Chapter 6

Summary, general discussion and future perspectives and conclusion
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Multiple sclerosis (MS) is generally considered to be an autoimmune disease targeting the human central nervous system (CNS). Adaptive immune responses towards myelin and/or neuro-axonal proteins are well-known phenomena occurring in MS patients. Indeed, current therapeutics targeting the immune system have been shown to be very effective in reducing relapse-rate and the formation of new inflammatory white matter (WM) lesions. Much of the current knowledge about the (auto)immune aspects of the disease was produced using a widely applied animal model for MS, autoimmune experimental encephalomyelitis (EAE). This model consists of animals (mainly rodents) that are immunised with brain homogenates, myelin or neuro-axonal proteins, and mimic some of the pathological aspects found in MS autopsy specimens. Several currently used effective MS therapeutics, like glatiramer acetate, mitoxantrone and natalizumab, have been developed based on initial, crucial EAE experiment. However, in the best case, current MS treatment slows down disease progression. None of the farmaca, however, have proven to be able to halt or reverse disease progression. Altogether, MS animal models (especially EAE) have been proven to be a powerful tool in MS research, but more detailed knowledge about MS disease progression is hard to infer from MS animal models. Observations regarding disease progression rely almost completely on in vivo magnetic resonance imaging (MRI) and post-mortem studies (but then of course in a cross-sectional way). An increasing number of studies have shown that grey matter (GM) involvement in the disease differs from WM involvement. For example, in the chronic phase of MS, WM pathology remains more or less stable in terms of its extent, whereas GM pathology becomes much more prominent and even accelerates. What causes this phase shift between GM and WM damage with progressing disease is not clear, but it has been shown that the pathology of GM and WM damage is very different in MS. The aim of this thesis was to delve into this matter and gain more insight into GM pathologic changes in MS. What are they composed of? How exactly do they differ from WM pathology? What is their origin? Can they be imaged by available MRI techniques? And, importantly, what is their clinical relevance? These questions and the research performed in an attempt to find the answers, is divided under the following sections in this thesis: pathology, pathogenesis, imaging, and clinical relevance.

6.1 Summary

Chapter 2.1 – Cortical demyelination, especially subpial lesions (type III) can be very extensive in MS. They often extend over multiple adjacent gyri and it has been shown that they become more prominent in disease progression. Interestingly, cortical lesions (CLs) do not contain significant numbers of lymphocytes, they lack blood-brain barrier disruption and do not show signs of complement activation, all features that are common in WM lesions. Furthermore, it has been shown that there is no correlation between the extent of cortical demyelination and WM pathologic changes in chronic MS, suggesting that cortical demyelination is a process that to a large extent may be independent of ongoing WM pathology. Because of their topographical distribution at the surface of the cerebral cortex, it has been proposed that subpial CLs might result from diffusion of myelinotoxic substances derived from leptomeningeal inflammatory infiltrates. We therefore characterised meningeal inflammation in a large sample of chronic MS autopsy specimens and investigated possible global and regional correlations between meningeal inflammation and subpial cortical demyelination. More specifically,
we quantified leptomeningeal infiltrates of T-cells, B-cells, macrophages, dendritic cells, T-helper cells, (activated) cytotoxic T-cells and plasma cells and related these to global and regional subpial cortical demyelination. Significant leukocyte infiltration was found in the meninges of MS patients compared to non-neurological controls. However, the extent of subpial cortical demyelination did not correlate with the presence or extent of meningeal inflammation. Furthermore, no differences were found regarding leukocyte infiltrates in leptomeningeal tissue overlying subpial CLs compared to those adjacent to normal-appearing cortex.

Chapter 2.2 – Although myelin debris has been observed within MS lesions, in cerebrospinal fluid (CSF) and in cervical lymph nodes of MS patients, the route of myelin debris transport out of the brain after demyelination has been largely unclear. We observed myelin debris in the leptomeninges and perivascular spaces of MS patients, and investigated whether this myelin is largely extracellular or whether it was located mainly within macrophages or dendritic cells. By using specific immunohistochemical staining methods for the detection of various myelin proteins, including proteolipid protein, myelin basic protein, myelin oligodendrocyte glycoprotein and 2',3'-cyclic nucleotide 3'-phosphodiesterase we observed a high amount of mainly extracellular myelin in the meninges of MS patients. This finding was highly MS-specific, as the extracellular myelin in the meninges and perivascular spaces was not observed in various other neurological disease nor in non-neurological controls. As the myelin debris observed in our study was immunopositive for virtually all myelin proteins, we suggested that these particles might not have been taken up and degraded by macrophages in our chronic MS material. Our findings may change the concept that demyelination irrevocably leads to the uptake and degradation of the myelin by phagocytes in chronic MS. Based on our findings, we postulate that the meninges and perivascular spaces contribute to the drainage route of (damaged) myelin out of the brain in MS patients and that this detached myelin is largely ignored by the immune system in chronic MS patients.

