Progression of multiple sclerosis

The role of microglia and neurons

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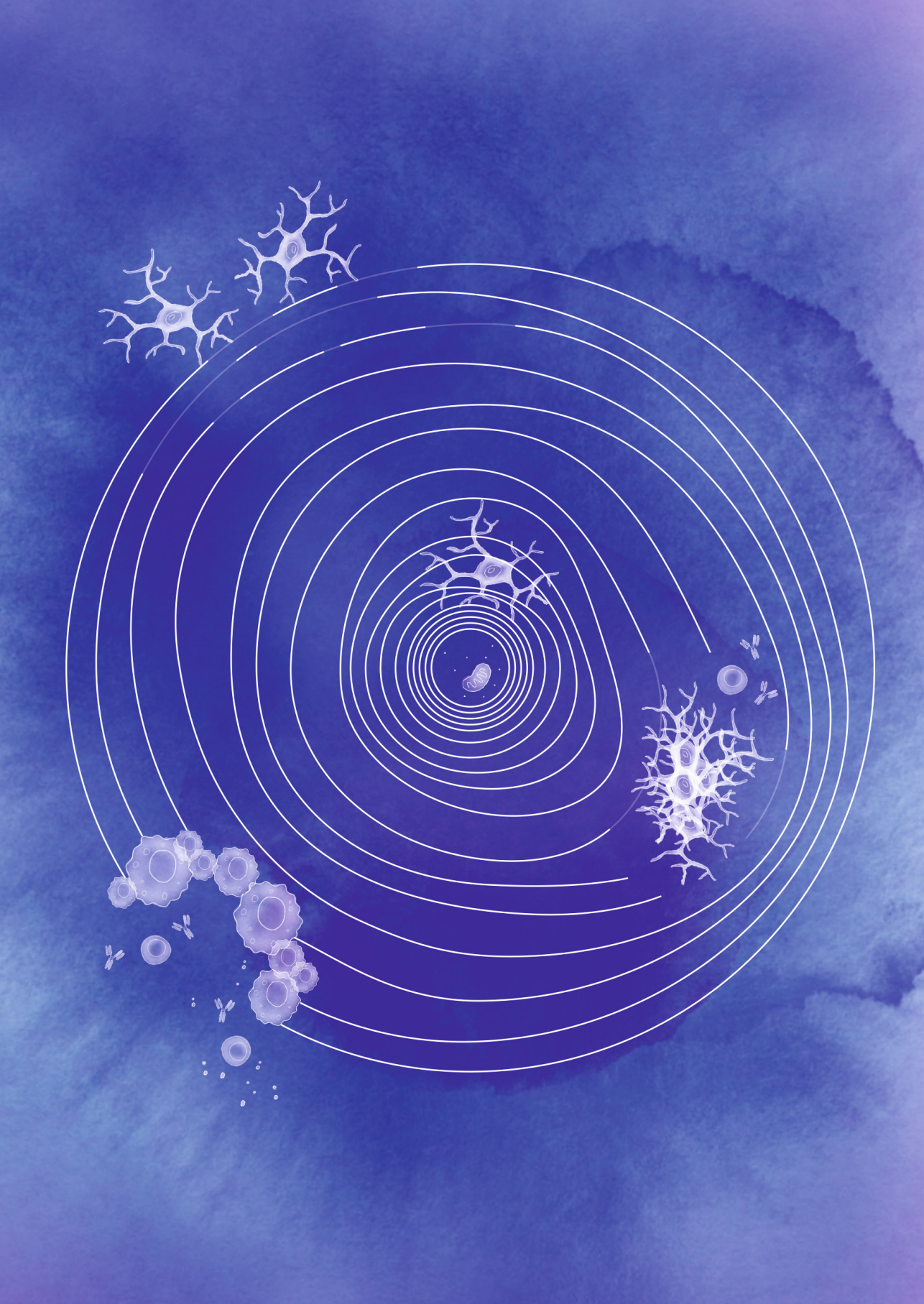

CHAPTER 10

General discussion

Multiple sclerosis (MS) is a chronic inflammatory disorder in which focal demyelination occurs throughout the central nervous system (CNS) [1]. Bi-directional communication between microglia and neurons plays an important role in MS pathology. Microglia are essential in regulating neuro-axonal health, and neurons and their axons can regulate microglia homeostasis. Altered neuro-axonal communication with microglia can therefore cause activation of microglia, and consequentially trigger or exacerbate neurodegeneration. Understanding how changes in neuro-axonal communication with microglia are contributing to pathological progression in MS may elucidate therapeutic targets to prevent initiation and expansion of MS lesions, and to promote regeneration. The proposed view of MS lesion formation, expansion and repair summarizing the findings of this thesis is displayed in **Figure 1**.

INITIATION OF LESION FORMATION

The observation of changes in MS normal-appearing white matter (NAWM) as putative origin of MS lesions formation is not novel. In 1989, an MRI study showed alterations in the NAWM compared to healthy control white matter (WM) [2], which later were in part attributed to focal microglial activation in the absence of clear demyelination [3]. Importantly, abnormalities detected by MRI in NAWM distant from WM lesions could not be attributed to axonal pathology [4], indicating that microglial activation occurs prior to axonal damage. More recently, abnormalities in the NAWM in MS seen on MRI were followed over time and shown to predict the likelihood of developing subsequent MS lesions [5]. Accordingly, there are subtle transcriptional changes in microglia in MS NAWM compared to control WM [6]. Top differentially expressed genes related to lipid metabolism and phagocytosis were also upregulated in active MS lesions indicating early demyelination by microglia in NAWM. Since microglia adapt to local changes in the CNS [7–9], subpopulations with distinct cellular states may differentially contribute to MS pathology. The mechanisms by which these changes can lead to progressive multifocal changes as observed in MS has been uncertain. In this thesis, some of these mechanisms have been unveiled, including changes in check-point molecule expression, myelin structure, axonal mitochondria distribution, and microglia clustering in the context of adaptive immune activation.

