From THE DEPARTMENT OF CLINICAL NEUROSCIENCE
Karolinska Institutet, Stockholm, Sweden

INNATE IMMUNITY IN PROGRESSIVE MULTIPLE SCLEROSIS

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Karolinska Institutet

Stockholm 2015

Published by Karolinska Institutet.
Printed by AJ E-print AB

ISBN 978-91-7676-124-3

Innate Immunity in Progressive Multiple Sclerosis

THESIS FOR DOCTORAL DEGREE (Ph.D.)

AKADEMISK AVHANDLING

som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i **Kugelbergsalen**, NeuroHuset R2:U1, Karolinska Universitetssjukhuset, Solna.

Friday November 13th, 2015, at 12:00

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One hand wash the other, both wash the face

DEDICATED TO MY BELOVED ONES

ABSTRACT

Multiple sclerosis is (MS) is a chronic inflammatory autoimmune disease of central nervous system (CNS) leading to demyelination, axonal damage and neurological handicap, often affecting young adults. A majority of patients with MS initiate their disease with clinical bouts and relapses, but with time convert to a progressive course with dampened signs of CNS inflammation but increasing neurological deficits. This thesis is focused on highlighting the differences in levels of key immune mediators, neurofilament-light (NFL), and kynurenine pathway in different phases of MS and in an animal model of neurodegeneration.

In **Study I**, we determined levels of NFL, complement C3 and activity of the two main acetylcholine hydrolyzing enzymes, AChE and BChE, in cerebrospinal fluid (CSF) from patients with MS and controls. Levels of C3 were higher in MS patients compared to controls and correlated with MS disease disability and NFL. The BChE activity was correlated with C3 and NFL in individual samples suggesting a potential link between intrathecal cholinergic activity and complement activation. The results motivate further studies on the regulation and effector functions of the complement system in MS, and its relation to cholinergic tone.

In **Study II**, we identified a strong naturally occurring *cis*-regulatory influence on the local expression of complement receptor 2 (Cr2) in the rat spinal cord and increased soluble CR2 (sCR2) in the CSF of nerve injured rates. In transgenic mice loss of Cr2 resulted in increased loss of synapses in the axotomized motor neuron pool. In humans increased sCR2 levels were detected in the CSF of patients with MS as compared to controls, identifying CR2 as a potential novel biomarker of CNS inflammation. These results propose a new role for CR2/sCR2 as a modulator of innate immune reactions and synaptic plasticity in the CNS.

In **Study III**, we determined levels of tryptophan (TRP), kynurenine (KYN), kynurenic acid (KYNA) and quinolinic acid (QUIN) in CSF. The absolute QUIN levels and the QUIN/KYN ratio were increased in MS during relapse (RRMS). Interestingly, secondary progressive MS (SPMS) displayed lower TRP and KYNA, while primary progressive (PPMS) patients displayed increased levels of all metabolites, similar to a group of inflammatory neurological disease controls. In addition, MS patients with active disease and short disease duration were prospectively evaluated for neuropsychiatric symptoms. Depressed patients displayed higher KYNA/TRP and KYN/TRP ratios, mainly due to low TRP levels. These results demonstrate that clinical disease activity and differences in disease courses are reflected by changes in KP metabolites. Increased QUIN levels of RRMS patients in relapse and generally decreased levels of TRP in SPMS may relate to neurotoxicity and failure of remyelination, respectively.

In **Study IV**, we analyzed the main monocytes subsets and/or expression of the chemokine receptors CCR2 or CX3CR1 in relation to different MS disease courses, and after treatment with dimethyl fumarate (DMF). In contrast to the prior studies we could not detect significant quantitative or qualitative differences in the monocyte population between different MS disease stages. DMF treatment resulted in a heterogeneous response, with both expansion and reduction of non-classical monocyte subsets in a proportion of patients.

In summary and in context of current knowledge, my findings suggest that later stages of MS is characterized less of adaptive and innate cellular alterations in the periphery, also supported by the relative lack of efficacy of current therapies in MS directed mainly at modulating the adaptive immune defense. However, findings of altered complement expression and metabolic changes involving the KP may reflect low grade widespread tissue responses that can exert effects on synaptic remodeling and neuronal transmission. These pathways deserve attention as potential therapeutic targets in later stages of MS.

