Diversity of microglia

Their contribution to multiple sclerosis lesion formation

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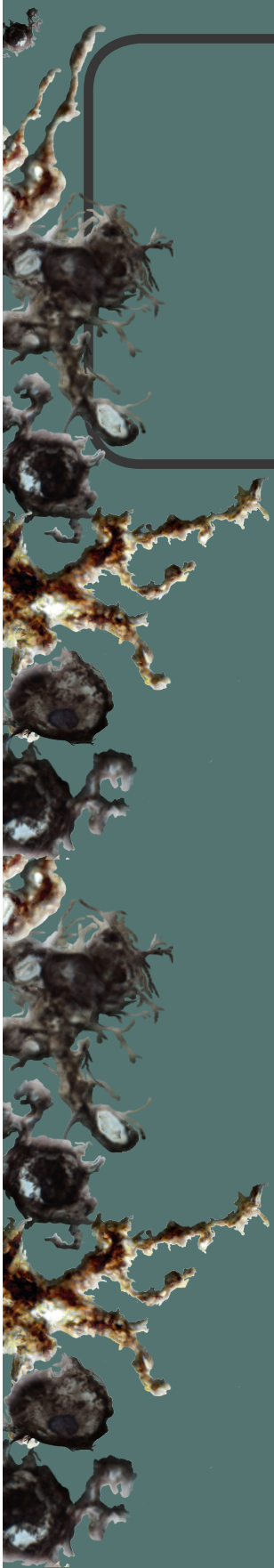

Chapter 1

General introduction

1. Multiple sclerosis – a chronic neuroinflammatory disease

1.1 Diagnosis and prevalence

Multiple sclerosis (MS) is a chronic neuroinflammatory disease, characterized by myelin loss and axonal damage, which leads to scar formation in the central nervous system (CNS)[1–3]. Clinically, MS patients present with visual, motor and sensory problems, but also develop symptoms related to cognition and fatigue[4]. In Europe, 108 in 100.000 people develop MS, which is often diagnosed at a young age, between 20-40 years, and the prevalence is higher in woman[4,5]. Main diagnostic criteria are the appearance of white or grey matter lesions with dissemination over time and space, detected by magnetic resonance imaging (MRI), and the presence of intrathecal antibodies, detected as oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF)[6,7]. The clinical course of MS is classified in various subtypes. The majority of patients (85%) is diagnosed with relapsing–remitting (RR) MS, having periods with acute relapses followed by (partial) neurological recovery, called remission. On average 20 years after disease onset, the majority of patients with RRMS advance to the progressive form, which is named secondary progressive (SP) MS and is classified as steadily worsening of neurological symptoms without the presence of relapses. In addition, 10-15% of the MS patients are diagnosed with progressive MS at disease onset, classified by worsening of symptoms without any relapses[5].

The cause of MS is unknown, but traditionally, MS is considered to be an autoimmune disease, whereby innate and adaptive immunity directed against myelin are involved. However, the antigen(s) that evoke(s) an immune response is unknown. In addition, environmental factors, such as viruses, lack of sunlight exposure, smoking and diet, are known to increase the odds to develop MS[8]. Furthermore, human leukocyte antigen (HLA)-DR and other genes related to immune function have been identified as risk factors for MS, and a combination of both genetic and environmental risk factors might contribute to the development of MS[8].

1.2 Therapeutic targets

Most disease-modifying therapies that are used in the clinic to treat RRMS focus on immune modulation and target the adaptive immune system by reducing infiltration of peripheral lymphocytes into the CNS or inhibiting their activation[9,10]. Furthermore, drugs that promote remyelination have been developed, but their efficacy is still under investigation in clinical trials[9]. An important drawback of most therapeutic drugs that are approved so far is their limited effect in the progressive, advanced stage of MS[9]. Therefore, there is an urgent need for therapies treating progressive MS. Recently, B-cell depletion therapies, such as ocraluzimab, showed for the first time a reduced clinical and radiological progression in patients with PPMS[11–13]. However, the long-term efficacy of B-cell depletion therapies to treat MS remains incompletely elucidated. Importantly, treatments that have been approved so far only reduce the symptoms of MS, but no effective therapy to successfully treat this complex disease has been found yet.