Decreased expression of check-point molecule CD200 and its receptor CD200R in the MS NAGM

Microglia in MS are chronically activated, and show partial loss of homeostasis in peri-lesion regions and NAWM [6,10]. We investigated the protein expression of CD200, which is mainly expressed by neurons and to a lesser extent by

oligodendrocytes. CD200 is a check-point molecule: binding of CD200 to its receptor, CD200R, on microglia keeps microglia in a homeostatic state [11,12]. Previously, it was shown that CD200 expression was lower in MS NAWM compared to control WM [11,12]. In experimental autoimmune encephalomyelitis (EAE), a mouse model for MS, CD200 expression is already down-regulated prior to any clinical symptoms [13]. In **Chapter 2** we show that in the normal-appearing grey matter (NAGM), CD200 expression is lower in layer 1 and layer 2 of the NAGM in MS compared to control grey matter (GM). The expression of CD200 was negatively correlated with cortical lesion rate. We hypothesize that the altered expression of CD200 may cause an altered activation state of microglia due to the loss of inhibition, and could lower the threshold for microglia to become activated and initiate demyelination. This indicates that the CD200-CD200R axis may play a pivotal role in susceptibility of GM lesion formation in MS.

Interestingly, the number of cells expressing CD200R was lower in MS NAGM compared to healthy GM. However, the low expression of CD200R by microglia makes interpretation difficult. In our study it remains unclear if there are less microglia in the MS NAGM cortex or if the microglia cells have lost their CD200R expression. CD200R blockage *in vitro* leads to increased secretion of inflammatory cytokines by macrophages and increased neuronal death in co-culture with hippocampal neurons. *In vivo*, blocking the CD200R leads to an spontaneous activation of microglia and aggravated clinical course of EAE, accompanied by increased infiltrates of T cells [14,15]. If cells in the MS NAGM have reduced CD200R expression, this may be related to the increased inflammation in the GM of people with MS, as seen in previous studies [6,16,17].

Myelin decompaction precedes demyelination in MS NAWM

Changes to the structure of myelin may be a second mechanism contributing to MS lesion formation susceptibility. Myelin derived from MS NAWM tissue is phagocytosed more efficiently than myelin derived from controls, which implies that there are already changes to myelin preceding demyelination [18]. With Nile red fluorescence spectroscopy, even in absence of histological myelin thinning or loss, a different polarity of the myelin in the NAWM in MS can be observed compared to healthy control myelin, indicating physiochemical alterations of the myelin sheath [19]. This may be due to an altered lipid metabolism in the MS NAWM, which leads to an increase in phospholipids and decrease in sphingolipids, that has been modelled to result in an increased repulsive force between myelin sheaths [20]. In **chapter 3**, we show, in line with previous research [21], that the nodes of Ranvier were disorganized. The elongation of the paranodal region could indicate that the myelin is wrapped less tightly around the axon. Indeed, we show that the G-ratio is smaller in MS compared to healthy controls,

indicating that the myelin diameter relative to the axon diameter is thicker in MS compared to controls. This relatively thicker myelin diameter was attributed to a less compact wrapping of the myelin in MS leading to gaps between the myelin lamellae.

Interestingly, the length of the paranode and the myelin decompaction were positively correlated to the number of activated and phagocytic microglia and to the number of T cells. *In vitro*, pro-inflammatory cytokines can cause elongation of paranodes [21]. Furthermore, pro-inflammatory cytokines *in vitro* can cause lipid metabolic defects leading to decreased sphingolipids and increased phospholipids [22], as seen in MS NAWM. Therefore, we conclude that the loss of homeostasis of microglia and the increased number of T cells in the NAWM in MS may trigger the ultrastructural alterations to the axon-myelin unit in MS. Alternatively, biochemically altering myelin structure in mice can elicit a demyelinating inflammatory immune response [23]. Together, altered myelin structure may be a trigger to initiate demyelination.

Increased axonal mitochondria may oxidize myelin and initiate demyelination

The observed myelin alterations may impact axonal energy demand. Decompaction of the myelin in the NAWM in MS implies that the axon is less well isolated, which may impact the action potential velocity. Additionally, the juxtaparanode has elongated in the NAWM in MS and has become overlapping with the paranode. The overlap of the juxtaparanode with the paranode may indicate that the potassium channels are unmasked, which can also cause alterations to the action potential velocity of the axon [24]. Mitochondria are highly dynamic organelles of the cell that are responsible for energy production. Although most axonal mitochondria are stationary, they will rapidly redistribute to sites of pathological stress and mitigate this stress through mitochondrial fission and fusion [25,26]. The density, shape, and size of axonal mitochondria are an indirect indicator of the axonal energy required to potentiate action potentials. In **chapter 3**, we show an increased number of axonal mitochondria in MS NAWM. It remains elusive if the increased number of mitochondria indeed indicates a higher axonal energy demand, or if it indicates a blockage of the transport of mitochondria.

