Progression of multiple sclerosis

The role of microglia and neurons

van den Bosch, A.M.R.

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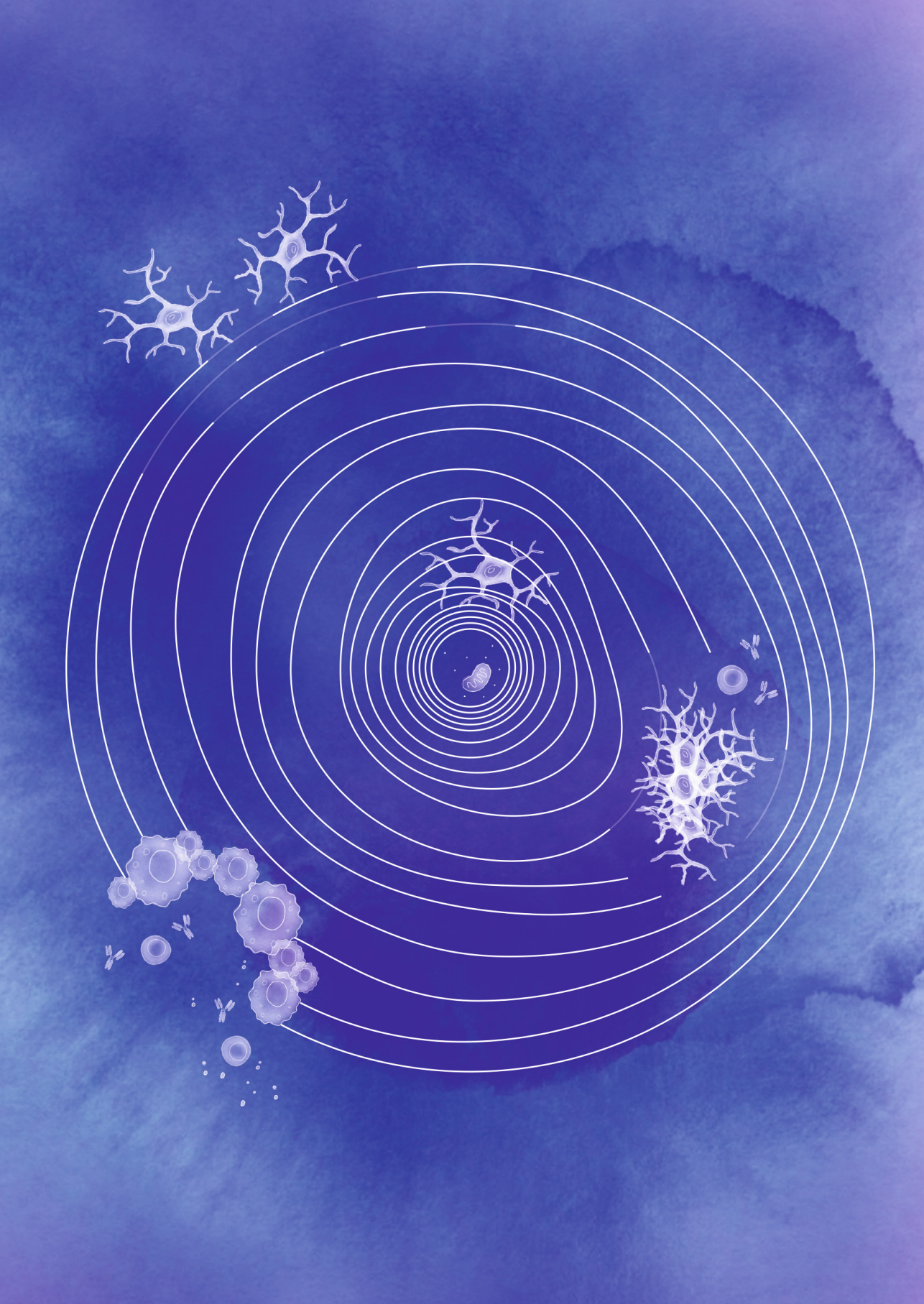