1.3 Neuropathology

From a neuropathological perspective, MS lesions are classified into different subtypes, defined by immunohistochemical stainings that visualize intact myelin and activated microglia/macrophages (**Figure 1**). Myelin intactness is visualized by myelin proteolipid protein (PLP) staining, and microglia/macrophage activation and morphology is visualized by HLA-DR staining[1,14,15]. Four types of white matter (WM) lesions are defined: active, mixed active/inactive (mixed or chronic active), inactive and remyelinated lesions (shadow plaques). Furthermore, also cortical grey matter (GM) lesions are classified, defined by their location in the cortex[14]. It has been proposed that GM lesions are different from WM lesions, demonstrated by a low number of infiltrating lymphocytes and a low number of activated microglia/macrophages in GM lesions[16,17]. Besides the presence of lesions, reactive sites have also been found in MS tissue, characterized by intact myelin and the presence of accumulating ramified HLA-DR$^+$ microglia. These reactive sites can contain clusters of HLA-DR$^+$ microglia, so-called nodules. Furthermore, normal-appearing MS tissue without lesions or reactive sites exists, characterized by ramified microglia and absence of demyelination, based on PLP staining. However, this normal-appearing tissue already shows alterations in myelin composition and axonal integrity as compared to control WM[18,19]. In sum, different MS lesion subtypes have been described, whereby microglia activation relates to lesion activity, and it has been demonstrated that MS lesion pathology is highly heterogeneous among patients[1,14].

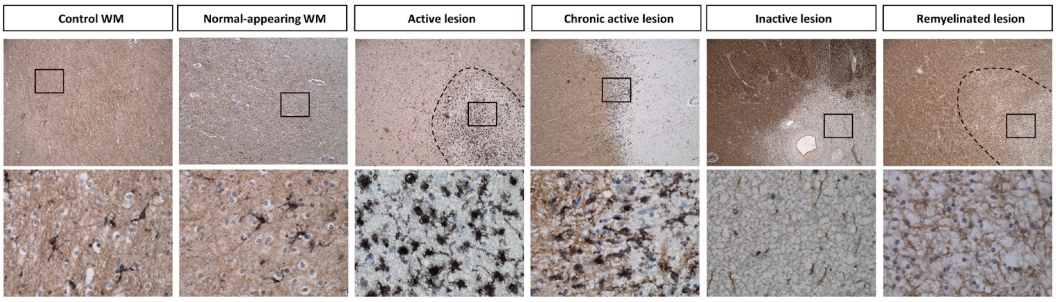

Figure 1 | Characterization of MS lesions by myelin protein PLP and microglia/macrophage activation marker HLA-DR. Immunohistochemistry pictures show myelin intactness and microglia/macrophage activation, stained by myelin proteolipid protein (PLP; brown) and human leukocyte antigen (HLA)-DR respectively (black), to identify control white matter (WM), normal-appearing WM, active, chronic active, inactive and remyelinated MS lesions. Second row of pictures is zoomed in on the square, indicated in the pictures on the first row, to show HLA-DR stained microglia/macrophage presence or absence and morphology. Lesions were characterized based on criteria published by Luchetti *et al*., 2018[14].