The size of axonal mitochondria were comparable in MS NAWM and control WM. When mitochondria fragmentation occurs, increasing the number of mitochondria, while the surface area remains the same, the calcium buffering capacity increases. Unfortunately, although calcium buffering is considered neuroprotective, it also leads to an increase of free radicals, which when in pathological amounts can cause tissue damage and oxidize lipids, such as myelin

[27–30]. In MS NAWM, the level of oxidized phospholipids is higher compared to control WM [31]. We hypothesize that in regions where pathological levels of free radicals are produced, the myelin will become oxidized, and this will trigger demyelination.

Some microglia nodules form 'mini lesions'

Microglia nodules were described in relation to MS pathology for the first time in 1993 [32,33] and are regularly considered to precede MS lesion formation [34–42]. They are associated with axons undergoing Wallerian degeneration [37] and with encapsulation of activated complement deposits [34,35]. Microglia nodules are engaged in phagocytosis [43] and express both pro- and anti-inflammatory cytokines, such as tumour necrosis factor (TNF), interleukin (IL)-1β, and IL-10 [44,45]. In **chapter 4**, we show that MS donors with microglia nodules have a more exacerbated pathology than MS donors without microglia nodules, as they have a higher reactive site load, higher lesion load, higher proportion of active lesions, and lower proportion of inactive and remyelinated lesions. Therefore, microglia nodules are pathologically relevant. The question then remains whether microglia nodules are forming in a response to Wallerian degeneration due to damage caused by pathology or are themselves instigators of MS lesions. Microglia nodules are not restricted to MS, since these are also found in relation to Wallerian degeneration in brain donors with traumatic brain injury, ischemia, or stroke [37,42], where microglia nodules line up around complement-opsonized axons similar as in MS [34,35,42]. Therefore, differences between microglia nodules in MS and stroke should elucidate MS-specific characteristics of microglial nodules and their possible contribution to MS lesion formation. Using RNA sequencing, we show in **chapter 4** that although microglia nodules in MS share some commonalities with microglia nodules in stroke, there are many differentially expressed genes that are indicative for lesion formation in MS. Excitingly, microglia nodules in MS, and not in stroke, express genes that have previously been associated with MS lesion pathology and lipid metabolism, possibly indicating demyelination. In **chapter 4**, we show that microglia nodule have indeed phagocytosed oxidized phospholipids and encapsulate partially demyelinated axons, forming 'mini-lesions'.

Lymphocytes are involved in progression of microglia nodules into lesions

There must be fate-determining factors which determine whether a microglia nodule will progress into an MS lesion, or if it will resolve. One of these factors could be interaction with cells of the adaptive immune system. In **chapter 4**, we show that microglia nodules in MS are in a more inflammatory environment, as there are B cells, immunoglobulin (Ig) producing B-lineage antibody secreting cells and activated T cells in close proximity to microglia nodules in MS and not

in stroke. Ig produced by B cells can break the immune-tolerance of microglia [46], which indicates that these microglia nodules in MS are perhaps easier to activate through pro-inflammatory cytokines. Furthermore, phagocytosis of oxidized phospholipids, together with activation by pro-inflammatory cytokines, can lead to a hypermetabolic and hyperinflammatory phenotype. Indeed, gene expression indicates that microglia nodules in MS are under metabolic stress, and the mitochondria network of microglia nodules in MS is hyperfused, which is indicative of hypermetabolism. Lastly, the association of microglia nodules with C1qB combined with the presence of Ig leads to formation of the membrane attack complex, which can lead to osmolysis of the tissue. Therefore, presence of pro-inflammatory cytokine-secreting T cells and Ig-producing B-cell blasts makes a microglia nodule in MS more prone to lesion formation.

LESION PROGRESSION

Molecular pathways underlying disease progression of MS are not yet fully understood. Consequently, there are no suitable biomarkers currently available that can monitor lesion formation, smouldering lesion expansion, remyelination, or scar formation. Therefore, predicting the disease course of people with MS is challenging. Although the overlap is substantial, people with MS are commonly categorized based on dominant clinical descriptors as relapsing-remitting (acute attacks followed by recovery), primary progressive (gradual worsening from onset), and secondary progressive (relapsing-remitting at onset but gradual worsening later in the disease course) [47]. These clinical entities are challenged by recent views that people with relapsing-remitting MS also show progression independent of relapses [48], and a substantial part of people with primary-progressive MS also show relapses [49]. Although several mechanisms contribute cumulatively to disability progression, expansion of white matter lesions has been demonstrated as a relevant correlate of disability progression. On MRI, lesion evolution can be monitored with conventional techniques, such as T2-weighted and gadolinium-enhanced T1-weighted sequences, over time within patients. Lesions can shrink over time, reflecting noninflammatory processes, such as regeneration and repair, become larger over time, or turn into chronic black holes, correlating with permanent demyelination and severe axonal loss [50,51]. In this thesis, we aimed to understand the molecular mechanisms underlying lesion de- and remyelination, to create a better understanding of disease progression.