Chapter 2.3 – The pathogenic mechanisms underlying subpial cortical demyelination are not known. CLs in the post-mortem setting are largely non-inflammatory, although in a subset of the lesions activated microglia can be found at the edges of the lesions. To assess the clinical significance of CLs with and without rims of activated microglia and to assess possible associations with other pathological features we investigated clinical and pathological features of 41 MS patients. 22 MS patients were selected for the presence of extensive subpial cortical demyelination (termed ‘CL group’) and 19 MS patients with only little demyelination of the cerebral cortex were also selected (termed ‘non-CL group’). In a subset of the CL group (12 patients) a proportion of the CLs harboured rims of activated microglia at the edges of the lesions (termed ‘RAM-CL group’). In the rest of the patients in the CL group no activated microglia were found at all in the CLs (termed non-RAM CL group). Interestingly, MS patients harbouring RAM CLs were significantly younger at the time of their death compared to patients mainly harbouring nonRAM CLs or compared to patients without significant cortical demyelination. Furthermore, there was a significant positive correlation between the presence of RAM CLs and the number of chronic active WM lesions. Therefore, our data indicate that MS patients with RAM CLs have more active WM inflammation and experience a less favourable disease course.

Chapter 3.1 – Besides physical impairment, 40-65% of the MS patients also experience various degrees of cognitive deterioration. Processing speed and visuospatial memory
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are the most frequently reported to be abnormal in MS. Recent studies have shown that the hippocampus, a brain structure of critical importance for proper memory function, is severely affected in MS. As the cholinergic neurotransmitter system plays an essential role in learning and memory function, and the hippocampus is a major region of cholinergic input from the basal forebrain, we investigated different components of the cholinergic neurotransmitter system in the MS hippocampus. In MS hippocampus, activity and protein expression of choline acetyltransferase (ChAT), the acetylcholine synthesizing enzyme, was decreased, while the activity and protein expression of acetylcholinesterase (AChE), the acetylcholine degrading enzyme, was found to be unaltered. In contrast, in Alzheimer's disease (AD) hippocampus, both ChAT and AChE enzyme activity and protein expression was decreased. Our findings therefore revealed an MS-specific cholinergic imbalance in the hippocampus, which might be useful information in terms of future treatment development for memory problems in this disease and pharmaceutical dose evaluation, as was illustrated by the next chapter.

Chapter 3.2 – A recently conducted multi-center clinical trial by Krupp et al. using the cholinesterase inhibitor donepezil (10 mg/day) indicated no cognitive improvement when compared to placebo treated MS patients. In light of our results (see chapter 3.1), where we found decreased activity and expression of ChAT, but unaltered activity and expression levels of AChE, we suggested that a higher dose of donepezil might be warranted in order to restore the cholinergic imbalance and therewith possibly improve cognitive functioning in cognitively affected MS patients.

Chapter 4.1 – Gene expression analysis on CLs, normal-appearing cortex and control cortex was performed using microarray-based technology. In MS cortical sections (i.e. CLs as well as normal-appearing cortex) a striking upregulation was found of immunoglobulin-related genes. As it has previously been suggested that the oligoclonal bands in the CSF of MS patients may be the result of Epstein-Barr virus (EBV) infection we used highly sensitive quantitative polymerase chain reaction (qPCR) and immunohistochemical staining techniques in order to detect possible EBV transcripts and proteins respectively. However, no evidence for latent or lytic EBV infection in any of the investigated samples was found.