LIST OF SCIENTIFIC PAPERS

I. **Shahin Aeinehband***, Rickard PF Lindblom*, Faiez Al Nimer, Swetha Vijayaraghavan, Kerstin Sandholm, Mohsen Khademi, Tomas Olsson, Bo Nilsson, Kristina Nilsson Ekdahl, Taher Darreh-Shori, Fredrik Piehl.
Complement Component C3 and Butyrylcholinesterase Activity Are Associated with Neurodegeneration and Clinical Disability in Multiple Sclerosis
PLoS One. 2015 Apr 2;10(4):e0122048

II. Rickard PF Lindblom, **Shahin Aeinehband**, Alexander Berg*, Mikael Ström*, Faiez Al Nimer, Cecilia A Dominguez, Nada Abdelmagid, Matthias Heinig, Kerstin Sandholm, Johan Zelano, Karin Harnesk, Bo Nilsson, Kristina Nilsson Ekdahl, Norbert Hübner, Mohsen Khademi, Margarita Diez, Staffan Cullheim, Fredrik Piehl.
Complement Receptor 2 is a Novel Marker of Neuroinflammation with Neuroprotective Properties
Manuscript

III. **Shahin Aeinehband**, Philip Brenner, Sara Ståhl, Maria Bhat, Mark D Fidock, Mohsen Khademi, Tomas Olsson, Göran Engberg, Jussi Jokinen, Sophie Erhardt, Fredrik Piehl
Cerebrospinal Fluid Kynurenines in Multiple Sclerosis; Relation to Disease Course and Neurocognitive Symptoms
Brain, Behavior, and Immunity. 2015 Jul 17. pii: S0889-1591(15)00411-0

IV. **Shahin Aeinehband**, Roham Parsa, Fredrik Piehl
Monocyte Subset Frequencies and Chemokine Expression in Multiple Sclerosis
Manuscript

**These authors contributed equally to this work*

PUBLICATIONS NOT INCLUDED IN THIS THESIS

Kular L*, Liu Y.*, Ruhrmann S, Gomez-Cabrero D, **Aeinehband S**, James T, Górnikiewicz B, Tegnér J, Olsson T, Piehl F, Ekström T, Kockum I, Feinberg AP, Jagodic M.
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LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
APC	Antigen-Presenting Cell
BBB	Blood-Brain Barrier
BChE	Butyrylcholinesterase
BMI	Body Mass Index
bp	Base pairs
C1q	Complement component 1 q
C3	Complement Component 3
DA	Dark Agouti
CD	Cluster of Differentiation
CIS	Clinically Isolated Syndrome
CR2	Complement Receptor 2
CSF	Cerebrospinal Fluid
CNS	Central Nervous System
DC	Dendritic Cell
EBV	Epstein-Barr Virus
EAE	Experimental Autoimmune Encephalomyelitis
GWAS	Genome-Wide Association Study
HLA	Human Leukocyte Antigen
IFN	Interferon
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IL	Interleukin
MAC	Membrane Attack Complex
MHC	Major Histocompatibility Complex
MRI	Magnetic Resonance Imaging
MOG	Myelin Oligodendrocyte Glycoprotein
NFL	Neurofilament-light
NK	Natural Killer
OCB	Oligoclonal Bands
OND	Other Neurological Disease
PD	Parkinson's Disease
PPMS	Primary Progressive MS
RRMS	Relapsing-Remitting MS
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
PVG	Piebald Virol Glaxo
SNT	Sciatic Nerve Transection
SPMS	Secondary Progressive MS
VRA	Ventral Root Avulsion
WT	Wild-type

1 INTRODUCTION

1.1 MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS), with demyelination, neurodegeneration and brain atrophy as hallmarks [1]. A large variety of symptoms are associated with MS due to focal inflammatory attacks, and their character is dependent on where these lesions are located. Some symptoms are more common among most patients, including motor, visual and sensory disturbances [1]. In addition, neurocognitive comorbidities such as depression, anxiety, suicidality, fatigue, bipolar disease are more prevalent in MS patients and are factors that lowers the quality of life for many patients, while their molecular basis is not clearly understood [2-6].

The risk of acquiring MS depends on genetics, environment and geography. The average age of onset is approximately 30 years [7] and there is a 2-3 times overall higher risk for women to acquire MS, for unknown reasons [8]. The prevalence is higher in North America and Europe, compared to the rest of the world [9]. In Sweden, the risk of acquiring MS is 189 in 100 000 [10].