Concurrent pathophysiological mechanisms occur simultaneously in MS

Acknowledging the limitations as discussed above, clinical characterisation and treatment selection could be improved by stratification based on disease-

driving pathophysiological mechanisms rather than the traditional clinical descriptors [52]. Therefore, to understand disease progression in MS, all known pathology throughout the CNS should be taken into account. In **chapter 5**, we performed unbiased dimension reduction considering all pathological and clinical parameters known of individual people with MS at the NBB. We show that MS pathology is characterized by three distinct dimensions that are not correlated to each other, that are driven by lesion activity, microglia morphology, cortical pathology, nodules, and infiltrating lymphocytes. These concurrent pathophysiological mechanisms together influence disease progression. The dimensions disentangled MS pathology into 1) microglia activation with ongoing demyelination, 2) lesion formation and microglia activation without demyelination, and 3) loss of lesion activity and scar formation.

Cortical pathology is associated with WM pathology and disease progression

Cortical lesions, and particularly leukocortical lesions and intracortical lesions but less so subpial cortical lesions, are associated with subcortical demyelinating and inflammatory lesion activity [53]. In line with this, interestingly, in **chapter 2**, we show that expression of check-point molecule CD200 in the GM was positively correlated with the expression of CD200 in the WM. Accordingly, the WM pathology was also more severe in MS donors with lower CD200 expression in the GM. This indicates that mechanisms of lesion initiation in the GM and the WM may have donor-specific similarities. In **chapter 5**, we show that cortical pathology is associated with all three dimensions of pathology related to disease progression. The cortical lesion rate is positively correlated to dimension 1, which is associated with microglia activity and ongoing demyelination, and to dimension 3, which is associated with loss of lesion activity and scar formation. Interestingly, dimension 3 is mainly associated with subpial lesions, which are, in contrast to other cortical lesion types, indeed not correlated with WM lesion activity [53].

Similarly to CD200, CD47 is also a check-point molecule, which is mainly expressed by neurons and to a lesser extent by oligodendrocytes. Binding of CD47 to its receptor, SIRPα, keeps microglia in a more homeostatic state. Previously, loss of CD47 has been implied in the uncontrolled internalization of myelin, and has been hypothesized to boost myelin uptake and promote demyelination [54]. In **chapter 2**, we show that in contrast to CD200, CD47 expression was similar in MS NAGM and control GM, however CD47 expression was lower in cortical peri-lesion regions and in cortical lesions compared to the NAGM. Previously, this was also found in the peri-lesion region of mixed lesions in the WM, where it was hypothesized to be involved in expansion of lesions. We hypothesize that CD47 is similarly involved in the expansion of GM lesions.

Foamy microglia morphology is associated with degeneration and smouldering lesion expansion

Reflecting on the dimensional reduction analysis, the dimension associated with foamy microglia in lesions associates with fastest accumulation of disability. In line with this, previously we have shown that the proportion of active and mixed lesions in the CNS correlates with a faster disability accumulation in MS [53]. Interestingly, the microglia/macrophage activity score is positively correlated with the lesion load and the proportion of active lesions. This indicates that foamy microglia in active and mixed lesions may be associated with lesion progression. In **chapter 5**, we show that the microglia/macrophage activity score is positively correlated with dimension 1, which was associated with microglia activation with ongoing demyelination, but negatively with dimension 2 and 3, which were associated with ramified microglia activation without demyelination and with loss of lesion activity, respectively. This indicates a biological difference between active and mixed lesions with ramified microglia compared to those with foamy microglia. Accordingly, in **chapter 6**, we show that only the proportion of active and mixed lesions with foamy microglia positively correlate with the neurofilament light chain levels in the cerebrospinal fluid (CSF), and not the proportion of those with ramified microglia. This indicates that active and mixed lesions with foamy microglia are associated with more acute axonal stress than those with ramified microglia. Indeed, with immunohistochemistry, we show that that acute axonal damage is most prevalent in active lesions with foamy microglia and the border of mixed lesions with foamy microglia and not in those with ramified microglia. Interestingly, although uptake of myelin debris by microglia can induce an anti-inflammatory phenotype that is beneficial to repair, excessive lipid uptake and loss of lipid efflux may drive foamy microglia towards a more pro-inflammatory phenotype that limits remyelination [54,55]. Possibly, lesions with foamy microglia are more degenerative than those with ramified microglia. In **chapter 7,** we show that, compared to mixed lesions with ramified microglia, the border and peri-lesion region of those with foamy microglia have a higher expression of genes that indicate degeneration. Lesions with foamy microglia have a disturbed iron metabolism, increased antigen presentation and Ig production, more oxidative stress, and more destabilization of microtubules. Additionally, the higher number of oligodendrocytes with enrichment of immune-related pathways, immune-oligodendrocytes, in lesions with foamy microglia may perpetuate sustained demyelinating activity, as these are cytotoxic targets [56]. As the gene expression in the peri-lesion region of mixed lesions with foamy microglia shows similarities with the border of these lesions with foamy microglia, we hypothesize that these lesions are expanding.