Chapter 4.2 – Micro-RNAs (miRNAs), small non-coding and single stranded RNAs, modulate post-transcriptionally the expression of genes. As miRNAs have been implicated in several neurodegenerative diseases and immune responses, we investigated by using microarray-based technology the global expression pattern of the currently known miRNAs and in parallel their predicted messenger RNA (mRNA) targets in subpial CLs and chronic active WM lesions. Compared to control tissue, 41 miRNAs were differentially expressed in chronic active WM lesions and 17 miRNAs in CLs. 6 miRNAs were differentially regulated in both chronic active WM lesions and subpial CLs. Additionally, 2222 mRNAs were differentially expressed in chronic active WMLs and 959 mRNAs in CLs. Differentially expressed miRNAs were selected for their predicted differentially expressed mRNA target using the algorithm from TargetScan Human. These pathway and network analyses revealed yet undiscovered miRNA-mRNA pathways which may have a significant role in lesion pathogenesis. Among these are pathways involved in myelin formation, neurite growth, mitochondrial functioning, mTOR pathway and inflammation. Subsequently, the expression of miRNA-219, the most strikingly downregulated miRNA in chronic active WM lesions (7.93x) as well as in CLs (4.80x) was validated by means of in situ hybridisation.
Interestingly, miRNA-219 was formerly implicated to be essential in myelination.34-36 Our data indicate widespread alterations in miRNA expression levels in chronic active WM lesions and subpial CLs and may pave the way for further innovative research regarding the identification of pathways involved in WM and CL pathogenesis.

**Chapter 5.1** – CLs occur frequently in MS, but are poorly visible on conventional MRI. The reason why some CLs are visible and others are not is currently unknown. Therefore, in this study we investigated whether CLs that are visible on conventional MRI differ from MRI-invisible CLs in terms of underlying histopathology and quantitative MRI (qMRI) measures. Here, we did not find differences between visible and invisible CLs in terms of histopathology or qMRI measures. However, MRI visible CLs were significantly larger when compared to their invisible counterparts. Furthermore, MRI visible CLs are associated with a higher total CL load suggesting that when CLs become visible on MRI, they merely represent the ‘tip of the pathological iceberg’.

**Chapter 5.2** – With the introduction of the 3D double inversion recovery (3D DIR) MR sequence a substantial increase of MRI detected CLs in MS patients was found when compared to more conventional MR sequences. Therefore, 3D DIR became a widely used MR sequence for the detection of CLs in the MS research setting. Multinational scoring criteria were developed to facilitate the evaluation of DIR images, but sensitivity and (pathological) specificity of the technique and the scoring criteria were not yet formally assessed by comparison to the gold-standard of histopathology. Therefore, we conducted a direct histopathology-to-MRI comparison for the 3D DIR sequence. Although we found that the sensitivity of 3D DIR is higher when compared to 3D fluid-attenuated inversion recovery (1.6-fold improvement), the overall sensitivity for detecting CLs still remains relatively low (18%). Especially subpial CLs were difficult to detect with 3D DIR. However, the 3D DIR sequence was found to be highly pathologically specific (90% of hyperintensities actually proved to be demyelinated CLs), indicating that (as an editorial accompanying our paper put it) the CLs that are picked up by 3D DIR are ‘few [in number] but true’.

**6.2 General discussion and future perspectives**

*Subpial cortical demyelination in MS*

Extensive supial cortical demyelination is a characteristic pathological hallmark in a subset of primary and secondary progressive MS patients.16,24 Based on their location at the surface of the cerebral cortex it has been repeatedly suggested that subpial cortical demyelination is a result of diffusion of myelinotoxic factors that are produced as a result of inflammation in the meninges.15,24 Furthermore, tertiary ectopic lymphoid-like structures in the meninges have been described in MS patients and were proposed to be linked to cortical demyelination and even neuronal loss.24,37-41 Intriguingly, the B-cells in these ectopic lymphoid-like structures were suggested to be infected (by 100%!) with EBV, suggesting that the persistence of EBV plays an important role in MS immunopathology.30

