# Chapter 6

## Summary, general discussion and future perspectives and conclusion

Multiple sclerosis (MS) is generally considered to be an autoimmune disease targeting the human central nervous system (CNS). Adaptive immune responses towards myelin[1-4] and/or neuro-axonal proteins[5-8] 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.[9-11] 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.[12,13] Altogether, MS animal models (especially EAE) have been proven to be a powerful tool in MS research,[14] 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.[15-20] 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[21,22]. 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.[16,19,23,24] They often extend over multiple adjacent gyri and it has been shown that they become more prominent in disease progression.[24] Interestingly, cortical lesions (CLs) do not contain significant numbers of lymphocytes,[15] they lack blood-brain barrier disruption[20] and do not show signs of complement activation,[18] 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,[17] 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,[15,19] although in a subset of the lesions activated microglia can be found at the edges of the lesions.[19] 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.[25] Processing speed and visuospatial memory

are the most frequently reported to be abnormal in MS.[26] Recent studies have shown that the hippocampus, a brain structure of critical importance for proper memory function, is severely affected in MS.[27,28] 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.[29] 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[30] 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,[31-33] 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 lympoid-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.[42] 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,