The main cause of MS is still unknown. However it is established that there is a big genetic component involved and that a large number of susceptibility genes interplay with environmental triggers which result in MS. Previous infections, especially Epstein-Barr virus (EBV), have also for long been suspected to trigger MS and cannot be ruled out as a potential cause [11]. Genome-wide association studies have been very successful tools in finding susceptibility genes in MS [12]. As of today, 110 genes that confer susceptibility to MS have been identified [13], in addition to the human leukocyte antigen (HLA), which is the strongest and most well established risk locus [14]. For instance, the presence of the class-II allele HLA-DRB1*15:01 is the strongest risk allele with an odds ratio (OR) of 3.10 [15, 16] and absence of the protective class-I HLA-A*02:01 allele has an OR of 1.37 [16, 17], while non-HLA associations contribute less, with OR values around 1.1. Other factors that increase the risk of acquiring MS include; active or passive exposure to tobacco smoke [18-21], low sun exposure in turn causing low vitamin D levels [22] and high body mass Index (BMI) [23, 24] at young age. With regard to tobacco smoke, it was recently shown that patients with early disease that continued smoking after their diagnosis had a reduced time to conversion to a progressive disease course [25].

The most common characteristic of MS is the presence of focal lesions in the brain and spinal cord, which also sets the basis for diagnosis. Before a MS diagnosis can be determined, the McDonald criteria has to be fulfilled, which states that there has to be spreading of lesions both in space and time [26]. Brain lesions can be detected with magnetic resonance imaging (MRI) and is the main tool for identifying and distinguishing different types of changes in the CNS, inflammatory lesions accompanied by blood-brain barrier (BBB) damage (gadolinium enhancing lesions), T1 weighted MRI showing permanent axonal loss [27], T2 weighted MRI showing the total amount of lesions including inactive ones, demyelination and remyelination

[28, 29]. All of these aspects are important in order to get a closer understanding of the clinical picture, for diagnosis, for following disease progression for clinical purpose or for measuring the outcomes of drug treatment. In addition, MRI has been essential in research and has contributed by giving insights about the pathology. Analysis of cerebrospinal fluid (CSF) also contributes to the clinical diagnostic work up, where oligoclonal bands (OCB) are present in a majority of patients, aiding diagnosis of MS by ruling out other neurological disorders [30]. The OCB indicates local inflammation and presence of differentiated B cells in the form of immunoglobulin G (IgG) producing plasma cells. The specificity of these IgGs is unknown and their contribution to disease is not clearly understood.

1.1.1 Clinical course

1.1.1.1 CIS

When patients experience a first bout of clinical symptoms, without dispersion in time, they are defined as a clinically isolated syndrome (CIS) [31], which is usually a pre-stage to MS but in a minority not leading to a final diagnosis [31]. Abnormalities in a baseline MRI scan predict the subsequent development of MS in patients with CIS. In the long term, about 80% of patients with an abnormal MRI go on to develop MS, compared with only 20% of those with a normal MRI [32].

1.1.1.2 RRMS

Approximately 85 % of the newly diagnosed patients suffer from relapsing-remitting MS [33], which is characterized by episodes of inflammatory attacks and manifestation of clinical symptoms – relapse, followed by full or partial recovery from symptoms – remission [34]. Symptoms occur due to demyelinating processes, and to some degree also transection of axons [1]. Demyelinated axons could become partially restored by remyelination mechanisms in oligodendrocytes. However, these compensatory mechanisms have limited potential and could with recurrent demyelination and irreversible neurological symptoms become exhausted – leading to chronic demyelination and possibly the early signs of progressive disease [35, 36].

1.1.1.3 Progressive MS

Within 20 years, perhaps due to exhaustion of the repairing mechanisms or initiation of another type of pathological mechanisms, a large majority of patients with RRMS enters a secondary progressive disease course (SPMS), with chronic accumulation of neurological deficits not explained by relapses [37]. In addition, approximately 15 % of newly diagnosed patients display a progressive disease course already from onset; primary progressive MS (PPMS) [38]. Interestingly, the onset typically is after the 4th decade of life, which coincides with the age when most RRMS patients convert to SPMS [7, 39]. This has led to the hypothesis that PPMS is “amputated” from the usual relapsing-remitting symptoms [40].