In chapter 2.1 we described the presence of significant meningeal inflammation in MS patients.52 The predominance of T-cells we found in the meninges of MS patients was also reported by others.37,43 Despite a reported frequency of tertiary lymphoid follicles in the meninges of secondary progressive MS cases of ~40%, no lymphoid-like structures were found in our material. Remarkably, up until now, lymphoid-like structures have never been observed in our elaborate and well-characterised MS autopsy material.44,45 Hence,
an association between the occurrence of lymphoid-like structures and (the presence or extent of) subpial cortical demyelination is absent in our material. How to explain these discrepancies between our study\textsuperscript{42} and the findings reported by others? Several possible technical and/or methodological issues have been proposed.\textsuperscript{46} Suboptimal preservation of the meninges during tissue processing, rough cutting and/or handling of the tissue sections during cutting, as well as insufficient sampling have all been proposed to lead to a potential bias in our study.\textsuperscript{46} Despite extensive sampling of MS tissue samples for many years,\textsuperscript{47} including whole brain coronal sections, we indeed cannot completely rule out that we have missed lymphoid-like structures in the meninges of our MS autopsy specimens. In our opinion, however, this explanation seems to be unlikely, since the prevalence of tertiary lymphoid follicles was reported to be \textasciitilde40\% in secondary progressive MS patients and should therefore have been found in our elaborate MS sample. Another possibility is that the research from our international colleagues was based on MS material from a subpopulation which shows a more active inflammatory disease progression. Another factor which strengthens our findings is that we have investigated significant numbers of MS autopsy cases, including those with aggressive disease courses and even in those patients no lymphoid-like structures were ever observed. The presence of tertiary lymphoid-like follicles were reported to be linked to more severe cortical demyelination in these patients,\textsuperscript{39} however, a recent report by the same group revealed that the number of tertiary ectopic lymphoid follicles was not associated with the extent of cortical demyelination\textsuperscript{38} and that meningeal inflammation was only modestly linked to cortical demyelination in the forebrain.\textsuperscript{38} Furthermore, in line with our results, regional associations between meningeal inflammation and cortical demyelination were never reported. Therefore, if meningeal leukocyte infiltrates are indeed responsible for the induction of cortical demyelination in MS, one may expect such an association. Interestingly, it has been shown that extensive subpial cortical demyelination is also a pathologic hallmark in primary progressive MS patients (see chapter 2.3 and Bø et al.,\textsuperscript{16} Bø et al.,\textsuperscript{15} Peterson et al.\textsuperscript{19} and Kutzelnigg et al.\textsuperscript{24}), however these patients were never reported to harbour follicle-like structures in their meninges.\textsuperscript{30,38-41,48} 

A recent biopsy study based on material from early MS patients with tumefactive lesions, also indicated significant meningeal inflammation in these patients.\textsuperscript{49} Although, as proposed by the researchers, meningeal inflammation may have induced the frequently observed (active) cortical demyelination (which was mainly seen in mixed GM-WM lesions), it could also very well be that the meningeal inflammation found in this study reflects higher overall inflammatory activity in these patients and is therefore a relatively patient-bound phenomenon. Furthermore, if meningeal inflammation is primarily responsible for the induction of (extensive) subpial demyelination\textsuperscript{30,37-41,49} one would expect subpial cortical demyelination to be a more prominent feature in relapsing-remitting and acute forms of MS, but this does not seems to be the case based on autopsy studies.\textsuperscript{24,50} Furthermore, a critical point could be raised about the ‘type’ of subpial lesions selected in the previously mentioned studies. Although subpial CLs are often studied, these studies may possibly be biased by investigating the more inflammatory type I lesions (mixed GM-WM lesions) that reach the cortex’ surface with the most striking example found in the study perfomed by Dal Bianco et al.\textsuperscript{37} So, although meningeal inflammation is evident in MS, the remaining question is still whether the presence of meningeal inflammation and subpial cortical demyelination ‘per se’ are causally related or whether they are just
guilty by association? Therefore, subpial cortical demyelination may be the result of yet unknown mechanisms and the T-cell mediated meningeal inflammation as found in our study may instead reflect overall WM disease activity.