Chapter 1

Introduction and outline of the thesis

MULTIPLE SCLEROSIS

Diagnosis

Multiple sclerosis (MS) is the most prevalent neuroinflammatory disorder among young adults, affecting 2.8 million people worldwide [1,2]. Individuals with a genetic predisposition for the onset of MS are more vulnerable to penetrance of environmental risk factors [3]. To date, over 200 single-nucleotide polymorphisms (SNPs) have been associated with increased risk of MS. Most of the susceptibility associated genes are related to immunological pathways, and the major histocompatibility complex (MHC) region is the main genetic determinant [4]. Accordingly, disease-modifying therapies that reduce the relapse rate are immune-modulatory and target the adaptive immune system [5]. However, our understanding of the risk factors and mechanisms underlying disease progression remains limited, resulting in an unmet need for therapies capable of halting and reversing progression of MS [6].

People with MS have focal, demyelinating lesions throughout the central nervous system (CNS) [7], which can be detected by magnetic resonance imaging (MRI) [8]. Moreover, more than 95% of people with MS have immunoglobulin (Ig) G oligoclonal bands in the cerebrospinal fluid (CSF), persisting throughout the course of the disease and indicating continuous intrathecal antibody production [9]. Diagnostic criteria for MS include evidence of grey matter (GM) or white matter (WM) lesions in at least two separate areas of the (CNS) that occurred at different time points. In some settings, oligoclonal bands can substitute for demonstration of lesions in time [10].

MS is a highly heterogenic disease, both clinically and pathologically [11]. During life, pathological disease progression manifests as progressive loss of walking ability and is reflected by biomarkers such as MRI [8] and soluble biomarkers in blood and CSF [12]. The rate of disability progression is variable between patients and is largely unpredictable. Traditionally, people with MS have been categorized as distinct clinical phenotypes, i.e. relapsing-remitting, secondary progressive, or primary progressive. However, accumulating evidence suggests that the clinical course of MS is better considered as a continuum, reflected by concurrent pathophysiological processes that vary across individuals over time [6]. The formation of new focal demyelinating lesions and ongoing inflammation and demyelination are pathophysiological processes that contribute to progression of MS which we investigated in this thesis. Unravelling of the molecular mechanisms of various pathophysiological processes contributing to MS progression may lead to the identification of new therapeutic interventions and to the validation of biomarkers that can predict the disease course.

MS lesion pathology

MS lesions can occur throughout the CNS and are histologically characterized by focal inflammation and demyelination. A number of classification systems have been introduced, among which the Bö/Trapp system, the De Groot/Van der Valk modification, the Lucchinetti/Lassmann/Brück system, the Vienna consensus, and, most recently, the Kuhlmann system [13–18]. The Bö Trapp system focused on microglia density, stratifying between active, chronic active (or mixed active/inactive), and inactive lesions. The Lucchinetti/Lassmann/Brück system further subdivided active and chronic active lesions based on oligodendrocyte destruction and on the presence of myelin degradation products within the microglia into early or late demyelinating lesions. The De Groot/Van der Valk modification combined these two systems, and furthermore described clusters of microglia as pre-active lesions (or nodules). The Vienna consensus included inflammation based on both microglia and perivascular infiltrates and on active demyelination based on myelin degradation products, distinguishing between entire lesions or plaque margins. The Kuhlman classification system combined the former classification systems, while reducing complexity by not stratifying for early or late demyelination, and included the presence of remyelination,. This unified classification system serves as a fundamental framework for categorising MS lesions based on histopathological similarities of the myelin and microglia, aiming to provide a cohesive understanding of MS pathology [19]. Nonetheless, it is important to recognize that beyond this classification system the biological landscape of MS lesions is likely far more intricate and multifaceted. Each MS lesion subtype may harbour numerous additional biologically relevant characteristics, contributing to considerable heterogeneity within and across lesion types.

