to pro-inflammatory (M1) microglia in preactive lesions could be the first event in the transition from preactive to active MS lesions. This would imply that microglia profiles in preactive lesions could predict whether a preactive lesion would resolve or progress into a full-blown damaging lesion. In this thesis we performed a detailed study to test this hypothesis.

### Possible triggers of microglia activation in preactive lesions

While many triggers of microglia have been proposed, it is not known which factors activate microglia in preactive MS lesions. The absence of signs that pertain to BBB disruption in the vicinity of preactive lesions suggests an intrinsic trigger within the CNS rather than a factor derived from the periphery. In the CNS damaged neurons produce a wide variety of factors that activate microglia, e.g. ATP, cytokines and stress proteins. The profile of factors produced by damaged neurons and indeed other CNS cells may occur as a consequence of infections, toxin exposure, protein misfolding and metabolic disturbances, which may affect all neural cell types.

Although astrocytes play a prominent role in the pathogenesis of MS, it is unlikely that astrocytes are major players in the development of preactive lesions. In general, reactive astrocytes are not observed close to preactive lesions. Although this does not mean that astrocytes are not involved indirectly, for example
by affecting other cells, it is more likely that they contribute to pathological processes at later stages.

Various studies indicate that neurons possess the capacity to control microglia activation, both via direct receptor-ligand interactions as well as soluble factors and electrical activity. Neuronal and axonal damage leads to dysregulation of these controlling mechanisms and in this way influences microglia by not providing the ‘off’ signals, resulting in an activated state. In addition, abnormally truncated, folded or aggregated proteins, a distinct feature of many neurodegenerative diseases, are a well-known trigger for microglia. However, axonal bulbs, a pathological sign of axonal damage, are not associated with preactive lesions in established MS. Nevertheless, this cannot exclude that subtle neuronal and axonal imbalances e.g. changes in ion homeostasis and mitochondrial dysfunction in regions distant from preactive lesions could influence microglia.

Current data point towards oligodendrocytes to have the leading role in preactive lesion formation. Oligodendrocytes, the myelin producing cells in the CNS, are extremely vulnerable to stress, notably oxidative stress, due to their high metabolic rate, high levels of intracellular iron and relatively low amounts of anti-oxidative enzymes. Gene expression studies on NAWM from MS patients revealed specific upregulation of genes that reflect cell survival mechanisms in oligodendrocytes, including activation of the HIF1α and STAT6 signalling pathways. In addition, oligodendrocytes in preactive lesions express the anti-apoptotic heat shock protein HSPB5. This indicates that oligodendrocytes are in an alerted state, raising the question whether the activated microglia induce oligodendrocyte stress or whether microglia are triggered by the stressed oligodendrocytes. To address this, the expression of HSPB5 by the oligodendrocytes is extremely interesting, since HSPB5 has been reported to activate microglia in vitro. Recombinant HSPB5 induced an immune-regulatory response in cultured human microglia. HSPB5 acts as a TLR2 agonist with CD14 as an essential co-receptor, this limits its signalling function to dendritic cells and microglia. Microglia in preactive lesions express the TLR2 and CD14 receptors, moreover they express various molecular markers that were identified in HSPB5 stimulated microglia by genome-wide transcript studies. This suggests that microglia activation in preactive lesions is, at least partly, driven by HSPB5 derived from stressed oligodendrocytes. The immune-regulatory phenotype after HSPB5 stimulation implicates that oligodendrocytes invoke microglia to help and restore tissue homeostasis. Whether stressed oligodendrocytes produce other factors that are involved in this process or what initially stresses the oligodendrocytes is, however, currently unknown.

**Thesis: aim, hypothesis and outline**

**Aim**

Although there are effective therapies to treat the relapsing-remitting phase of MS, none of these approaches are absolute i.e. lesions formation and neurodegeneration continues. Thus, an urgent need for new drug targets remains. Preactive lesions are considered to be the first, reversible, steps in MS lesion development. The aim of this thesis is to gain more insight in the mechanisms driving preactive lesion formation and to investigate why some preactive lesions
progress into active demyelinating lesions while others do not. Understanding the underlying pathways that lead to preactive lesion formation may provide novel clues for therapeutic approaches.

**Hypothesis**
The hypothesis tested in this thesis is that stressed oligodendrocytes, expressing HSPB5, activate microglia in an immune regulatory manner (M2), to restore tissue homeostasis.

In some lesions this repair mechanism fails leading to the formation of an active demyelinating lesion. In this scenario a second signal ‘switches’ anti-inflammatory (M2) microglia present in a preactive lesion to a pro-inflammatory (M1) state, that subsequently leads to the production of pro-inflammatory cytokines, the attraction of leukocytes and tissue damage as observed in active MS lesions (Figure 5)

**Outline**
So far the presence of preactive lesions has only been described in post-mortem material of human MS patients. At what particular disease stage preactive lesions develop and their relation to acute relapses and remissions of neurological disease, or indeed progressive neurological disease is unknown. For this reason we investigated the presence of preactive lesions in three different manifestations of EAE (relapsing-remitting EAE, secondary progressive EAE and progressive EAE), induced in differently aged mice from the same mouse strain (chapter 2).

The finding that the small heat shock protein (HSP) HSPB5 can activate microglia in vivo and that oligodendrocytes express this protein in preactive lesions, strongly suggests that stressed oligodendrocytes are key players in preactive lesion formation. However, whether stressed oligodendrocytes express any other members of the small HSP family is unknown. Since small HSPs respond differently to stress events, expression patterns may point to new ideas of what triggers oligodendrocyte stress in preactive lesions. In chapter 3 we examined four small HSP known to be expressed in the brain.

The central role of oligodendrocytes in preactive lesion formation is relatively surprising, since oligodendrocytes were originally thought not to be actively involved in immune responses. Only recently accumulating data suggest a more
prominent function for oligodendrocytes in the CNS innate immune system. In chapter 4 we reviewed evidence for the active role of oligodendrocytes in immuneresponses and the cross talk between oligodendrocytes and microglia. Knowledge of this cross talk may uncover novel pathways of immuneregulation in the brain.

As mentioned above, a ‘switch’ from an anti-inflammatory (M2) to a pro-inflammatory (M1) activation status of microglia within preactive lesions might be the key to the development into a full-blown active demyelinating lesion. This would suggest that transitional lesions would consist of pro-inflammatory (M1) microglia. In chapter 5 we performed a systematic study to determine the phenotype of microglia within preactive lesions.

Genome-wide micro-array-based transcript profiling of HSPB5-stimulated human primary microglia revealed that HSPB5 can induce an immunoregulatory phenotype, but not a classical anti-inflammatory (M2). Two pronounced upregulated transcripts were pentraxin 3 (PTX3) and interleukin 1 beta (IL-1β), which we studied in more detail in chapter 6 and 7 respectively.

Changes within the CNS microenvironment are crucially involved in the activation status of microglia. The molecular composition of this microenvironment can prime microglia, resulting in a magnified or re-programmed response. Priming of microglia with interferon-gamma (IFNγ) and lipopolysaccharide (LPS) as a secondary event has been studied extensively. In chapter 8 we examined the influence of IFNγ priming on HSPB5 stimulated microglia.

Finally, we conclude with chapter 9 where the results of the previous chapters are summarised and put into perspective, enabling us to give an update on the current knowledge of preactive MS lesions.

References
GENERAL INTRODUCTION

CHAPTER 1


GENERAL INTRODUCTION