For a long time MS was primarily considered a disease of the white matter, without neurodegenerative components responsible for loss of neurons and axons [41]. Today, we know that injury to axons occur as early events in disease, and that their loss correlate with

irreversible neurological disability [42]. In contrast to RRMS, the BBB of progressive patients appear more intact, as suggested by the relative absence of contrast enhancing MRI lesions [43, 44]. Thus, acute lesions characterized by a ruptured BBB, permitting inflammatory infiltration, are often found in RRMS patients in relapse. While the lesions found in PPMS and SPMS are inactive demyelinated areas with a large degree of axonal loss, absence of oligodendrocytes and few inflammatory cells [45, 46]. Cortical lesions are more common in progressive MS, and these type of injuries occur to large degree in the absence of infiltrating cells, which has led to the hypothesis of a soluble factor produced by inflammatory cells trapped in the meninges that produce soluble factors able to cause damage by diffusing to cortical areas [47, 48]. Other mechanisms has also been suggested, such as diffuse microglia activation, age-dependent iron accumulation, mitochondrial injury and ROS [49, 50].

1.2 THE IMMUNE SYSTEM

The adaptive immune system is shaped through life by the pathogens we encounter. The adaptive immune system is slow but specific and can mount a response towards any foreign antigen, novel or evolutionary conserved. T cells and B cells, collectively known as lymphocytes, develop in the thymus and bone marrow respectively, are the major players in the adaptive immune system. The maturation of lymphocytes include a process known as somatic recombination, where a pool of receptors are formed by stochastic rearrangement of gene segments resulting in a large pool of receptors with different specificities. Thus, each individual cell has a unique receptor, and only clonal expansion of the same progenitor cell could produce the same receptor [51-53]. However, the immune mechanisms of lymphocytes are large dependent of antigen presentation by antigen presenting cells from another part of the immune defense; the innate immune system, which represents the first line of protection against invading pathogens. It recognizes certain structures that have been conserved through evolution, but is unable to adapt to recognize novel antigens. The fundamental cells and factors of the innate immune system include monocytes, macrophages, dendritic cells (DCs), natural killer cells (NK), granulocytes, complement factors and microglia in the CNS.

The role of inflammation is to effectively clear the host from pathogens, without causing potentially detrimental damage to healthy tissue. Neurons in the CNS proliferate to a very little extent and inflammation could cause irreversible damage, which is why the CNS to a high extent is isolated from the peripheral blood and lymphatic system by the BBB [54, 55].

1.2.1 Complement

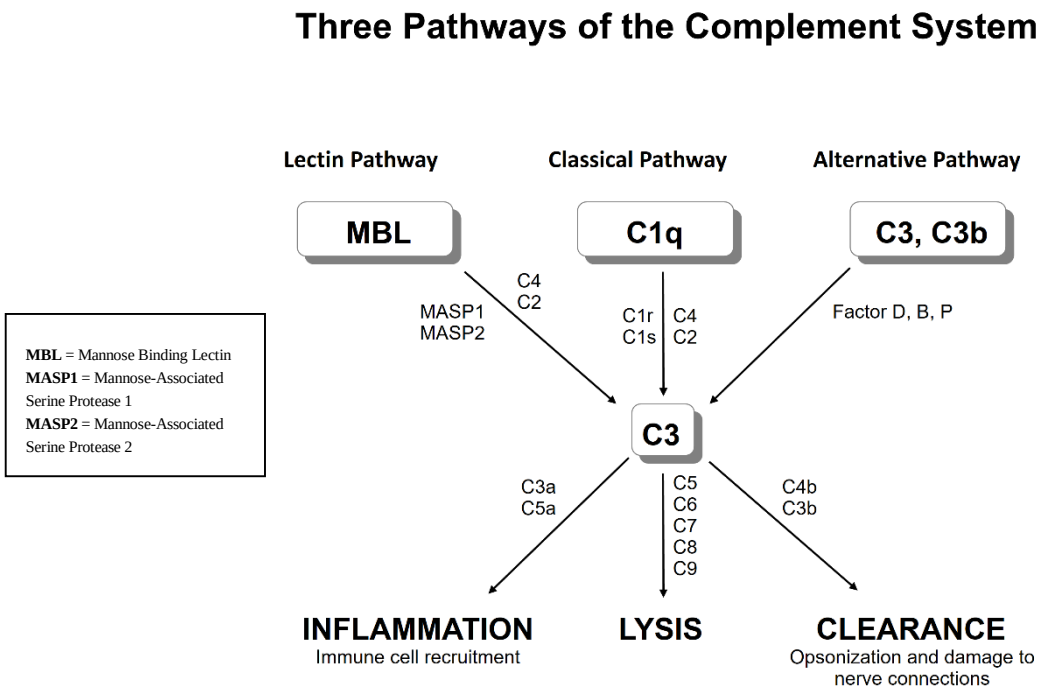
The complement family consists of about 30 soluble and cell-bound proteins, mainly synthesized in the liver. Complement has an important role in innate immunity [56], where they remain in an inactive form until they encounter pathogens or other triggers. Complement factors can also be produced locally in the CNS, by microglia, astrocytes and neurons [57-62].

