CHAPTER 10

English & Dutch summary
Multiple sclerosis (MS) is a chronic disorder of the brain, spinal cord and optic nerve (the central nervous system - CNS), which generally manifests between 20 and 40 years of age. The occurrence of MS differs between countries and population groups, in the north of Europe almost 1 in 1000 people are affected, while the disease is almost absent around the equator. The exact cause of MS is unknown so far, but most likely it is a combination of genetic and environmental factors.

Multiple sclerosis literally means many (multiple) scars (sclerosis) in the CNS, which are the characteristic pathology features of MS. The scars are the result of recurrent immune reactions that cause damage to the myelin, a fatty layer around the nerve fibers that protects and insulates them. Myelin is necessary for optimal signal transduction from the nerves in the brain towards the rest of the body, essential for crucial body functions. When this signal transduction hampers neurological deficits occur, like muscle weakness, paralysis, sensory problems and vision loss. The neurological signs people with MS suffer from depend on the nerves that are affected, therefore symptoms can vary a lot between individual MS patients. In most cases the condition starts with episodes of sudden worsening (relapses) alternated by periods of recovery (remission). This is called relapsing-remitting MS (RRMS). However, over time (approximately 10-15 years after diagnosis) symptoms become gradually persistent and repair no longer occurs. This stage of the disease is referred to as secondary progressive MS (SPMS). In a minority of people with MS the symptoms gradually accumulate without clear episodes of improvement, immediately from the start of the disease. This course is described as primary progressive MS (PPMS). In general PPMS starts at an older age than RRMS, more comparable to the age at which RRMS develops into SPMS.

Although there are various drugs that can control the relapses in MS, none are able to stop the progression completely. Thus, there is no therapy available that can heal MS.

Studying the actual areas of damage (the plaques or lesions) and the attempts of the brain to repair this damage in the CNS in post-mortem material of MS patients can give new insights in the search for treatments. From a neuropathological perspective lesions can be classified based on the degree of inflammation i.e. the activity of both the innate and adaptive immune cells. A demyelinated area completely filled with myelin-filled macrophages and adaptive immune cells is called an active lesion. Chronic active refers to lesions in which the active inflammation forms a rim around an empty center with myelin damage. When this rim of inflammation also disappears a scar-like area remains. This type of lesions is called inactive. Lesions form and regress during the course of the disease, with inactive lesions as end stage. Sometimes repair can occur, which is called remyelination. However this occurs less often as the disease develops and damage remains.

How these lesions really start is currently unknown, although it seems that the first steps of lesion development take place within the CNS itself. Well before any obvious myelin damage or leukocyte infiltration is observed, subtle alterations are seen in the CNS parenchyma. Post-mortem examination of these focal changes show clusters of activated microglia, the primary immune cells of the CNS, in
otherwise normal appearing tissue. Transitional stages are also seen in which only a few myelin-filled macrophages are associated with the clusters of activated microglia suggesting the initial stages of myelin damage. This notion is supported by positron emission tomography (PET) scan techniques revealing that these focal changes occur several months before appearance of destructive actively demyelinating lesions. This suggests that these microglia clusters may well be the first stage in lesion formation, and accordingly they are called preactive lesions.

Preactive lesions are observed during pathological examination in 67% of MS patients. The presence of preactive lesions is independent of MS form (RRMS, SPMS and PPMS) or disease duration. The fascinating aspect of these lesions is that the number is significantly higher compared to active lesions, suggesting that not all of these lesions will develop into full-blown damaging lesions. This implies that some intrinsic regulation exists which is able to stop lesion formation at this early stage. However, it is currently unknown what these controlling mechanisms are and what goes wrong when a preactive lesion does develop into an active lesion. The studies performed in this thesis aim to gain more insight in the mechanisms driving preactive lesion formation and the processes underlying the transition from preactive into active lesion. Understanding these pathways could lead to novel therapeutic targets that are able to encourage the natural resolving power of preactive lesions and stop active lesion formation, and thus halt damage in MS.