Lymphocytes contribute to demyelinating activity

Only few lymphocytes populate the non-diseased human brain [57,58]. In MS NAWM, the number of T and B cells is higher compared to control WM, and there is a further enrichment of T and B cells in MS lesions [57,59,60]. B-cell presence in lesions is associated with a more severe clinical MS, higher proportion of mixed lesions and T-cell clustering [61]. Most lymphocytes are peri-vascular, although some are found in the parenchyma [62,63]. In MS, some B cells develop into antibody-secreting cells, and are responsible for intrathecal and local immunoglobulin (Ig) production [57,62]. This is in line with the presence of oligoclonal bands in the CSF of people with MS [64]. Recently, spatial transcriptomics has shown that there may be a higher number of B cells in the MS brain than previously believed. These B cells lack the commonly used B cell marker CD20, and highly express CD79a and CD38, implying their development into Ig producing cells [62]. This is of special interest, as Ig can break the immune tolerance of microglia, making them more prone to activation by cytokines [46]. Additionally, Ig can activate the complement cascade, ultimately leading to formation of the membrane attack complex [65]. In **chapter 4**, we show that activated T cells secreting cytokines and Ig-producing B cells may be involved in progression of a nodule into an MS lesion. Accordingly, in **chapter 5**, we show that perivascular and parenchymal T cells and plasma cells in the brain stem as well as presence of cuffing is associated with a higher score on dimension 1, indicative of microglia activation and ongoing demyelination throughout the CNS and with clinically more severe MS. We additionally show that absence of cuffing is associated with a higher score on dimension 3, indicating loss of demyelinating activity and scar formation. Strikingly, in **chapter 7**, we show that especially in mixed lesions with foamy microglia Ig is produced by plasma blasts. We propose that mixed lesions with foamy microglia are expanding and are degenerative based on their gene expression profile and presence of acute axonal damage. Together, this indicates that CNS-resident populations of T and B lymphocytes are associated with both initiation of demyelination and lesion formation, as well as lesion expansion and ongoing demyelination through sustained microglia activation.

LESION REPAIR AND REMYELINATION

Remyelination can be extensive in MS despite having a long disease course [53,66]. It is thought that many active and some mixed lesions have the potential to remyelinate, which means that not all active and mixed lesions have equal remyelinating potential [67].

Axon stability is important for regeneration

In **chapter 5,** we show that dimension 1 is negatively correlated with lesions with ramified microglia, and positively correlated acute axonal damage. In line with this, dimension 3, associated with loss of microglia activation, is associated with lower levels of acute axonal damage. In **chapter 6**, we show that mixed lesions with ramified microglia are associated with less acute axonal damage than those lesions with foamy microglia. Accordingly, in **chapter 7**, we show that in the border and peri-lesion region of mixed lesions with ramified microglia, there is higher expression of genes associated with synaptic functioning, cytoskeleton organisation, neurite outgrowth, and stability of the microtubule network, compared to those with foamy microglia. In contrast, in lesions with foamy microglia, there is higher expression of genes associated with repulsion of axonal growth. Therefore, ramified microglia may support axon regeneration, and the environment of these ramified lesions compared to foamy lesions may be more permissive to regeneration and remyelination. Taken together, this indicates that the morphology of microglia in these lesions could be an indicator for their remyelinating potential.

Preservation of myelin stability and more remyelination in mixed lesions with ramified microglia

In **chapter 7**, we show that in the border of mixed lesions with ramified microglia, there is a higher expression of genes that are associated with myelin stability, myelin ensheatment, and remyelination, compared to the border of mixed lesions with foamy microglia. On a protein level, we show that there is a comparable number of $SOX10^{+}QKI^{+}$ and $SOX10^{+}ABCA2^{+}$ oligodendrocytes in the border of mixed lesions with ramified microglia compared to the NAWM, and a loss of these oligodendrocytes in the border of mixed lesions with foamy microglia. This indicates that in these lesions with foamy microglia, there is a loss of myelin integrity and stability. As there is no difference in the number of total oligodendrocytes, it is likely that the myelin in the border of mixed lesions with foamy microglia has lost integrity, therefore insulating the axons less efficiently, but is still present. As myelin has inhibitory effects on the ability of oligodendrocyte progenitor cells to differentiate into mature remyelinating oligodendrocytes, and therefore clearance of instable myelin in the border of mixed lesions with foamy microglia is an essential step for efficient remyelination [68–70].

GENETIC SUSCEPTIBILITY AND PATHOLOGY

Lastly, genetic constitution of individuals could also contribute to differences in disease progression and lesion expansion. From twin and familial clustering studies is has become clear that genetics play a role in MS. The theory of common disease-common variant indicates that diseases with high prevalence in a population, such as MS, are caused by several small, frequently occurring, genetic variations. This implies that many single-nucleotide polymorphisms (SNPs) will each have a small effect toward the disease [71,72].

rs3135388 is associated with microglia activation without demyelination
To date, over 200 SNPs have been associated with increased risk of MS. Most of these associated loci are related to immunological pathways, affecting B cells, T cells, natural-killer cells, myeloid cells and microglia [73,74]. In **chapter 5** we found that the polygenetic risk score, which is an estimate of an individual's genetic liability to develop MS, is associated with dimension 2 of disease progression, that was associated with microglia activation without demyelination. This dimension is considered to reflect initiation of lesion formation due to the association with microglia nodules. This association is driven by the rs3135388 SNP. This tagging SNP of the HLA-DRB1*15:01 allele, confers a greatly increased susceptibility to develop MS [75,76].