Interestingly, and this is related to chapter 2.2 and chapter 2.3, the mechanisms underlying (pure; i.e., type III) subpial cortical demyelination may indeed differ from what has been previously proposed. In chapter 2.2 we described the abundant presence of extracellular myelin particles in the meninges and perivascular spaces of (exclusively) MS patients. Remarkably, in contrast to what we e.g. observed in stroke patients, intracellular myelin particles were observed on only very rare occasions in the MS meninges. Isotype specific control stainings were carefully and repeatedly performed and found to be completely negative. Luxol-fast blue positive particles with typical morphology of myelin particles were also abundantly observed in the MS meninges, which together with the negative findings in non-neurological controls, makes our observation of myelin particles in the meninges of MS patients highly unlikely to be biased by technological and/or methodological errors. Taking the current scientific knowledge about the pathophysiology of MS into account, it is difficult to elaborate about an explanation for the abundant presence of extracellular, apparently non-immunogenic myelin in the MS meninges. In my opinion this observation can be explained by two different things or by a combination of both. First, it may be that the myelin in MS brains is (biochemically) altered such that the immune system (c.q., microglia and macrophages) is not able to efficiently clear the myelin from the CNS. Subsequently, the myelin debris will drain via the interstitial fluid and the perivascular spaces into the subarachnoid space/CSF. Interestingly, several biochemical alterations of the myelin have been described in the WM and GM of MS patients and during demyelination, including protein carbonylation, oxidation and citrullination.51-60 It could be that the myelin is altered from the beginning of the disease or with disease progression. Therefore, since our findings were primarily based on material from chronic MS patients, it would be very interesting to see whether extracellular myelin particles can also be found in the beginning of the disease (i.e. early relapsing-remitting MS) or in acute MS cases. Although, abundant myelin scavenging macrophages have been clearly reported in acute WM MS lesions in relapsing-remitting MS and acute MS cases, making the finding of extracellular myelin perhaps less likely.61,62 Second, it may be that not the myelin but the immune system is altered over time, leading to a reduced clearance of (damaged) myelin from the MS brain. As the inflammatory character of the disease is declining during MS disease progression, it would be very interesting to see whether changes occur in the immune system of MS patients with prolonged disease duration. Interestingly, the role of immune senescence has for example been proposed in AD.63 Senescent microglia seem unable to digest beta-amyloid plaques and neurofibrillary tangles in AD.63 This was recently confirmed in vitro.64 Therefore, it has been proposed that in AD, neurodegeneration occurs secondarily to microglial senescence. It would be interesting to see whether microglia senescence also a plays a role in neurodegeneration in the MS cerebral cortex. For example, by investigating the clinical and immunological responses after EAE induction in ‘aged’ rodents or to investigate possible biochemical and epigenetic changes in the immune system of progressive MS patients compared to recently diagnosed MS patients or more ideally in follow-up studies.

Interestingly, it has been shown that activated microglia (in the form of nodules) fail to phagocytose degenerating myelin at the edges of (slowly expanding) chronic active WM
MS lesions in secondary progressive MS patients. This degenerating myelin occurs in the presence of deposits of C3d, an opsonin formed during complement activation (via the alternative complement cascade). These microglia/C3d/myelin profiles were not observed in newly forming MS lesions of acute MS cases or other neurodegenerative diseases, indicating a highly MS-specific phenomenon. In relation to this, it has recently been shown that (serum) complement factor H, a glycoprotein that regulates the formation and function of complement C3 and C5 convertase enzymes, is a highly sensitive and specific biomarker for distinguishing between relapsing-remitting and primary and secondary progressive MS. Serum factor H levels were significantly increased in progressive MS, possibly highlighting the role of the innate immune system in the progressive disease phase. In relation to this, recent exciting data indicated that the priming of microglia depend on (C3) alternative complement cascade activation and that (alternative) complement cascade activation together with microglia priming occur in perilesional areas of MS patients. In our preliminary work we confirmed that at the edges of these chronic active WM lesions, there is indeed myelin disruption without the massive uptake of these myelin destruction products by activated microglia (Fig. 1). Furthermore, microglia/C3d/myelin profiles could also be observed in some rims of activated microglia (RAM) CLs (Fig. 2). Remarkably, no immunoglobulin deposition was found in these areas (data not shown), possibly suggesting an antibody-independent way of complement activation. Interestingly, in chapter 2.3 a highly significant positive correlation was found between the presence of chronic active WM lesions and the presence of RAM CLs (Spearman ρ = 0.74; P < 0.0001), indicating a possible common pathophysiological pathway. Whether axonal damage or myelin disruption is the first event in the activation of the complement cascade remains to be elucidated and warrants further investigation. In AD, beta-amyloid plaques are also associated with activated microglia and C3d deposition, also without signs of phagocytosis or IgG deposition. Therefore, future studies will have to prove, preferentially by means of electron microscopy, whether the activated microglia at the edges of the RAM CLs indeed lack myelin degradation products or not and thus may play a pivotal role in the formation of subpial cortical demyelination or not. This may give insight whether CLs are more likely to be the result of an adaptive immune response or is the result of an innate immune response. Or perhaps of both. If the activated microglia in these lesions do not participate in the demyelinating process, studies should focus on alternative functions of these cells, e.g. possible neuroprotective functions.