Chapter 1 provides a general introduction of the different aspects of MS. The clinical features, possible causes, and the pathological mechanisms operating in the disease are discussed, as well as the therapies that are currently FDA approved for MS treatment. Moreover, the pathological features such as the different lesion types and what is currently known about the preactive lesions have been described. This knowledge has led to the working hypothesis and research questions raised in this thesis.

So far the presence of preactive lesions has only been described in post-mortem tissue of MS patients. Pathological examination however provides only a snap shot of the pathological status at death. During what stage of the disease preactive lesions develop and their relationship to relapses, remission or progression of the disease is therefore currently unknown. For this reason we investigated the presence of preactive lesions in autoimmune encephalomyelitis (EAE), an animal model of MS.

EAE is the most commonly used experimental model for MS. The disease can be induced in various animal species although rodents are more commonly used. Depending on the animal and immunization protocol used, the clinical courses and pathology observed differ. In chapter 2 we examined the presence of preactive lesions in EAE in Biozzi mice. In MS as mentioned above the progressive forms of the disease occur in older people. For this reason we examined EAE in different aged mice. The clinical course of EAE between young (8-12 weeks) and old (1 year) mice was found to be markedly different. Young mice present clinical features of RRMS, relapses are alternated by periods of remission. This is called relapsing EAE (RREA). After several months these mice develop a progressive course, referred to as secondary progressive EAE (SPEAE). In contrast, old mice develop progressive disease immediately from onset (progressive EAE – PEAE).
Pathological examination of the young and old mice however revealed that preactive lesions are present in all three manifestations of EAE. This is in agreement with the observations made in MS patients.

Preactive lesions are observed in areas of normal appearing tissue. However, when examined microscopically, the microglia clusters are associated with stressed oligodendrocytes, the myelin-producing cells of the CNS. Cells subjected to stress events are well-known to produce a range of proteins in an attempt to rescue the cell from death. Depending on the stress event, different stress proteins are produced. Thus, knowledge of the proteins that are produced by stressed cells can provide information of stress the cells are subjected to. Previous research showed that oligodendrocytes in preactive lesions express HSPB5. HSPB5 belongs to the small heat shock protein family, that consists of 11 stress proteins with similar structures and functions. In chapter 3 we examined whether oligodendrocytes in preactive lesions express other small heat shock proteins (HSPB1, HSPB6, HSPB8, HSPB11) in addition to HSPB5. Although this study showed that HSPB1, HSPB6 and HSPB8 are upregulated in active MS lesions, they are not observed in preactive lesions, while HSPB11 is not associated with MS lesions. The expression of only HSPB5 is characteristic for oligodendrocytes in preactive lesions. This finding thus provides novel clues about the event that could cause stress in oligodendrocytes in preactive lesions.

The close connection between activated microglia and stressed oligodendrocytes in preactive lesions suggests an intimate interaction between these two cell types. Yet, the questions remain as to whether activated microglia induce the oligodendrocyte stress or whether the stressed oligodendrocytes trigger microglia activation and the subsequent formation of the microglia clusters? Chapter 4 reviews the current knowledge about the complex interplay between oligodendrocytes and microglia. While oligodendrocytes were originally thought of as immunologically inert and merely seen as bystander victims of immune responses, recent data point out that they are capable of actively producing proteins that could trigger and regulate the immune system. In this way oligodendrocytes are able to activate microglia. To protect the CNS in an optimal way microglia are able to adapt different activation states (phenotypes). When adapting a pro-inflammatory phenotype, microglia are harmful for the oligodendrocytes. However, when they have an anti-inflammatory phenotype they fulfill reparative functions and protect oligodendrocytes. The phenotype microglia obtain depends on the immune proteins they are exposed to. Since oligodendrocytes can produce these immune regulatory proteins, they can, in a way, determine their own fate.