The complement system is a potent activator of the immune system, which is why the cascade is highly regulated and a large proportion of the complement genes exert regulatory functions [63, 64]. There are nine central components in the complement cascade (C1-C9) that are

formed by subsequent proteolytical reactions, turning an inactive protein, known as an zymogen, into an active form [63]. These events can be divided in four major steps. First are the early complement components which are activated through three different pathways; the classical, alternative and lectin pathway which all converge to the central complement component C3. Following the formation of C3, further activation becomes possible through its cleavage by the activation of C3 convertases, resulting in C3a and C3b. If the third event is activated, it results in the formation of C5 convertase which cleaves C5 into C5a and C5b. Finally, C5b could initiate the terminal pathway which is the final step in the cascade and involves assembly of the late phase proteins C5b-C9, known as the membrane attack complex (MAC) [63].

To control the activation of complement, host cells express complement receptors on their surface that regulates the cascade [65]. Some examples include CR1 [66], CR2 [67], CD46 [68],CD55 [69] and CD59 [70].

Complement activation is associated with three distinct mechanisms. The first mechanism involves the small complement fragments that are cleaved and released upon activation, aiding the recruitment of additional immune cells to the inflammatory site. These small fragments known as anaphylatoxins include C3a, C4a and C5a, exert pro-inflammatory effects once released. The second mechanism, opsonization, also known as “coating” of cells, facilitates the elimination of pathogens. C3b, C4b and C5b are the larger fragment of their precursors, and can opsonize the surface of bacteria, causing surface receptors on phagocytic cells or B cells to recognize it, and thereby enhancing phagocytic activity. In this fashion, opsonization lowers the threshold in immune activation and creates a stronger response. The third mechanism is exerted by MAC, which efficiently cause cell lysis and elimination of pathogens by creating small pores in the bacterial cell wall [63]. To highlight the importance of the complement system and its central component C3, individuals lacking C3 suffer from recurrent bacterial infections.



1.2.2 Role of the immune system in MS

Immunology in MS is a complex matter with cells from both the innate and adaptive immune system in interplay with each other. It has been debated whether MS is a disease caused by dysregulation in the immune system, or if it is neurological with inflammation as a secondary consequence [71]. However, a large body of evidence suggests the former. A majority of the susceptibility genes that have been identified are related to T helper (Th)1 cell-mediated pathways and the adaptive immune system in general [13, 16, 72]. And the strongest genetic associations to MS, the HLA class I and class II molecules, are required for antigen presentation to CD4+ and CD8+ T cells. Further support for the notion that MS is caused by the immune system comes from experiments in the most widely used animal model of MS, experimental autoimmune encephalomyelitis (EAE). EAE is induced by subcutaneous injection of a myelin component mixed with an adjuvant, causing $CD4^+$-mediated autoimmune disease [73, 74]. EAE can also be induced by adoptive transfer of encephalitogenic CD4+ T cells into a naive recipient [73-75].

Historically, autoreactive CD4+ Th1 cells, with their signature cytokine interferon gamma (IFN-γ), have been associated with the inflammatory attacks causing lesions in the CNS of MS patients [74, 76]. In addition, Th17 cells producing IL-17 and IL-22 have been increasingly recognized as pathogenic in MS. The expression pattern of molecules important for recruitment and adhesion is different between Th1 and Th17 cells, which is why each of them promote inflammation in different parts of the CNS [77, 78]. It has been shown that Th17 cells can promote disruption of the BBB and cause inflammation [79]. In addition, MS patients in relapse have increased numbers of Th17 cells in the peripheral circulation and these numbers are

reduced after treatment with beta-interferons [80], one of the most common first-line treatments for early disease [81].

