Chapter 6

Summary, general discussion, future perspectives
For the research described in this thesis, we have aimed to further improve the visualization and detection of abnormalities in the cortical and non-neocortical grey matter (GM) in multiple sclerosis (MS), to investigate the relation between white matter (WM) and GM damage, and to study the clinical effects of GM damage.

6.1 | Improved detection of GM abnormalities
Histopathological studies have shown that GM demyelination is common and extensive in MS. [1,2] However, investigation of the clinical correlates of GM damage in MS has been limited by the fact that in vivo visualization of cortical demyelination by standard MRI techniques has been difficult. [3] This difficulty partly comes from the fact that, in contrast to white matter lesions, cortical lesions are non-inflammatory. They lack lymphocyte infiltration, complement deposition and blood-brain barrier leakage. [4-6] Hence, more advanced imaging techniques were applied to improve cortical lesion visualization with MRI. In our center, a multi-slab three-dimensional (3D) double inversion-recovery (DIR) technique was introduced and was shown to enable a five-fold increase of cortical lesions detected, compared with more standard MRI techniques such as T2 and FLAIR. [7] The study described in chapter 2.1 was conducted to investigate signal and contrast properties of an improved, single-slab version of this 3D-DIR technique, with a substantially reduced acquisition time making it clinically applicable. Its performance in detecting both cortical and WM lesions was compared to other single-slab 3D sequences (FLAIR, T2-and T1-weigthed). Images were acquired in 16 MS patients and scored by two independent raters. 3D-DIR showed the highest detection of intracortical and mixed WM-GM lesions, which to a certain extent reflected a better discrimination from juxtacortical lesions. Studies that compared the ability of different MR sequences in detecting cortical lesions previously suggested that part of lesions visible on 3D-T1 sequences are not visualized by 3D-DIR, and that 3D-T1 sequences may thus provide complementary information about GM demyelination. [8-10]

The patients described in the above mentioned study participated in a follow-up study after a median interval of three years (chapter 2.2). One of the goals of this second study was to describe the longitudinal behaviour of cortical lesions, as depicted with 3D-DIR. A large majority (91%) of focal cortical
hyperintensities visible in MS patients at baseline were still visible three years later, providing confidence to the consistency and specificity of the DIR findings. Furthermore, we found that the cortical lesion number but not the WM lesion number at follow-up was significantly higher in secondary progressive (SP) than in relapsing-remitting patients, which confirms results of a study with a shorter follow-up duration. [11] This latter finding suggests that focal cortical pathology is more prone to develop during the SP phase of the disease and may even occur (partly) independently from WM lesion accumulation at that stage. It is also consistent with post mortem results, which previously showed that although GM demyelination is already present in RR and acute MS, it becomes much more prominent in the chronic disease stage. [2]

3D-DIR awaits histopathological verification in a post mortem study to reveal the proportion of cortical grey matter lesions that still remains undetected by this technique. Furthermore, it is important to understand what specific property of cortical lesions determines their conspicuity on MRI. Possible clues come from a recent post mortem MRI study from our group (Seewann et al., submitted), using conventional T2-weighted and 3D-FLAIR MR sequences (Figure 1). It was shown in this work that cortical lesions that were visible on MRI did not differ from MRI-invisible cortical lesions in terms of underlying histopathology, but that the MRI-visible lesions were simply larger than their invisible counterparts. This suggests that (ultra-)high-field MRI with optimized resolution and signal-to-noise / contrast-to-noise ratios, should be able to detect a larger portion of cortical demyelination. So far, few studies have been performed in which a possible improvement of cortical lesions detection at 7T or even higher field strengths was assessed. Those studies that are available were often limited by a lack of comparison with clinical field strength or with (optimized) histopathology. [12,13]

The results from the two studies described in chapter 2 signify that the single-slab version of 3D-DIR currently is the most effective technique when aiming to investigate direct effects of focal cortical grey matter lesions on clinical measures; this has been assessed in this thesis and will be further discussed in section 6.3.
6.2 | Detection of hippocampal lesions and implications for the study of memory impairment

Memory impairment is a prominent deficit within the spectrum of cognitive deficits in MS. Pathology of the hippocampus in MS was expected because of this structure’s pivotal role in memory function. The study described in chapter 3.1 used hippocampal tissue samples of MS patients and of non-neurologic controls, which were subsequently immunohistochemically stained for myelin to study the occurrence, extent, and localization of hippocampal lesions. Lesions were found in 15 of the 19 MS cases investigated, were most often mixed intrahippocampal-perihippocampal, and importantly, were more frequent in cases with reported cognitive impairment. As for the localization of the lesions, the cornu ammonis (CA) 1 and the molecular layer of the dentate gyrus were relatively often affected by isolated intrahippocampal lesions, whereas CA2 was always spared from demyelination.