Chapter 5 focusses on the question why some preactive lesion develop into full blown active lesions, while others resolve. The answer may be found in the phenotypes of the activated microglia in preactive lesions. As discussed above, microglia are capable to adapt an activation state that is harmful for the environment (pro-inflammatory microglia, also referred to as M1 microglia) as well as one that has protective and reparative functions (anti-inflammatory microglia or M2 microglia). We hypothesized that the preactive lesions may exhibit two forms – either resolving or progressive. The resolving preactive lesions would thus consist
of anti-inflammatory microglia. Their protective and reparative capabilities would recover oligodendrocyte stress and restore tissue homeostasis. When this repair mechanism fails, microglia could switch into a pro-inflammatory phenotype to remove the damaged cells. However, this pro-inflammatory response goes hand-in-hand with tissue damage and the attraction of inflammatory cells from the circulation (active lesion). Phenotyping of activated microglia in preactive lesions, however, revealed that they did not have a classical pro- or anti-inflammatory phenotype but rather exhibited another phenotype. Thus, based on the current pro-and anti-inflammatory phenotype markers used to identify different types of microglia \textit{in vivo}, we could not predict which preactive lesions would develop into an active lesion.

The stress protein HSPB5 may contribute to the phenotype of microglia observed in preactive lesions. Previous studies pointed out that HSPB5 is able to activate microglia in an experimental setting. Extensive analysis of HSPB5-stimulated microglia revealed that HSPB5 induces an immune-regulatory and protective phenotype, although not a classical anti-inflammatory (M2) phenotype. Rather, the HSPB5 activated microglia produce a broad range of proteins to fulfill these protective functions, including the proteins pentraxin-3 (PTX3) and IL-1β. PTX3 is a protein that is known to have immune controlling and protective functions. In chapter 6 we studied the expression of PTX3 in MS lesions and examined whether it could have a therapeutic effect in EAE. Our data show that PTX3 is expressed by microglia in MS lesions and is already present in preactive lesions. PTX3 is expressed in human microglia triggered by different TLR ligands yet, the administration of systemic PTX3 during EAE in Biozzi mice did not alter disease severity or course. Thus, while PTX3 may have local functions during neuroinflammation in facilitating myelin phagocytosis, this study does not point to a role for PTX3 in controlling MS.

In chapter 7 we describe the presence of IL-1β in MS lesions and compare this to the expression patterns observed in rhesus macaque monkeys (\textit{Macaca mulatta}) with EAE. In post-mortem tissues from animals with EAE IL-1β staining was observed in resident microglia and differentiated macrophages rather than monocytes. This suggests that the production of IL-1β occurs within the CNS itself. Although MS lesions are not as fulminant as the lesions observed in rhesus EAE, IL-1β was also observed in activated microglia during lesion formation. IL-1β positive microglia were already detectable in preactive lesions, although only in a minority. IL-1β plays an important role during inflammation. The selective presence of IL-1β in some preactive lesions may point to a contribution of IL-1β to the switch into active lesion development. Future research is necessary to determine whether IL-1β indeed is crucial for lesion formation.

The data presented in chapter 8 show that when human microglia are exposed to the pro-inflammatory protein interferon-γ (IFNγ) prior to HSPB5-stimulation, the protective activation state otherwise induced by HSPB5 is abrogated. IFNγ is able to reprogramme microglia into a phenotype with pro-inflammatory damaging characteristics. Using microarray approaches on cultured human microglia we identified markers triggered by IFNγ. These markers of the reprogrammed microglia can be detected in active demyelinating MS lesions, but not in preactive lesions. This discrepancy between the phenotypes observed in preactive and
active lesions suggests that IFNγ plays an important role in the origin of damage observed in active lesions.

In chapter 9, the general discussion, we have summarized our findings described in the various chapters, put these in perspective, and re-evaluated our hypothesis based on the presented results.

In this thesis we used a multidisciplinary approach to study the multifarious aspects of preactive lesions. Our data suggest that stressed oligodendrocytes, via HSPB5, alert microglia. Microglia migrate towards the stressed oligodendrocytes and become activated in a reparative way. However, when the microglia are not able to restore tissue homeostasis, they change their phenotype into a pro-inflammatory one to remove the damaged cells and attract immune cells from the circulation to strengthen the immune response. Yet, this inflammatory reaction harms also the surrounding tissue, an active lesion.

The knowledge acquired about the underlying mechanisms of preactive lesion formation and development provides promising opportunities to manipulate preactive lesions therapeutically and in this way stop lesion development and consequently damage, in people with MS.