rs10191329 is associated with exacerbated pathology
Intriguingly, none of the SNPs associated with increased risk of onset of MS have been found to be associated with clinical MS severity [77]. Lately, the International Multiple Sclerosis Genetics Consortium and the MultipleMS Consortium identified rs10191329 as a SNP associated with earlier age of disability accumulation [78]. In **chapter 8**, we show that homozygous rs10191329 SNP carriers have a higher cortical lesion rate and a higher lesion load. In **chapter 5**, we show that homozygous carriers of rs10191329 all score high on dimension 1, associated with microglia activation and ongoing demyelination and with a faster accumulation of disability. Accordingly, in **chapter 9**, we show that homozygous rs10191329 carriers compared to non-carriers have a higher proportion of active and mixed lesions with foamy microglia. Furthermore, we show that homozygous rs10191329 carriers have a decreased neuronal density in the NAGM and have more acute axonal stress in the NAWM. WM lesions of homozygous rs10191329 carriers have a higher number of T cells than non-carriers. With nuclear RNA sequencing of oligodendrocytes and neurons we identified mitochondrial changes in homozygous risk carriers compared to homozygous non-risk carriers, which may be indicative of stressed cells. Collectively, these findings consistently indicate that homozygous rs10191329

carriers have a more severe pathology showing more lesions with higher levels of acute tissue damage.

The rs10191329 SNP is located in the *DYSF-ZNF638* locus [75]. A causal relationship between MS progression and the gene products of this locus remains to be show. Dysferlin is a mediator of plasma membrane repair in response to membrane damage [79]. We show that it is mainly expressed by oligodendrocytes in the WM and by neurons in the GM. The number of dysferlin$^+$ cells in the NAWM and NAGM is increased in homozygous rs10191329 carriers compared to non-carriers, which may be associated with the increased acute axonal damage. ZNF638 is a factor implicated in viral transcriptional silencing in association with the human silencing hub complex [80]. Similar to dysferlin, we here show that ZNF638 is also mainly expressed by oligodendrocytes in the WM and by neurons in the GM. The increased number of ZNF638$^+$ cells in the NAWM and NAGM may imply a failure to silence viral DNA, possibly due to alternative splicing.

SUMMARY

Data presented in this thesis indicates that loss of homeostasis of microglia and decompaction of myelin sheaths precede lesion formation, and that microglia nodules are the first starting point of MS lesions. Different pathophysiological processes are simultaneously occurring in people with MS, which combined with the genetic make-up are indicative for the clinical and pathological progression of the patient. Lastly, especially lesions containing foamy microglia, and less so those containing ramified microglia, are degenerative, with ongoing demyelination triggered by Ig-producing plasma blasts. Lesions containing ramified microglia, on the other hand, may hold the key to regeneration and remyelination.

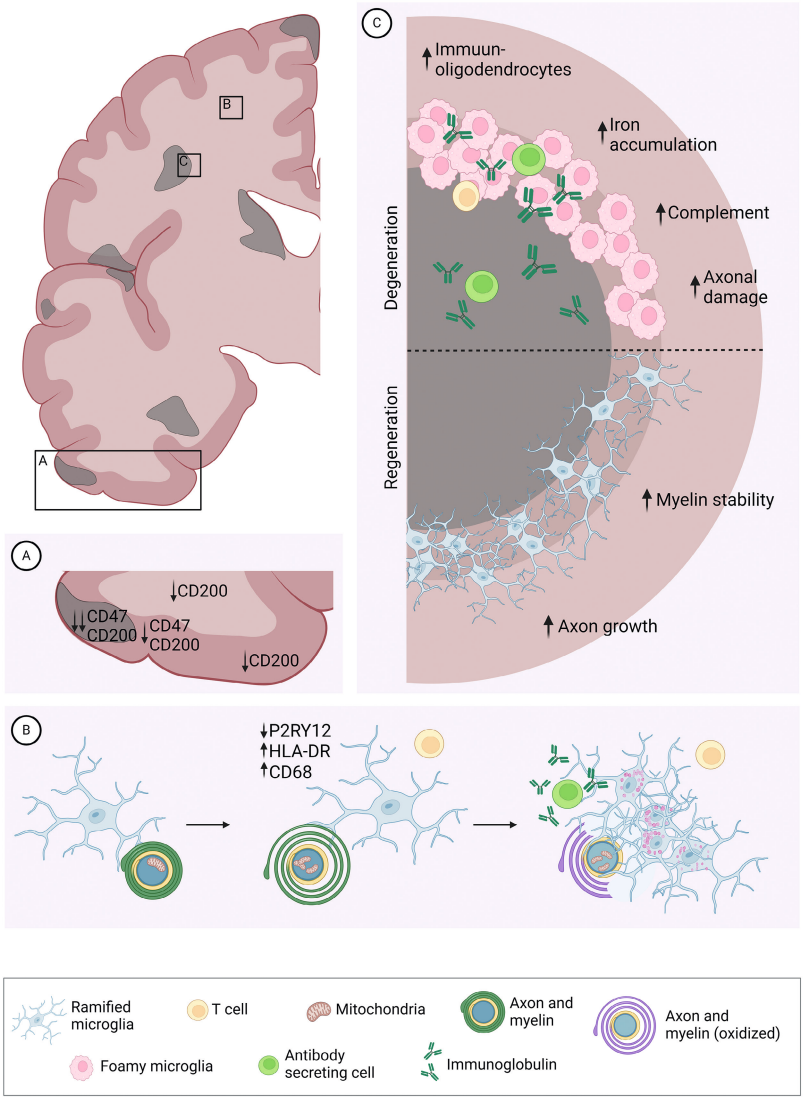

Figure 1: Summary of the findings of this thesis. A) In normal-appearing white and grey matter, CD200 expression is decreased. In grey matter lesions and peri-lesion regions, CD47 and CD200 expression is decreased. B) In the normal-appearing white matter there are more T cells and activated microglia which is correlated to less compact wrapping of the myelin and an increase of axonal mitochondria. Microglia nodules form, clear up oxidized phospholipids, and are activated by T cells and by immunoglobulin produced by antibody secreting cells, leading to 'mini-lesions'. C) Mixed active/inactive lesions with foamy microglia are likely expanding and are characterized by complement activation, immunoglobulin production, immune-oligodendrocyte destruction, and axonal damage. Contrastingly, those with ramified microglia are likely remyelinating or becoming inactive and are characterized by higher myelin stability and axonal growth.