1.4 Innate and adaptive immunity

An important feature of MS is the combination of neurodegeneration and inflammation, whereby cells of the innate and adaptive immune system play a key role in MS pathology[15,20–23]. In the relapsing–remitting stage of MS, the blood-brain barrier (BBB) is damaged, which promotes entering of leukocytes into the brain[24,25]. Both T and B cells are present in active lesions in early and late MS, with the highest numbers found in early MS, whereas a higher number of antibody-secreting (plasma) B cells is found in late MS[21,22]. It is not known what exactly causes disruption of the BBB, but it is a fea-

ture of the early phase of MS. Contrary, it has been suggested that patients with progressive MS have a relative intact BBB, as shown by MRI studies that hardly encounter gadolinium-enhanced lesions in progressive MS, together with the limited success of relapsing MS therapies to treat progressive MS patients[3,25]. Additionally, only tissue-resident memory T cells are detected in active lesions of progressive MS brain donors, providing evidence for a closed BBB[22,26]. However, these findings are still inconclusive since several studies show alterations in tight junctions of the BBB in progressive MS[27,28], but it needs to be demonstrated if these alterations result in BBB leakage and facilitate infiltration of peripheral immune cells. If the BBB indeed is relatively intact in progressive MS this may indicate that the immune response is compartmentalized in the CNS[22], therefore an important role arises for immune cells present in the CNS in progressive MS.

Next to cells of the adaptive immune system, also innate immune cells play an essential role in MS. Microglia are resident phagocytes of the CNS and in MS, next to macrophages, they take up myelin and contribute to demyelination[1,20,29–31]. In active and mixed MS lesions, microglia are found in the lesioned center or rim respectively, and classification of lesion activity is based on their presence and morphology, as they can appear as ramified, round or foamy, detected by HLA-DR staining[14]. After prolonged uptake of myelin, detected by myelin oligodendrocyte glycoprotein (MOG) or oil-red O staining in active MS lesions[32], microglia appear as foamy, lipid-laden cells and might play an important role in immune regulation. It is still a debate whether activated microglia can start demyelination or whether an adaptive immune response is crucial to initiate the process of myelin breakdown.

2. Microglia – the phagocytes of the brain

2.1 Origin and signature genes

Microglia constitute up to 16% of the total cell numbers in the CNS and are professional phagocytes that maintain brain homeostasis, but also contribute to brain pathology[33,34]. In MS lesions, they are not the only myeloid cell population present, since also monocyte-derived macrophages can infiltrate the CNS due to BBB damage. Microglia, together with perivascular and meningeal macrophages, originate from the yolk-sac and populate the CNS around embryonic day 9.5 in mice, and in gestation week 4 in humans[35–39]. They are long-lived cells that maintain self-renewal by coupled apoptosis and proliferation, which is spatially and temporally regulated[40]. In contrast, monocyte-derived macrophages originate from the fetal liver, and during adulthood they are replaced by monocytes that derive from the bone marrow[36,39]. Despite their different origin, for a long time it has been a challenge to distinguish microglia from infiltrating macrophages, since markers to distinguish them were lacking. For this reason, many studies focused on distinguishing microglia from macrophages. The recent development of omics tools finally enabled the identification of microglial signature genes in mouse models[41–43], and recently also in human[44,45]. When comparing isolated microglia to whole brain tissue or to other tissue-resident macrophages in mice or human, *P2RY12*, *TMEM119*, *CX3CR1* and *ADGRG1* appear as core signature genes[42–45](**Figure 2**). These signature genes are exclusively expressed when microglia are present in the CNS environment, they disappear after only a few hours

in culture[44], highlighting the importance of studying the profile of microglia direct after isolation. Furthermore, hematopoietic stem cell-derived macrophages that repopulate the brain after BBB impairment acquire a microglia identity and express genes as *Tgfbr1* and *Tmem119*, which makes it still difficult to distinguish microglia from infiltrating macrophages under neuropathological conditions[46].