Figure 1 | Comparison between MRI-visible and invisible cortical lesions. 1: Cortical lesions were assessed on proteolipid-protein stained tissue sections, and marked as visible (red) and invisible (blue) after comparison with the corresponding MRI images. 2: Corresponding proton density-weighted MR image of the same brain slice. 2a: MRI-visible lesion. Note the subtle signal intensity increase that could be detected after direct comparison with the stained tissue section. 2b: MRI invisible lesion.
These latter findings fit with data known from the neurological literature. The CA1 subregion is affected in neurologic conditions such as medial temporal lobe epilepsy, Alzheimer disease, and hypoxic disease. Interestingly, hippocampal atrophy measured in vivo in RRMS patients was also found to predominantly affect CA1. [14] How regional susceptibility for damage within the hippocampus can be explained in MS is not entirely clear, but it may be related to a higher vulnerability of CA1 neurons to glutamate excitotoxicity. Our group is currently investigating this hypothesis and first indications for its plausibility come from observations showing that several neurotransmitter pathways are dysregulated in the MS hippocampus, among which glutamatergic homeostasis (E-J Kooi et al., in progress).

GM demyelination outside the cortex has not been solely observed in the hippocampus, but also in the thalamus and basal ganglia, [15] spinal cord [16] and hypothalamus. [17] Whether demyelination of the GM in all of these areas share a common pathogenic pathway, remains to be clarified.

Ideally, relations between hippocampal pathology and cognitive decline should be investigated in vivo. Therefore, in vivo hippocampal lesion visualization was addressed in chapter 3.2, using coronally reformatted 3D-DIR. A surprisingly high number of 14 out of 16 MS patients had at least one hippocampal lesion. Scoring of false-positives was avoided by inclusion of an age-matched control group, as was done for the studies in chapter 2. The mean number of hippocampal lesions found was comparable to that found in the post mortem study discussed above, i.e. on average 2 tot 3 lesions per patient. Half of the hippocampal lesions could be retrospectively detected on T2-weighted images, indicating that, as with cortical lesions, 3D-DIR is the technique of choice for detection of MS hippocampal lesions.

6.3 | Clinical effects of GM damage and its relation with WM abnormalities
Various studies have pointed out that neocortical and non-neocortical GM pathology may have significant clinical impact: patients with a (neo)cortical presentation of MS were shown to exhibit severe cognitive disturbances [18] and hippocampal demyelination was associated with cognitive decline (chapter 2.1). Apart from these reports, however, little was known concerning the effects of GM damage on cognition in MS.
The great variability in cognitive symptoms between patients is one of the reasons that, compared to physical disability, cognitive decline in MS has less often been the topic of research. Nevertheless, certain functional cognitive systems are consistently and frequently reported to be involved in MS. [19] Because our studies into the effects of GM damage were unprecedented, we included cognitive tests for the most frequently involved cognitive domains in MS, but limited the number of tests to reduce patient burden. We specifically included a test for hippocampal function, the location learning test (LLT) for visuospatial memory. [20] Additionally, executive functioning was assessed for using the Stroop test. [21] Lastly, we included a sensitive, more general measure of cognitive decline, the letter digit substitution test (LDST) for processing speed of information. [22]

In chapter 2.2, the potential influence of cortical demyelination as depicted by 3D-DIR on cognition was investigated. Significant relations were found between increased cortical lesion number at follow-up on the one hand and measures of visuospatial memory and processing speed on the other hand. Because a significant association between WM lesion number at follow-up and cognition was additionally found, as well as a correlation between WM lesion numbers and cortical lesion numbers, the effect of cortical lesions on the cognitive measures in our study could not be fully discriminated from the effect exerted by WM lesions. Therefore, a recommendation for future research is to study selected patient groups with discordant GM and WM damage, enabling a greater comprehension of what part of cognitive decline originates from cortical lesions.