Based on the classification system described by Kuhlmann *et al* (2017) [19], various lesion types can be distinguished, as shown in **figure 1**. In the WM, reactive sites and four types of lesions are defined: active, mixed active/inactive (mixed), inactive, and remyelinated lesions. Reactive sites are regions with no demyelination and with accumulation of microglia and/or macrophages (hereafter microglia). Active lesions have partial demyelination and an accumulation of microglia. Mixed lesions have a demyelinated, hypocellular core and a border with accumulation of microglia. For active and mixed lesions, the microglia are scored as ramified, ameboid, or foamy. Inactive lesions have a demyelinated, hypocellular core with a lower density of microglia/macrophages compared to the normal-appearing WM (NAWM). Remyelinated lesions have sparsely myelinated axons with a similar microglia density the NAWM. Inactive lesions and remyelinated lesions are considered as two possible end-points for MS lesion development [11]. In the GM, three types of lesions are defined based on their location: subpial, intra-cortical, and leuko-cortical lesions. Subpial

lesions are lesions spanning the first layers of the cortex that can extend towards but not into the WM. Those without clear demyelination of the first layer of the cortex that do not extend into the WM are classified as intra-cortical lesions. Cortical lesions that extend into the WM are classified as leuko-cortical lesions.

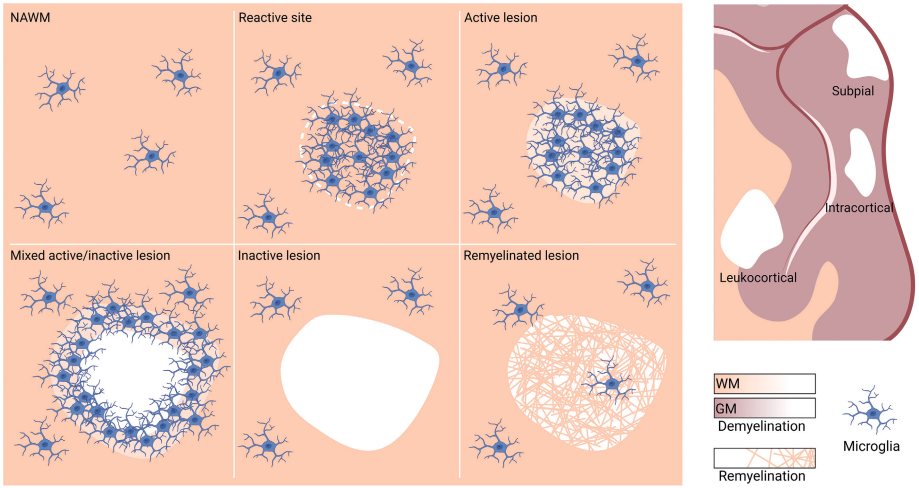

Figure 1: Schematic presentation of characterisation of MS lesions in the WM and GM based on activated microglia and myelin, according to Kuhlmann *et al.* (2017). NAWM: normal-appearing white matter, WM: white matter, GM: cortical grey matter.

For each donor, the reactive site load, lesion load, proportion of active, mixed, inactive, and remyelinated lesions, the cortical lesion rate, and the microglia/macrophage activity score can be calculated [11]. These donor characteristics vary between donors and are clinically relevant: the lesion load and the proportion of mixed lesions positively correlate with clinical severity [11]. Likely, different lesion types have a different propensity for ongoing tissue damage or repair, and consequently, they will vary in the likelihood of becoming either a scar that becomes an inactive lesion, or a remyelinated lesion. Understanding the upstream and downstream mechanisms that determine whether lesions will continue to expand, become inactive, or remyelinate can help identify new therapeutic targets to halt disease progression. Simultaneously, these insights may provide biomarkers to better predict the clinical disease course.

PROGRESSION OF MS

Lesion formation

Molecular changes in the brain tissue preceding MS lesion formation may serve as triggers for their formation. In this thesis, we aim to gain insight into these alterations in the NAWM and normal-appearing GM (NAGM), as this is likely to provide valuable understanding of key mechanisms driving the initiation of MS lesion formation.

Neuronal cell bodies and their dendrites lie in the GM, and their associated axons reach into the WM. These axons are myelinated by oligodendrocytes. The myelin sheath is characterized by a multilamellar structure of multiple lipid-rich plasma membranes, as shown in **figure 2**, that enables rapid saltatory conduction. The charge of phospholipids and sphingolipids ensures compact wrapping of the myelin sheaths [20]. Each myelin internode becomes flanked by nodes of Ranvier [21].