Microglia core signature		Disease-associated microglia signature
Microglia *vs* other CNS cells		Diseased *vs* homeostatic state
P2ry12	*C3*	*Apoe*
Tmem119	*CSF1R*	*Axl*
Fcrls	*SPP1*	*Ccl2*
Olfml3	*SLCO2B1*	*Spp1*
Hexb	*CX3CR1*	*Msr1*
C1qb	*P2RY12*	*Itgax*
C1qa	*C1QB*	*Clec7a*
Csf1r	*C1QC*	*Gpmnb*
P2ry13	*IFNGR1*	*Chi3l3*
Cx3cr1	*HSPA1B*	*Cxcr4*
Gpr34	*HLA-DRA*	*Cxcl16*
Tgfbr1	*GPR34*	*Lgals3*

Figure 2 | Microglial core signature genes defined in mouse and human, and in disease. Selection of microglia core signature genes, identified by comparing microglia gene expression to other CNS cells or whole brain tissue in both mouse (microglia versus oligodendrocytes, astrocytes, cortical and hippocampal neurons)[41,42], and human (microglia versus cortex tissue)[44,45]. Selection of common disease-associated microglia genes identified in animal models for AD, ALS and EAE[47]. Data was obtained from published RNA-sequencing datasets[41,42,44,45,47].

2.2 From homeostasis to immune response

Microglia play an essential role in neural development during embryogenesis and in the postnatal CNS, by secreting neurotrophic factors, regulating synaptic pruning and plasticity, and supporting myelination[34,48–51]. Furthermore, they are crucial players in maintaining brain homeostasis during adulthood by taking up apoptotic cells or cellular/myelin debris[33,52](**Figure 3**). Microglia are highly dynamic, constantly survey the brain and express a wide range of receptors to recognize and respond to changes in their microenvironment[33,51,53,54]. These receptors comprise purinergic, chemokine, Toll-like, interferon and Fc-receptors[53]. Upon tissue injury or inflammation, microglia migrate to the site of injury, become activated and can secrete a wide range of neurotoxic or neuroprotective mediators.

Recently, microglia have become a major topic of interest in the field of neurodegenerative diseases since risk genes for Alzheimer's disease (AD), Parkinson's disease and MS are highly expressed by microglia[44], including the MS risk genes *HLA-DRA* and *MERTK*[44]. Furthermore, it has become clear that microglia are not a homogenous population in the CNS. Using single-cell

sequencing approaches, various mouse microglial populations under homeostatic and neurodegenerative conditions were defined, whereby each population might have a unique role in CNS pathology[47,55]. Recently, distinct microglial subsets were also defined in human brain tissue, identified in MS lesions[56,57].

Notably, the core signature genes of microglia have been linked to homeostasis, since expression of *P2ry12*, *Tmem119*, *Cx3cr1* and *Adgrg1* was reduced in animal models for neurodegenerative diseases, such as AD, amyotrophic lateral sclerosis (ALS) and experimental autoimmune encephalomyelitis (EAE)[47,55]. Contrary, genes involved in lipid processing and phagocytosis, like *Axl*, *Msr1* and *Itgax* were higher expressed in this so-called disease-associated microglia population[47,55] (**Figure 2**), indicating that activated microglia play an essential role in clearing apoptotic cells and myelin debris. Microglial signature genes are useful for characterizing unique activation states of these cells in relation to development, ageing or pathology.