FUTURE DIRECTIONS

As usually in science, our findings have led to a multitude of new questions. Future research addressing these questions can validate, strengthen, and deepen our knowledge on the role that microglia and neuro-axonal communication have in initiation of lesion formation, expansion and repair. In this section, I would like to elaborate on some interesting future directions we can take from here. Excitingly, potential therapeutic targets have come from the studies in this thesis, some of which I want to highlight in this section.

In the first part of this thesis, pathways involved in initiation of lesion formation were discussed. CD200 emerged as an interesting mechanism to dampen microglia activation and to prevent loss of homeostasis. However, the mechanism driving the reduction of CD200 in MS remains unclear. As the decreased expression in the NAGM is limited to the first two cortical layers, those that are closest to the meninges, perhaps soluble factors in the CSF or in the serum are mediating this expression of CD200. Investigating the expression of CD200 *in vitro* in neuronal cells, such as SY-SH5Y cells, before and after incubation with CSF, cytokines, and Ig, would be of interest. Next, *in vitro* studies should study the effect of loss of neuronal CD200 on microglia in a co-culture of neuronal cells with microglia, by studying their cytokine secretion and potency to phagocytose myelin. Increasing the expression of CD200 in the same co-culture should then decrease the inflammation and their phagocytic potency. If CD200 upregulation dampens microglia activation, this may also rescue the disorganisation of the paranode and juxtaparanode domain [21].

The tubular mitochondria in microglia nodules in MS imply hypermetabolism and hyperinflammation. We have proposed that the activation of microglia nodules by phagocytosis of oxidized phospholipids together with the activation by cytokines has led to a hypermetabolic and hyperinflammatory state. This needs to be functionally validated, e.g. through metabolic measurements with a Seahorse analysis of microglia that have phagocytosed oxidized lipids and have been activated through pro-inflammatory cytokines. If they are indeed hypermetabolic, removal of one of the activators should prevent the microglia from becoming hypermetabolic and hyperinflammatory through this pathway. In that case, oxidative species and oxidized lipids are interesting targets. Superoxide dismutase (SOD) is an antioxidant that converts the superoxide generating hydrogen peroxide, which then is catalysed by glutathione (GSH) into water [81]. In some MS lesions, the expression of SOD is increased, likely reflecting a protective response [82]. Perhaps, general upregulation of SOD or GSH through viral vectors can successfully catch some of the oxidative species before the myelin is oxidized. The Keap1-Nrf2 pathway is activated by oxidative

stress to activate transcription of antioxidant genes [83]. Upregulation of Nrf2 may therefore also be an interesting therapeutic target to prevent oxidative stress and damage. Alternatively, antibodies targeted against oxidized phospholipids, such as E06, may prevent the phagocytosis of the myelin. However, inhibiting phagocytosis of damaged myelin may be more detrimental than beneficial, as this will likely inhibit remyelination [68]. The hypermetabolism of microglia nodules itself may present an interesting therapeutic target. In the hypermetabolic state, both oxidative phosphorylation as well as glycolysis are maximally utilized, which leads to longevity of the cell, inflammation, and production of oxidative species. It is possible to inhibit oxidative phosphorylation by inhibiting complexes of the mitochondrial electron transport chain (ETC). Metformin, a treatment for diabetes type 2, is an example of a therapeutic target that can block the first complex of the ETC and reduce the production of oxidative species, which may have a beneficial effect [84,85]. In the third part of this thesis, gene expression analysis of oligodendrocytes and neurons of homozygous carriers of the progression risk SNP rs10191329 and homozygous non-risk carriers indicated mitochondrial changes which may imply cellular stress. Similarly to the microglial nodules, a Seahorse analysis on isolated oligodendrocytes and neurons of risk carriers and non-risk carriers or on CRISPR-cas9 gene edited cell-lines will shed more light on the functional implications of the increased mitochondrial gene expression.

In the second part of this thesis, pathways involved in lesion expansion and lesion repair were discussed. Here, CD47 emerged as an interesting target to halt lesion expansion. As this is also the case for WM mixed lesions thought to be expanding [12], targeting CD47 may be beneficial for both WM and GM. Similar to CD200, functional tests are still needed to gain a better understanding of the molecular mechanism of CD47, and how down- or upregulation of this check-point molecule influences microglia activity, inflammation, and phagocytic propensity.