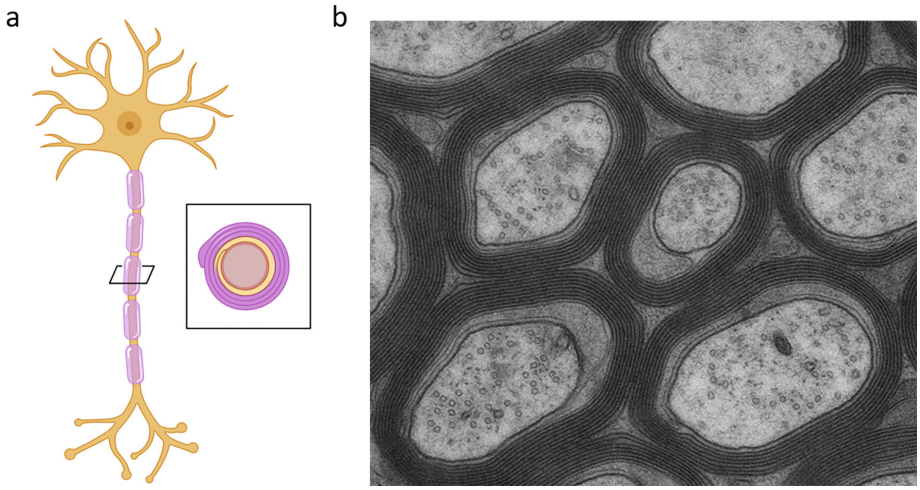

Figure 2: a) Animation of a neuron and it's axon in yellow, with the multilamellar myelin in pink, created with Biorender.com. b) Electron microscope cross section image of the axons of the mouse optic nerve. Oligodendrocytes wrap around axons, forming multilaminar lipid-rich myelin sheaths. Copyright by Prof. K.A. Nave, MPI for Experimental Medicine, Göttingen. Adapted from https://www.mpg.de/5786721/glial-cells-metabolites.

Myelin collected from post-mortem MS brains is phagocytosed more efficiently compared to myelin from healthy control donors [22], indicating that the myelin itself in MS is fundamentally altered, which may trigger demyelination. Previously,

compared to myelin of healthy control donors, changes in the molecular composition of myelin membranes and structural organisation have been observed. Wheeler *et al.* showed a perturbed lipid metabolism that resulted in a disturbed lipid composition with an increase in phospholipids and decrease of sphingolipids [20]. Musse *et al.* showed deamination of the myelin basic protein. Both changes in lipid composition have been modelled and predicted to result in an increased repulsive force between myelin sheaths, which may impact the saltatory conduction [20,23]. In the NAWM, there is a higher presence of oxidized phospholipids (oxPLs) compared to healthy control WM [24]. Microglia bind to oxPLs through scavenger receptors and Fcγ receptors, leading to internalisation and, depending on the volume, transition to foamy cell, and through Toll-like and complement receptors, triggering pro-inflammatory signal transduction [25,26]. Therefore, in the NAWM in MS, oxPLs can trigger demyelination and sustained inflammation.

Microglia are yolk-sac-derived myeloid cells that populate the brain during embryonic development [27,28]. They are the phagocytes of the CNS and play crucial roles in various physiological processes. Homeostatic microglia are involved in neurodevelopment and regulation of myelin integrity, immune surveillance, remodelling of myelin and synapses, and vascular regulation [29,30]. In MS, microglia play a significant role in disease pathogenesis. Activated microglia are highly dynamic and can be both pro-degenerative or pro-regenerative, depending on stimuli from the cellular (micro)environment [29,31]. Microglial immune activity is controlled through seclusion from the circulation, cell-bound and soluble restraining factors, and transcriptional regulators. These immune checkpoint mechanisms control microglial function by efficiently and tightly regulating microglial responses to inflammation [32]. Microglia in the WM and GM have distinct characteristics, which may partially be attributed to neuronal membrane-bound immune checkpoint molecules, such as CD200 and CD47 [33–35]. Microglia have a highly diverse morphology which dynamically changes, as visualized in **figure 3**, with distinct states of activation [30]. Resting, homeostatic microglia have a ramified morphology. Upon activation and uptake of debris, microglia can become more rounded and ameboid or even foamy after excessive lipid uptake. Furthermore, microglia in the NAWM can form small clusters, or nodules. It is hypothesized that a subset of these nodules may progress into MS lesions, depending on stimuli from their (micro)environment [36,37]. In the NAWM compared to healthy WM, microglia downregulate homeostatic markers, including P2RY12 and TMEM119, and upregulate pro-inflammatory mediators [38]. Moreover, microglia in the NAWM already have an increased expression of genes associated with lipid-metabolism, which is further upregulated in MS lesions. Microglia in the NAGM have an increased expression of genes