Because of the abovementioned difficulties concerning in vivo detection of cortical lesions, MRI studies have frequently investigated the clinical effect of cortical atrophy. Cortical grey matter atrophy was shown to correlate significantly with clinical disability, [23] including cognitive impairment, [24] but these studies were limited because of their relatively small sample size. Therefore, GM and WM atrophy were investigated in a large cohort (N=927) of MS patients (chapter 4.1). Regression models were used to investigate GM and WM atrophy and lesion volume information as explanatory variables for physical impairment, as measured with the EDSS, and cognitive impairment, as measured with the paced auditory serial addition task (PASAT) for sustained attention. We found that GM atrophy was the strongest predictor
of both physical disability and cognitive impairment, stronger than the other MRI variables, which emphasizes the clinical relevance of this measure and may have importance for future clinical trial design. Additionally, more research is warranted into the clinicocognitive significance of regional cortical vulnerability, since cortical thickness studies suggested that in particular the temporal and frontal cortical regions are affected. [25]

The finding that atrophy or thinning may have a predilection for certain cortical regions has fuelled the discussion concerning the origination of GM damage. Some researchers have put forward that it arises primarily in the GM, whereas others suggest that GM damage is largely secondary and arises as a result of ongoing damage in the WM. Retrograde neuroaxonal degeneration is a supposed pathway for secondary GM damage, which may arise due to ‘virtual hypoxia’ [26] or glutamate imbalance of WM axons. [27] Although the assumption that GM damage arises secondarily to accumulating WM damage has its merits, MRI and histopathology studies [28] indicate that GM and WM abnormalities may show largely independent patterns. We investigated this particular question in the same large cohort as abovementioned (chapter 4.1). Using multiple linear regression, GM atrophy was found to be predicted by T2 lesion volumes. This finding may be an argument for the hypothesis that GM damage results secondarily from damage to WM tracts. We also found in this study that GM atrophy becomes much more prominent in the SP phase as compared to the RR phase, whereas white matter atrophy was similar between different disease phases, confirming histopathological [2] and recent longitudinal in vivo findings in smaller samples. [29]

Our findings, and those of others in smaller patient samples, that GM atrophy predicts disability and accumulates throughout the entire disease, may stimulate the use of this measure in future (clinical) studies. It is therefore important to shed more light on the pathological substrate of GM atrophy. In a histopathological study, [30] neuronal loss was found inside cortical lesions but could not explain the overall cortical thinning that also occurred outside focal lesions, suggesting that part of the thinning may be caused by factors in the normal-appearing GM (NAGM). Unfortunately, little is known about the extent of histopathological abnormalities outside areas of focal cortical demyelination. Future in vivo studies could aim to further understand what is measured with GM atrophy by using 3D-DIR to distinguish the contribution of focal cortical lesions on (regional) cortical volume changes.
The lack of specificity of frequently used MRI measures for WM damage, such as T2 lesion volume and WM atrophy, may also contribute to their incapacity to explain clinical impairment. We therefore used a different quantitative MRI measure, diffusion tensor imaging (DTI), to investigate the attribution of WM damage to cognitive impairment (chapter 4.2), in combination with a novel software tool called tract-based spatial statistics (TBSS). This allowed for a data-driven and voxelwise localization of WM damage. Thirty MS patients were compared to 31 age-matched healthy controls and were found to have a lower fractional anisotropy (FA) in a number of brain regions, including in parts of the corpus callosum and in the fornices. The latter may be secondary to damage in the hippocampus, but may also be due to focal demyelination in the fornices, the existence of which has been described previously.[17] Radial diffusivity increases and a less pronounced increase of axial diffusivity were found in regions of reduced FA. The added value of axial and radial diffusivity with regard to histopathological specificity has been suggested by animal research,[31,32] but needs further confirmation in MS patients. Patients in this study showed normal visuospatial memory performance, just-normal attention, and impaired processing speed. The latter was found to be associated with abnormal diffusion measures in the corpus callosum, which shows that at least part of the cognitive decline in MS can be explained by using more advanced MR measures of white matter damage. This is in particular true for cognitive domains that are heavily dependent on communication between distant brain areas and therefore on intact long WM tracts, such as processing speed of information. Nevertheless, many aspects of cognitive decline cannot be fully understood when GM damage is not taken into account.

Properties of the brain that limit effects of damage may be especially dynamic in the early phase of the disease. Possible responses comprise repair of damage, for instance by remyelination; and functional compensation, which involves the use of altered behavioural strategies. A different possible response of the brain is adaptation, which involves recruitment of other cortical brain areas and parallel pathways through synaptic changes and unmasking of latent corticocortical connections.[33] Functional MRI has the potential to depict this adaptive reorganization, and thereby to explain poor relations between measures of structural damage and clinical function. In two studies in this thesis that are discussed below (chapters 3.3 and 5.1), functional connectivity
(a measure of inter-regional cortical correlations of neuronal variability) was investigated using fMRI acquired during rest. One of the reasons to study resting state fMRI is because task-related increases of neuronal metabolism are small compared to the total brain metabolism; [34] task-fMRI may therefore only reveal the proverbial tip of the iceberg. Taking brain activity into account that occurs in the absence of external stimulation may thus lead to a better understanding of brain function and adaptation in MS patients. In the first study functional connectivity of an a priori defined region of interest was investigated, whereas in the second study a data-driven analysis was applied.