Hippocampal pathology and cognitive problems in MS

In chapter 3.1 we observed a reduction in the activity and protein expression of the acetylcholine synthesizing enzyme, ChAT, but an unaltered activity and expression of AChE in the MS hippocampus. This cholinergic imbalance was fundamentally different from what we found in the AD hippocampus where both enzymes were clearly down-regulated. Our findings may give a rational explanation why a clinical trial with a standard dose of the AChE inhibitor donepezil failed to restore memory problems in MS patients (see chapter 3.2). Furthermore, we and others also proposed to treat more severely cognitive impaired MS patients. In AD, AChE inhibitors have been shown to only have a modest therapeutic effect. Therefore, as proposed in AD, it may be worthwhile to select possible responders and non-responders to AChE inhibitors. For example, by selecting cognitive impaired MS patients based on damage in cholinergic neurotransmit-
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Figure 1. Complement deposition and microglia activation in proximity to degenerating myelin sheaths at the border of chronic active white matter lesions. (A-C) Immunofluorescent double labelling of anti-proteolipid protein (PLP; myelin) (green) with anti-C3d immunostaining (complement component; red). (A) Anti-PLP immunostaining revealed the presence of a degenerating myelin sheath at the border of a chronic active WM lesion (arrow). (B) Anti-C3d immunostaining revealed the presence of complement deposition at the border of chronic active WM lesions (arrows). (C) Merged image revealed that the complement was deposited on the degenerating myelin sheaths (arrows) and not on intact myelin sheaths (arrowheads). (D-F) Immunofluorescent double labelling of anti-PLP (myelin) (green) with the microglia marker anti-CD45 (red). (D) Anti-PLP immunostaining revealed a degenerating myelin sheath at the border of a chronic active WM lesion (arrow). (E) Merged image revealed that the complement was deposited on the degenerating myelin sheaths (arrows) and not on intact myelin sheaths (arrowheads). (F) Merged image revealed that activated microglia surround degenerating myelin sheath without the massive uptake of the myelin.
ter pathways as visualised with (high field) MRI. As cholinergic neurons from the basal forebrain also project to other cortical and deep grey matter areas it would be interesting to see whether these areas are also affected in MS brains.

Our data indicated that there is also a cholinergic deficit in non-demyelinated MS hippocampi (although that specific sample size was small (n = 3)), therefore future studies should reveal whether the cholinergic imbalance in MS hippocampi might also occur completely lesion-independently. We hypothesised that the loss of cholinergic input may be the result of (active) lesions in the fornix. Interestingly, lesion-independent pathological changes in the MS cortex have already been described by several other research groups. For example, in non-lesioned MS cortex dysfunctional mitochondria, a loss of GABA-ergic interneurons, activated neuroprotective pathways and reduced expression of several mitochondrial antioxidants (Witte et al. 2012) have been observed. Although several pathologic changes in GM MS lesions have been described, including synaptic loss, neuronal loss and glial cell loss, evidence is accumulating that GM pathologic changes may also occur lesion-independently.

**Gene expression analyses on MS lesions**

In chapter 4.1 we conducted microarray experiments on cortical sections from normal-appearing cortex, CLs and control cortex in order to gain more insight into the
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pathophysiological mechanisms underlying grey matter demyelination. No drastic changes were found between gene expression levels in CLs and normal-appearing cortex. As lymphocytes are hardly found in the MS cortex it was surprising to detect an upregulation of several immunoglobulin-related genes in MS cortical sections. Getting back to the tissue revealed contamination of the cortical sections with meningeal tissue as well as the presence of B-cells and plasma cells. In chapter 4.2, where also microarray experiments were conducted, we carefully checked for the contamination with meningeal tissue in order to avoid potential bias. As the meninges were previously proposed to be a niche for EBV infection in MS,30,48 we were able in chapter 4.1 to check for possible EBV infection in the MS meninges with highly sensitive techniques (i.e. qPCR). However, no signs of latent or lytic EBV infection were found by us and later also not by other researchers,44,96,97 making the role of EBV infections in the brains of MS patients a highly controversial topic.98