associated with glycolysis and iron homeostasis, possibly reflecting microglia reacting to iron depositions [35].

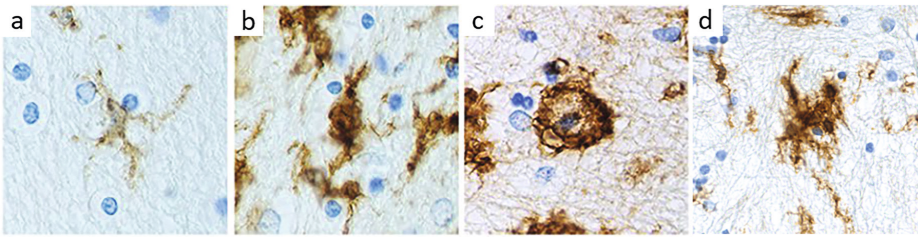

Figure 3: HLA-DR immunohistochemistry showing microglia with a) ramified, b) ameboid and c) foamy morphology, and d) a cluster of ramified microglia forming a nodule. Adapted from Hendrickx *et al.*, 2017, J. Neuroimmunol, doi: 10.1016/j.jneuroim.2017.04.007.

T and B cells are lymphocytes essential for the adaptive immune response. In contrast to myeloid cells, these cells respond on specific, unique antigens rather than molecular patterns. When activated, T and B cells proliferate and effector molecules, such as cytokines. Activated cytotoxic T cells release cytotoxic molecules and engage death receptors on the surface of target cells. B cells activated by antigens bound to their B cell-receptor can develop into specific antibody-secreting cells [39]. In the healthy CNS, low numbers of T and B cells populate the perivascular space. However, in MS, in particular T and B cells are triggered to infiltrate the parenchyma in association with local inflammation [40]. In the NAWM, there is an increase in the number of T and B cells compared to healthy control WM, both perivascular as well as parenchymal, and an increase in the number of antibody-secreting cells resulting in an elevated production of immunoglobulins [39,40,42]. In the NAGM, only few lymphocytes are found. However, in the meninges, larger numbers of B cells persist and can contribute to meningeal inflammation, which is associated with altered microglia morphology and function [41]. IgG produced by clonally-expanded plasmablasts from the WM and CSF of MS patients can bind myelin and trigger rapid demyelination *in vitro*. Possibly, myelin in MS is opsonized by IgG, which may lead to the more efficient phagocytosis by microglia [22]. The pro-inflammatory cytokines and immunoglobulins released by T and B cells can activate microglia, which can then become pro-inflammatory, and contribute to demyelination and MS pathology [43].

In summary, the NAWM and the NAGM are not so normal. Many early changes are seen in myelin composition, microglia activity, and lymphocyte accumulation,

which can have detrimental effects on overall CNS homeostasis and may even lead to the initiation of MS lesion formation.

Lesion expansion and failure of remyelination

In line with the broad clinical spectrum of MS, the pathological manifestation of the disease is also highly heterogeneous between and within patients [11,44,45]. Mechanisms driving this donor and lesion heterogeneity are not yet fully understood. Serial MRI studies have shown that MS lesions during life are highly dynamic; in expanding lesions both demyelination and remyelination are observed, giving rise to high variability of lesions [46,47]. Inflammatory and demyelinating lesion activity is widely present even in the late stage of the disease. In post-mortem tissue, the majority of lesions are either active or mixed, and the proportion of mixed lesions is correlated with clinical disease severity [11]. Remyelination, on the other hand, can also be extensive in MS, with wide heterogeneity between donors [48].