Iron dysregulation has been described in multiple sclerosis pathology, although it's exact role in disease progression is not yet clear [86]. If iron in the border of mixed lesions has accumulated in microglia, this may lead to microglial dysfunction and ultimately ferroptosis [87]. Ferritin is an iron storage protein that sequesters ferrous iron, playing an important antioxidant role in cells [88]. We here show that there is a higher expression of ferritin in the rim and peri-lesion region of mixed lesions with foamy microglia, accompanied by a higher density of ferritin^{+} microglia in these regions. This is indicative of more prominent iron dysregulation in mixed lesions with foamy microglia than those with ramified microglia, both in the border and the peri-lesion region. Further studies are necessary to understand what cells are accumulating iron in MS pathology,

and what the functional effect is. Iron chelators such as desferrioxamine (DFO) suppress ferroptosis by reducing the availability of iron, and may be an interesting therapeutic target [89]. Alternatively, blocking nuclear receptor coactivator 4 (NCOA4) through compound 9a, should prevent the delivery of iron-bound ferritin to autophagosomes for lysosomal degradation and ferrous iron release [90].

A common mechanism of disease progression in both the first and second part of this thesis is Ig. Ig are found near microglia nodules, where they may be involved in breaking the immune tolerance of microglia and activating the complement cascade. Furthermore, Ig production is more prevalent in mixed lesions with foamy microglia than in those with ramified microglia, and depending on the target of the Ig produced this may promote ongoing demyelination. The opsonized structures were heterogenic between donors, and this may translate into a different pathological or clinical development of MS. Therefore, it would be interesting to study the target of Ig found intrathecal, and to see if the same targets are opsonized in the brain. B cells are a well-known target for therapeutic intervention. Anti-CD20-mediated B-cell depletion, through rituximab, ocrelizumab, or ofatumumab, has a high level of success in limiting new events in relapsing MS. However, CD20 is not expressed on plasma cells, therefore immunoglobulin levels remain above the normal range after treatment [91]. Our data indicates that anti-CD79a-mediated B-cell depletion may be a more promising avenue. However, such monoclonal antibodies have poor blood-brain barrier penetrance [92], and effects within the brain may therefore be limited. Alternatively, Bruton tyrosine kinase (BTK) inhibitors are CNS-penetrant, and can target the maturation, survival, migration and activation of B cells and microglia. BTK inhibitors may therefore be a promising therapeutic approach, and are currently undergoing clinical trials [93].

We show that in lesions that are likely not expanding, there is more myelin stability and maintenance, and more axonal health, compared to lesions that are likely expanding. Genes involved in these processes that were higher expressed in the border of mixed lesions with ramified microglia compared to the border of mixed lesions with foamy microglia may provide interesting therapeutic targets to promote regeneration and remyelination, and inhibit lesion expansion. Among these genes are *QKI*, *ABCA2*, *APOD, BCAS1,* and *BOK*. Functional implications of up- or downregulation of these genes needs to be tested *in vitro* and *ex vivo*, before being tested *in vivo*.

Lastly, in the studies described in this thesis, we have capitalized on recent exciting revolutions in transcriptomics techniques that have now reached unprecedented resolution and sensitivity. In addition, we applied high- and ultra-resolution

microscopy. We have focused on optimizing protocols to apply these techniques on post-mortem human brain tissue. We adapted the protocol for cryo-tissue, and achieved high-resolution immunofluorescence visualization using super-resolution confocal microscopy and semi-automatic quantification with Imaris software. Possibly, future immunofluorescent studies may benefit from the use of multispectral light-emitting diodes (mLEDs) to minimize autofluorescence [94]. We successfully visualized and quantitatively characterized the axon-myelin unit at an ultrastructural level using transmission electron microscopy (EM). For future studies, scanning EM and 3D reconstruction of consecutive images could uncover additional interesting structures in the human brain [95]. Cryo-EM may reduce some fixation artefacts [96], and correlative-light-EM will facilitate the identification and characterization of specific interactions and regions of interest [97]. Lastly, we performed transcriptome-wide spatial transcriptomics at single-cell resolution. Various platforms are now commercially available, each with its own advantages and limitations. Some platforms offer genome-wide analysis, while others are probe-based approaches. StereoSeq (BGI) and Visium (10X Genomics) are genome-wide, whereas MERFISH (Vizgen), ISS (Cartana), and GeoMX DSP (Nanostring Technologies) are panel-based. The capturing area also varies. While most platforms offer a broad range of (customizable) chip sizes, those of Visium are limited to 6.5 x 6.5 mm, although each slide contains multiple chips, allowing multiple regions of interest to be sequenced per slide, which is cost-effective. The resolution varies significantly between platforms. Of the genome-wide platforms, StereoSeq offers the highest resolution, with capture sports of 200 nm. Visium on the other hand, has capture spots of 55 µm, and the recently launched Visium HD as capture spots of 2 x 2 µm. Because of its subcellular resolution, StereoSeq requires binning multiple spots based on an ssDNA image to achieve single-cell resolution, which poses bioinformatic challenges, whereas Visium will encompass multiple cells within a single spot. Results of both Visium and Visium HD are dependent on cellular density. Among the panel-based platforms, MERFISH provides single-molecule resolution, ISS offers single-cell resolution, and GeoMX DSP can achieve resolution as low as 10 µm. For our spatial transcriptomics project, we required discovery driven genome-wide analysis of large regions with varying cellular densities, therefore StereoSeq was the optimal choice. However, depending on the specific research question and tissue selection, other platforms might be more appropriate for other research questions. Continuous technological advancements in spatial transcriptomics hold significant promise for the future.

"Whereof one cannot speak, thereof one must be silent."
Ludwig Wittgenstein

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