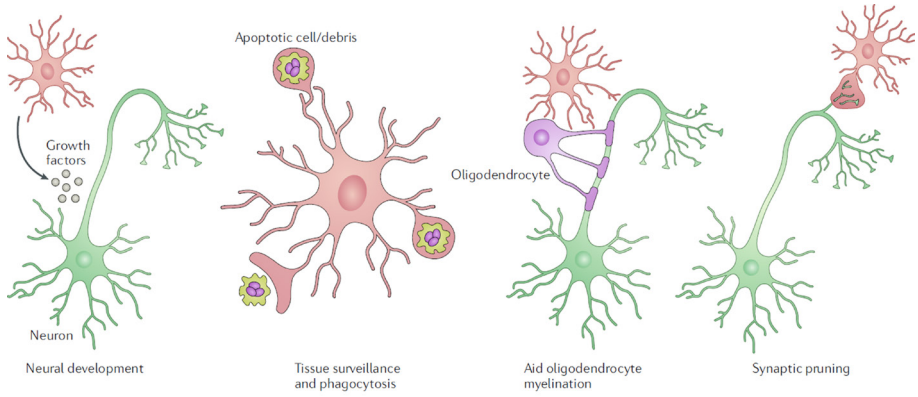

Figure 3 | Microglia have multiple roles in the CNS during embryogenesis and adulthood. Microglia are implicated in neural development (by secreting growth factors), in tissue surveillance (by removing apoptotic cells/debris), in myelination (by promoting oligodendrocyte maturation), and in synaptic pruning (by removing complement-opsonized synapses). Adapted from Dong & Yong, *Reviews Neurology*, 2019[52].

2.3 Tight regulation of activation

The activation of microglia is tightly controlled by inhibitory molecular interactions with neurons through CX3CR1–CX3CL1, CD200R–CD200 and SIRPα–CD47[54]. Interestingly, CD47 was downregulated around and in the rim of mixed MS lesions, suggesting reduced suppression of microglial activation in MS lesions[58]. Microglia respond upon CNS damage by phagocytosis of apoptotic cells and myelin debris, but are not necessarily immune activated upon activation. Of interest, our group previously has shown that primary human microglia are tolerogenic to microbial stimuli, such as lipopolysaccharide (LPS)[59], which might serve as a protective feature of microglia to prevent collateral neuronal damage caused by inflammation. Importantly, microglia might need an additional MS-specific stimulus to break their tolerance for microbial stimuli and start an immune response. Intrathecal antibodies, detected as OCBs in the CSF, are a diagnostic marker

for MS[6,60] and might target MS myelin-derived lipids or proteins[61,62]. These IgG antibodies are recognized by Fc-gamma receptors (FcγRs) expressed by microglia and are known for their role in immune activation in other myeloid cells[63–65]. Intrathecal IgG antibodies, which might form antibody complexes on myelin of MS patients, could be the MS-specific stimulus to break microglial tolerance thereby promoting immune activation. For a long time, there is an ongoing debate on whether microglia are protective or harmful once they are activated[20,52]. Since microglia are not a heterogeneous population, diverse populations with either beneficial or detrimental properties may exist next to each other with different roles in CNS pathology.

3. Microglial role in MS lesion initiation – use of post-mortem brain tissue

3.1 Microglia activation in MS

In MS, microglia are well-known for two main functions, as phagocytes that take up myelin, and as innate immune cells involved in inflammation. However, what triggers microglia to take up myelin and how do microglia become immune-activated in MS is not clear. Interestingly, *in vitro* studies show that microglia/macrophages that have taken up myelin become anti-inflammatory by expressing prostaglandin E2 synthase (PGES) and C-C motif chemokine ligand 18 (CCL18), and can promote neuronal repair[32,66]. More evidence for the role of microglia in facilitating neuronal repair comes from a study that describes the contribution of microglia to remyelination in MS[67]. Contrary, activated microglia/macrophages can also produce pro-inflammatory cytokines and chemokines to enhance inflammation by triggering the adaptive immune response, possibly by attracting and reactivating lymphocytes[20,52,68,69]. In addition to pro-inflammatory molecule production, activated microglia and macrophages can secrete reactive oxygen and nitrogen species that promote axonal and myelin damage, thereby contributing to MS pathology[70,71].

Recently, single-cell RNA-sequencing identified the profile of activated microglial subpopulations in active lesions in early MS defined by *CCL4*, *CTSD* or *SPP1* expression and reduced expression of homeostatic genes[56,57]. These findings emphasize on the heterogeneous nature of microglia in MS lesions. Importantly, these studies were performed in early MS, and considering that microglial activation is altered by age[45,72], the activation profile of microglia might be different between early or late MS disease stages, which is important to acknowledge when defining their potential role in MS lesion formation.