Then, in chapter 4.2 we determined for the first time differences in miRNA and mRNA expression profiles by means of microarray technology in chronic active WM lesions and subpial CLs. Pathway and network analyses revealed several interesting potential miRNA-mRNA pathways for future research. One of the most strikingly down-regulated miRNAs was miRNA-219, which has recently been shown to be essential for myelination.34-36 Oligodendrocyte precursor cell (OPC) proliferation is stimulated by the growth factor platelet-derived growth factor alpha (PDGFα). Recently, Dugas et al.34 and Zhao et al.36 clearly showed that the differentiation of OPCs into mature myelinating oligodendrocytes is regulated by upregulation of miRNA-219, leading to a repression of the PDGF receptor α gene in OPCs and upregulation of myelin genes. Interestingly, we found a high number of OPCs in CLs (Strijbis et al., in preparation). Although speculative, this may be linked to the down-regulation of miRNA-219. These high numbers of OPCs in CLs may explain the relative high remyelination degree of CLs.99 Why still the majority of CLs, despite extensive numbers of OPCs, fail to remyelinate, remains elusive for now, but ongoing oxidative damage/stress in CLs seem to be an interesting explanatory factor for the down-regulation of miRNA-219 and therewith halting the maturation of OPCs in fully mature myelinating oligodendrocytes.52,100 Future work is warranted to investigate whether miRNAs will be useful as potential therapeutic targets.

Imaging cortical lesions

Unfortunately, the majority of the CLs are missed with conventional MRI.101 In chapter 5.1 we found that the visibility of CLs mainly depends on lesion size and that visible CLs were associated with a higher overall CL lesion load. However, we cannot completely rule out that other histological parameters (e.g. synapse or dendritic loss) than investigated here contribute to the visibility of CLs on MRI. In chapter 5.2 we assessed the sensitivity and specificity of the relatively novel imaging technique 3D DIR. This sequence, suppressing both WM and CSF signals, was found to be highly pathologically specific but has a rather poor sensitivity for the detection of subpial CLs, although the sensitivity was still 1.6 fold higher than 3D FLAIR. Our current validation of the 3D DIR sequence for CL detection illustrates the need for a standard acquisition protocol, which will be crucial to improve inter-rater consistency and may facilitate the migration of the sequence to a more standard clinical and in research setting. Furthermore, the use of (ultra) high field MRI for the detection of CLs looks promising93,102,103 (see also Fig. 3) and should be further
elaborated in future studies in order to acquire more knowledge about the dynamics of CLs in time, and their exact clinical significance. To achieve this, the usefulness of high field MRI techniques for detecting CLs should always be validated with the gold-standard of histopathology.

**Concluding remarks**

The work described in this thesis underscores that GM pathology in MS is an important clinico-pathological feature and provides increased knowledge about:

- CL pathology.
- An unexpected insight that myelin destruction in MS may take place without (massive) phagocytosis in chronic MS and that myelin is located in perivascular spaces and leptomeninges.
- The clinical importance of activated microglia in subpial CLs and their possible interrelationship with chronic active WM lesions.
- The cholinergic neurotransmitter system in the MS hippocampus and offers a rational basis for the treatment of cognitive problems in at least a subset of the MS patients with AChE inhibitors.
- EBV infection which may after all not be a characteristic feature in MS brains and/or does not have a possible role in the immunopathogenesis underlying (subpial) cortical demyelination.
- Gene and miRNA expression levels in subpial CLs and chronic active WM lesions which may provide novel potential targets to combat MS disease progression.
- Detectable CLs on conventional MR images merely represent the pathological ‘tip of the iceberg’.
- The pathological sensitivity and specificity of the 3D DIR sequence for detecting CLs.

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**Figure 3.** High field (9.4 T) magnetic resonance imaging (MRI) on post-mortem multiple sclerosis (A) tissue revealed a subpial cortical lesion (CL) on a high resolution (B) T2-weighted image. (C) Tissue section immunohistochemically stained for myelin basic protein (MBP), indicating a large subpial CL. Reproduced from Schmierer et al., with permission from Oxford University Press.
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