There is also heterogeneity regarding the presence of B- and T-cell infiltrates in MS lesions. In some donors, B cells infiltrate the parenchyma in WM MS lesions, with the highest number of B cells found in active lesions [49,50]. Donors with B-cell infiltration have a more severe clinical disease, higher proportion of mixed lesions, and a higher number of T cells [49]. In some donors, perivascular cuffing of T cells is observed. These donors have more mixed lesions and a higher lesion load [51]. The number of B and T cells in lesions is further associated with axonal damage [50].

Many active and some mixed lesions have the potential to remyelinate [52], however, the myelin repair is often incomplete and therefore insufficient. Mechanisms underlying remyelination failure are not yet completely understood. In active lesions, failure to remyelinate occurs despite the presence of mature oligodendrocytes due to lack of myelin sheath formation. In mixed lesions, there is oligodendrocyte loss, and a hostile tissue environment (inflammatory microglia and lymphocytes) may play a role in remyelination failure [52]. The lack of remyelination in mixed lesions may be due to a block of the ability of oligodendrocyte progenitor cells (OPCs) to differentiate and start remyelinating [53]. Senescence and oxidative stress in OPCs with limited antioxidant capacity may be underlying the differentiation impairment and therefore underlying remyelination failure [54,55]. Although myelin internalisation by microglia fuels demyelination, the removal of damaged myelin also promotes remyelination, as myelin inhibits OPC differentiation [56].

Uptake of myelin debris by microglia can induce an anti-inflammatory phenotype that is beneficial to repair. However, excessive internalization of myelin may

drive foamy microglia towards a more pro-inflammatory phenotype [57]. This may indicate that active and mixed lesions containing ramified microglia may be more prone to remyelination while those with foamy microglia may be more inclined towards expansion.

POST-MORTEM MS BRAIN RESEARCH

Post-mortem brain research is often limited by tissue availability and quality. All tissue used in this thesis was obtained by the Netherlands Brain Bank (NBB). At the NBB, tissue from human brain donors with a variety of neurological and psychiatric disorders and of non-diseased donors is collected. The rapid autopsy program of the NBB ensures high-quality tissue, which combined with the extensive pathological and clinical data obtained per donor enables high quality scientific research. Brain donors sign up to the NBB during life. Of each donor, a detailed summary of the medical record and a neuropathological summary is made by trained medical staff. When the donor passes away, their body is transported to the Amsterdam UMC, location VUmc, where the autopsy takes place within 4-10 hours. Tissue is dissected following a standard protocol and an additional protocol according to the clinical diagnosis. For MS donors, lesions are dissected based on post-mortem MRI guidance in collaboration with the department of Radiology, Amsterdam UMC. For each dissected MS tissue block, the tissue is characterized for MS pathology. To date, 5,393 tissue blocks of a total of 251 MS donors have been characterized. Of each donor, the reactive site load, lesion load, cortical lesion rate, proportion of different MS lesion types, and microglia/macrophage activity score has been calculated [11,19,58]. The pathological characterization per donor in combination with their clinical variables is crucial to interpret the biological relevance of pathological findings.

During my PhD, together with collaborators, I was able to apply state-of-the-art quantitative techniques that have only scarcely been applied to human tissue before. We performed electron microscopy, high-resolution immunohistochemistry visualized with stimulated emission depletion microscopy (STED), single molecule array (SIMOA) on CSF and plasma samples, bulk next-generation RNA sequencing on laser micro-dissected tissue, and spatial transcriptomics using the Stereo-seq platform. Single nucleotide polymorphisms (SNPs) of MS donors were identified through genome-wide association studies (GWAS). These techniques allowed us to identify mechanisms driving MS lesion formation, lesion expansion, and failure of remyelination.