Our previous research showed that hippocampal demyelination occurs frequently in MS and can be imaged in vivo (chapters 3.1 & 3.2). The question arose at what time point and to which extent memory is affected by hippocampal damage. We hypothesized that hippocampal damage may exist without directly leading to impaired memory function, but that functional hippocampal-cortical connectivity may already be altered. Therefore in chapter 3.3, functional connectivity of the hippocampus with cortical areas of the brain was compared between 25 MS patients with intact spatial memory function and that of 30 healthy controls. Despite the absence of impaired spatial memory, decreased hippocampal functional connectivity with areas known to be involved in the memory process was found in MS patients, and was associated with occurrence of hippocampal atrophy. This indicates that disturbances of the communication process between the hippocampi and the cortical areas that enhances long-term memory can be already measured, before becoming clinically apparent. Future longitudinal studies are necessary to elucidate how functional connectivity changes and atrophy of the hippocampal memory system are related temporally, and whether these changes indeed predate memory impairment and atrophy. An ongoing study using a declarative memory encoding task shows that cognitively impaired MS patients have reduced brain activation in the right hippocampus and parahippocampal gyrus (Figure 2), and that they have lost increases of brain activation present in cognitively preserved patients (Hulst et al., in progress).

CIS patients are frequently found to have lesions that are asymptomatic, suggesting that part of focal damage can be compensated. That this partly occurs through adaptive reorganization has been indicated by task-fMRI studies in early MS patients. [35] In our most recent study, described in chapter
5.1, we questioned whether altered cortical resting state networks, indicative of cortical functional (dys)integrity, can already be found in the early phase of MS. Furthermore, we investigated whether these alterations are related to structural WM and GM damage. This was addressed using an independent component analysis of resting state fMRI data, comparing 14 CIS to 31 RR patients, and both patient groups to 41 healthy controls. CIS patients showed increased temporal coherences between brain regions (coactivation) in multiple networks, compared to controls or RR patients. By contrast, GM atrophy and WM diffusivity alterations were only found in RR patients, who showed a lower performance on measures of cognition compared to CIS patients and controls. The network differences found in CIS patients are thus suggestive of early cortical reorganization, but are lost with increasing brain damage and advancing

Figure 2 | A: The activated ventral and dorsal streams in the control group are shown, connecting the visual cortex and temporoparietal lobes to the hippocampus during encoding of correctly remembered items. B: Areas of reduced activation in cognitively impaired MS patients compared to controls are shown (right hippocampus and parahippocampal gyrus).
disability, indicating that cortical reorganization of resting state networks is a finite phenomenon in MS. Approaching clinicocognitive functioning from a residual capacity point of view offers possibilities in terms of rehabilitation and therapeutic strategies, that aim to extend adaptive reorganization (Figure 3).

**Figure 3** | A schematic representation of the relation between structural damage (accumulating axonal loss) and clinical impairment, which is dynamic and modified by functional reorganization. Our research and that of others indicate that during certain stages of the disease, functional reorganization prevents clinical change (patient group 1; medium amount of damage with high amount of functional reorganization, but little clinical disability). When the functional reserve is exhausted, clinical impairment builds up at a faster rate (patient group 2; high amount of damage, little amount of functional reorganization and high clinical disability). If therapies are able to lengthen the time frame of functional reorganization, the blue box will expand and the dashed vertical line representing the border between group 1 and 2 will shift to the right. Patients will then be longer in group 1 and exhibit little clinical change for a longer time period.

Advanced connectivity measures may offer new insights in adaptive reorganization. In an ongoing study (Schoonheim et al, in progress), we are applying a graph analysis to both magnetoencephalography (MEG) and fMRI data, comparing MS patients to healthy controls. Although the results are still preliminary, they suggest that graph analysis connectivity measures are gender-dependently altered in MS.
6.4 | Future perspectives

The results of the studies described in this thesis demonstrate that, although cortical lesion detection may still further improve by the implementation of (ultra-)high-field MRI (Figure 4), 3D-DIR remains the method of choice when studying GM lesions at conventional field strengths. Possible limitations for future research are that DIR acquisition protocols differ between centers, and that DIR images are not easy to interpret, which may lead to interrater variability. Preliminary results from a recent study, conducted with images and raters from different centers participating in the MAGNIMS study group, indicate that prospective data acquisition is necessary to assure sufficient image quality and homogeneity. Furthermore, before DIR can be applied in future multicenter pharmaceutical trials investigating possible effects of neuroprotective agents on limiting GM damage, agreement is needed on GM lesion scoring. MAGNIMS guidelines for cortical lesion scoring will be proposed in an upcoming paper (Geurts et al., in progress).