3.2 Normal-appearing MS tissue

MS post-mortem brain tissue without any signs of demyelination, characterized by intact myelin based on presence of PLP upon immunohistochemical staining, is ideal to study first changes related to MS pathology. Changes found in this so-called normal-appearing tissue are thought to be the first stage of MS lesion initiation[73–75]. Several studies have shown subtle changes in normal-appearing WM (NAWM) tissue by altered myelin lipid composition[18,76], apoptotic oligodendrocytes[73,74], reduced

axonal density[77,78] and axonal integrity[19,79], already showing first signs of myelin and axonal changes preceding MS lesion formation.

In addition to myelin and axonal changes, gene expression studies of NAWM MS tissue showed alterations in immunosuppressive as well as pro-inflammatory genes[80], and our group reported upregulation of scavenger receptor genes, involved in phagocytosis, already around chronic active MS lesions[81]. Moreover, our group has shown increased expression of phagocytosis and immunoglobulin genes in NAWM MS tissue, and also found NAWM-related microglial changes associated with immune suppression[63,82]. Interestingly, several other studies describe changes in NAWM MS tissue related to microglia clusters, referred to as nodules. These studies suggest that microglial nodules are the start of MS lesions as they express immune regulatory and anti-viral proteins, together with complement molecules that line up around damaged axons[69,83–85]. Together, early changes in NAWM MS tissue, observed in oligodendrocytes, microglia and axons, might relate to MS pathology and potentially can contribute to MS lesion initiation.

3.3 Studying post-mortem MS brain tissue

The studies that are described in this thesis were performed on post-mortem brain tissue that is provided by the Netherlands Brain Bank (NBB; www.hersenbank.nl). Pathological and clinical disease characteristics are available for each MS brain donor that comes to autopsy at the NBB and can be used to assess associations between MS pathology and clinical parameters[14]. For the studies on MS microglia described in this thesis, clinical and neuropathological parameters, including disease duration, severity and lesion loads, allowed us to study associations between disease parameters and the microglial profile in MS. Since most studies on the role of microglia in MS are performed in EAE or cuprizone animal models, more information is needed on the profile of human microglia in MS. Therefore, post-mortem brain material provides a unique opportunity to study the microglial profile in relation to MS pathology. In the past years, several protocols have been published describing isolation of purified microglia from post-mortem human brain tissue, based on their adherent properties in culture or antibody sorting techniques[59,63,86–89]. However, since clinical and autopsy parameters vary between donors, their impact on the microglial profile needs to be investigated in more detail in order to reliably translate changes in the microglial profile to donor neuropathology.

Since the majority of MS brain donors that come to autopsy at the NBB have progressive MS with a disease duration of on average 29 years[14], studying the microglial profile was limited to progressive MS. However, since the role of microglia in progressive MS is not completely understood, and the available therapeutic drugs show limited success in progressive MS, it is essential to fully characterize microglia in progressive MS, as they may serve as a potential therapeutic target to treat the progressive form of MS.

4. Thesis outline

The studies in this thesis provide information on the microglial profile in progressive MS and point towards their important role in MS pathogenesis and (potentially) therapy, since they can drive inflammation but on the other hand also protect the brain by removing myelin debris and facilitating remyelination. The scope of this thesis is to identify if changes in the profile of primary microglia isolated from post-mortem human brain tissue reliably reflects neuropathological changes (part 1). Subsequently, we characterized primary microglia in progressive MS to assess their contribution to MS lesion initiation (part 2) and development (part 3). In **Figure 4**, an overview of different techniques used to profile microglia in MS normal-appearing and lesioned tissue described in this thesis is displayed.