OUTLINE OF THE THESIS

The aim of the thesis is to unravel mechanisms of pathological progression in MS, specifically focussing on the role of microglia and neurons. Identification of key drivers in ongoing local-inflammation, neurodegeneration, and remyelination failure, has enabled the discovery of new therapeutic targets to prevent further damage or promote repair.

In part 1 of this thesis, entitled *loss of microglia homeostasis can lead to initiation of lesion formation,* we aim to identify key players in the formation of new lesions.

In **chapter 2**, we investigated the expression of the check-point molecules CD200 and CD47 and their receptors in the GM to unravel their putative role in MS lesion formation. We combined quantitative immunohistochemistry with qPCR to explore the expression of CD200 and CD47, and their receptors CD200R and SIRPα, in NAGM, cortical lesions, and in peri-lesion cortical regions.

In **chapter 3**, we studied possible early mechanisms of lesion formation by investigating the axon-myelin unit in NAWM in MS in relation to inflammation. We combined quantitative immunohistochemistry with electron microscopy, to investigate the amount of activated and phagocytic microglia and the amount of parenchymal and perivascular lymphocytes, the organization of the nodes of Ranvier, the density and diameter of axons, the area of the peri-axonal space, the diameter and compactness of myelin, and the frequency and size of axonal mitochondria.

In **chapter 4**, we explored a possible role of microglia nodules in the NAWM in lesion formation in MS. We combined immunohistochemistry with RNA sequencing of laser capture-dissected microglia nodules. We measured association of microglia nodules with presence of nearby T cells and B cells, immunoglobulin production, activation of the complement cascade, activation of lipid metabolism, phagocytosis of oxidized phospholipids, and shape of the axonal mitochondria network.

In part 2 of this thesis, entitled *lesions with foamy microglia are neuro-destructive and lesions with ramified microglia are regenerative*, we aim to unravel the regenerative and degenerative capacity of different types of lesions, stratifying between active and mixed lesions with ramified or foamy microglia.

In **chapter 5**, we identified three independent dimensions of pathology in MS using a data-driven approach, utilizing quantitative and qualitative

neuropathology data and clinical data. This enabled disentangling of different pathophysiological processes happening simultaneously in MS brains, which together determine clinical features of MS patients.

In **chapter 6**, we investigated the biomarker neurofilament light chain (NfL) in the CSF to identify the pathological correlate of NfL levels to better estimate disease activity and thereby disease course in people with MS. We combined a SIMOA assay with quantitative pathology of all fully characterized MS donors with CSF available of the Netherlands Brain Bank to study the correlation of NfL with the proportion and activity of lesion types.

In **chapter 7**, we performed high-resolution spatial transcriptomics combined with immunohistochemistry, to investigate the de- and regenerative capacity of mixed lesions with a border with ramified microglia compared to those with a border with foamy microglia within donors. We focussed on the centre, border, and peri-lesion regions, to study cell-type specific roles in de- or regeneration and in lesion expansion.

In part 3 of this thesis, entitled *genetic susceptibility for clinical severity is associated with more severe pathology*, we aim to biologically validate the severity locus SNP rs10191329 in the *DYSF-ZNF638* locus and investigate the pathological implications of this SNP using immunohistochemistry and bulk nuclear RNA sequencing.

In **chapter 8**, we contributed to a study of the International Multiple Sclerosis Genetics Consortium on the genetics of disability progression in MS. The consortium identified a SNP in the *DYSF/ZNF638* locus that was associated with clinical disease progression. In this chapter, we aimed to provide a biological validation of this SNP, by genotyping all MS donors of the NBB for the risk variant and by comparing pathological characteristics of donors carrying this risk variant to those without.

In **chapter 9**, we further elaborated on the pathological implications of the risk variant in the *DYSF/ZNF638* locus by combining immunohistochemistry and RNA sequencing of single cell types. We compared the neuronal density in the NAGM, the axonal density, and the degree of acute axonal stress in the NAWM, the localization and amount of dysferlin and ZNF638 in and around WM lesions, and the gene expression profile of oligodendrocytes and neurons.

In **chapter 10**, the findings and conclusions described in this thesis are discussed.

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