Figure 4 | High-resolution axial and sagittal 3D T1-weighted magnetization prepared rapid acquisition gradient-echo (MPRAGE) images from an MS patient, acquired at ultra-high-field strength (de Graaf et al., in collaboration with the 7 Tesla group UMC Utrecht, in progress). Arrowheads depict juxtacortical and mixed white matter-grey matter lesions. Currently, a combination of different 3D sequences, as well as quantitative sequences are investigated at 7 Tesla, and compared to images from the same patients acquired at lower field strengths.
The spatiotemporal relation between GM and WM abnormalities remains to be further elucidated. A previous study in PP patients showed that only a minority of focal measures of WM tract damage were related to nearby areas of GM atrophy. [36] Future studies should elaborate on this finding by relating measures of structural WM connectivity to focal cortical atrophy or reduced cortical thickness in relapse-onset MS.

Another question that needs to be assessed by future research is what is exactly measured with quantitative MRI of the cortex. In particular, how and to what extent measures of regional cortical atrophy are influenced by damage in focal cortical lesions should be further investigated, for example by combining cortical lesion probability maps based on 3D-DIR with regional cortical atrophy maps.

Although clearly some cognitive domains are more often impacted by the disease than other domains, cognitive test performance varies considerably between patients, and can even fluctuate due to fatigue or temporary clinical relapses within individual patients. It is therefore difficult to investigate cognitive decline as a whole in MS. Specific cognitive deficits in MS might be best understood in terms of ‘functional systems’. In this thesis, the cognition focus was largely placed on the hippocampal memory system. Important for future research into cognitive decline, is the availability of a cognitive test battery that can be assessed within limited time, but that provides more details with regard to the cognitive domains most frequently involved in MS. A complication arises from the fact that cognitive functions may influence one another to various extent. The relative dependence of cognitive functions that are ‘cortex-dependent’ on neuropsychological aspects that may more WM-related, such as processing speed and fatigue, needs to be explored. Furthermore, the results of the studies presented in this thesis suggest that models attempting to explain cognitive decline in MS should account for both WM and GM lesions (using T1, T2 and DIR), as well as for abnormalities in the ‘normal-appearing’ WM (using quantitative MRI such as DTI) and GM brain tissue (using atrophy measures).

In this thesis, hippocampal damage and decreased hippocampal functional connectivity was found to be apparent even before memory decline. In another study, CIS patients showed adaptive connectivity alterations in resting state networks that have been linked to different cognitive domains. Our knowledge
of the reserve capacity of the brain in MS can be improved by investigating the process of functional reorganization over time in longitudinal studies. The relative contributions of different adaptive mechanisms are likely to change across the disease. At what point do pathological changes reach a threshold, or in other words, when is the reserve capacity exhausted? Does memory decline subsequently set in? Can the period in which the effects of damage to clinical function are limited by reorganization, be extended by influencing the adaptive process through pharmacotherapy or other interventions? These are questions that need to be addressed by future studies.

6.5 | Conclusions
Advances in knowledge gained by the studies described in this thesis
- Single-slab 3D-DIR allows for the highest number of cortical lesions to be detected in vivo at conventional field strength
- Cortical lesions can be imaged reliably over time, and accumulate in particular in the SP phase
- Hippocampal lesions occur frequently in MS patients and can be imaged in vivo with 3D-DIR
- Cortical lesions are related with cognitive decline
- When measured with DTI, localized WM damage can be related to impaired processing speed
- Physical and cognitive impairment can be better explained by GM atrophy than by WM atrophy or T2 lesion volume
- Functional connectivity of the hippocampus is already decreased in patients with still intact memory
- Cortical reorganization of several resting state networks exist in CIS patients and may limit the clinical expression of damage early in the disease

This knowledge may act as a starting point for future research in MS into:
- The spatiotemporal relation between WM and GM abnormalities
- Effects of neuroprotective agents on limiting the development of GM lesions
- How reorganization of specific networks can be influenced
- How the interplay between damage, repair and adaptation influences cognitive function
REFERENCES