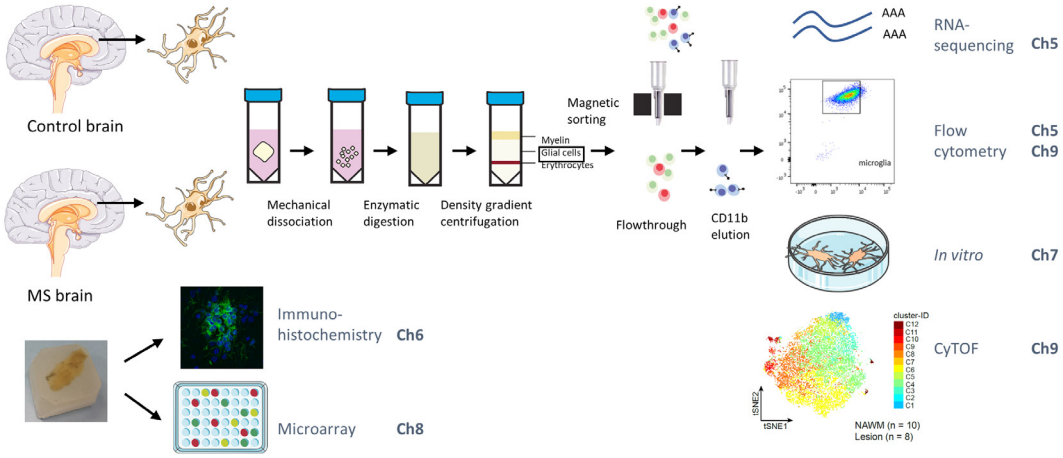

Figure 4 | Overview of microglia isolation method and techniques that were used to profile microglia in normal-appearing MS tissue and active MS lesions, described in this thesis. A protocol to rapidly isolate human microglia from post-mortem brain tissue was optimized, and the impact of ante- and post-mortem parameters was studied in order to translate changes in the microglial profile to donor neuropathology (described in Chapters 2 and 3). Subsequently, isolated microglia were directly analyzed to assess their potential role in MS lesion initiation, by RNA-sequencing (Chapter 5), flow cytometry (Chapters 5 and 9), *in vitro* assays (Chapter 7), and to assess their profile in active MS lesions by mass cytometry (CyTOF, Chapter 9). Whole-tissue analysis was performed on mixed and inactive MS lesions by microarray, to define mechanisms implicated in MS lesion activity (Chapter 8). Using immunohistochemistry, the phenotype of microglial nodules in NAWM tissue was determined to assess their contribution to MS lesion initiation (Chapter 6). Brain, microarray and microglia pictures were downloaded from Servier Medical Art by Servier licensed under a Creative Commons Attribution3.0 Unported License.

First, an optimized microglia isolation protocol and the impact of ante- and post-mortem donor variables on microglial profiling, together with the impact of culturing conditions on the microglial profile, is described in **Chapter 2**. In **Chapter 3** we describe several methods to isolate primary glial cells from post-mortem human brain tissue and subsequently discussed several downstream applications to profile microglia in relation to neurological and psychiatric diseases. Furthermore, to investigate the expression of individual microglial markers in more detail, we performed a meta-analysis to assess the transcription and protein expression of the phagocytic receptor BAI1 in monocytes, macrophages and microglia in mouse, human and zebrafish (**Chapter 4**).

Second, potential early microglial changes related to MS pathology were studied in normal-appearing tissue, to identify whether microglia contribute to MS lesion initiation. In **Chapter 5**, the transcriptional profile of human microglia was defined in control WM and GM, and in normal-appearing WM and GM MS tissue, by RNA-sequencing. Next, the phenotype of microglial clusters was determined in NAWM MS tissue using immunohistochemistry (**Chapter 6)**.

Third, we studied how primary human microglia become immune activated. Furthermore, we identified the microglial profile in active MS lesions, to assess their contribution to active MS lesion formation, together with the whole-tissue profile of MS lesions. In **Chapter 7** the immune activation profile of primary human microglia after co-stimulation with Toll-like receptor-ligands and IgG immune complexes was analyzed. The gene-expression profile of mixed and inactive MS lesions was identified by microarray analysis (**Chapter 8**), and the proteomic profile of human microglia was studied at single-cell level in control WM, NAWM and active MS lesions, using mass cytometry (CyTOF) (**Chapter 9**).

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