In order for naïve CD4+ T cells to become autoreactive and encephalitogenic, self-antigens from the CNS have to be presented to them by antigen presenting cells (APCs), such as DCs and macrophages [52]. The exact nature of self-antigen recognized by the T cell receptor (TCR) in MS are still elusive, but focus in animal models has been on myelin basic protein (MBP) [82], proteolipid protein (PLP) [83] and myelin oligodendrocyte glycoprotein (MOG) [84], which are components of the myelin sheath. It has been an enigma how peripheral cells could become autoreactive towards antigens presented in the CNS, since the CNS has been considered immune-privileged. However, the concept of immune-privilege has been questioned [85]. In support for the updated view of immune-privilege, are cases of the lethal disease progressive multifocal leukoencephalopathy (PML), caused by the Human JC Polyomavirus in MS patients treated with the monoclonal antibody natalizumab[86-88], which blocks the entry of lymphocytes to the CNS [86-88]. The JC virus is an opportunistic pathogen present in a majority of the population but only harmful in immunodeficient individuals, which speaks for the presence of surveilling leukocytes in the CNS. In a recent landmark study, it was shown that there is a direct route between CNS vessels and the peripheral lymphatic system, which could be a plausible explanation to this enigma [89].

A hypothesis about the immunopathogenesis of MS is that somehow T cells in the periphery become autoreactive, enter the CNS through expression of certain adhesion molecules, chemokine receptors and integrins, which facilitates their transmigration through the BBB [90]. A possible explanation of how autoreactive T cells emerge in the periphery is the concept of molecular mimicry. This is when T cells have recognized a past infection with structural or sequence similarities to a self-antigen, causing memory T cells later to cross-react with the self-antigen, in turn leading to autoimmunity as in MS [91-93].

T cells cross the CNS through venules and enter the perivascular space. The best characterized interaction is between Integrin alpha4beta1 (Very Late Antigen-4; VLA-4) expressed on T cells and the integrin receptor, vascular cell adhesion molecule-1 (VCAM-1) expressed by endothelial cells [94-96]. Moreover, it was recently announced that a new cell adhesion molecule has been found to be expressed on the surface of encephalitogenic Th-17 cells, aiding their transmigration through the BBB. [97]. Once in the CNS, T cells become re-activated by APCs that present the myelin components [98] which facilitates a pro-inflammatory environment and further recruitment of cells and factors that are actually responsible for causing damage, like infiltrating class I MHC-restricted CD8+ T cells, macrophages, resident microglia, complement or other factors produced by the innate immune system.

Moreover, experiences from successful drug treatment by eliminating B cells suggest that these cells could also have an important role in antigen presentation in MS [99], which is also supported by data from animal models of MS [100-102].

1.2.3 Innate immunity in progressive MS

In later phases of MS, when a majority of patients has entered a progressive disease course, the role of adaptive immunity declines and is has been hypothesized that there is a shift towards innate immunity [103]. One strong argument supporting this notion is that immunomodulatory drugs acting on the adaptive arm of the immune defense do not have any beneficial effects in progressive disease [104, 105]. However, there are some indications that a subpopulation of progressive MS with signs of active inflammation on MRI may have a beneficial effect of such drugs [106, 107]. Very recently, it was communicated that a larger phase III trial in PPMS with a novel anti-CD20 targeting monoclonal antibody (ocrelizumab) met its primary outcome; a reduced rate of progression in the active arm [108].

Once the inflammatory processes have been initiated, the neurodegenerative components have been suggested to continue, despite immunomodulatory drug treatment [105, 109]. This indicates that other mechanisms still not fully identified, could be involved in these processes and that they may act independent of the peripheral adaptive immune system. In progressive MS, it has been reported that there is an increased accumulation of inflammatory cells in the subarachnoid compartment, and these cells are trapped, or compartmentalized, behind an intact BBB [110, 111]. In about 40 % of cases with PPMS, cell aggregates has been found, consisting of B cells, plasma cells and other cells suspected to be DCs [112, 113]. These cells could be involved with mechanisms that release soluble factors promoting cortical lesions commonly found in progressive patients, and these cortical injuries could be responsible for the chronic and accumulating disabilities that are characteristic for progressive disease courses [48, 114]. These findings could give a possible explanation to the lack of response of current immunomodulatory drugs in progressive patients.

Macrophages engulf myelin debris in close proximity to lesion sites in MS brains [115, 116]. Traditionally, it has been challenging to segregate macrophages from microglia, since they were both identified by the same surface markers, e.g. CD11b, CD45 and F4/80. However, with the advancement of technology, it is now possible to study these cells separately in experimental conditions [117-120].

Microglia are similar to macrophages and constitute about 10-15 % of the cells populating the CNS, where they serve as the resident innate immune system of the CNS. In contrast to macrophages that are bone-marrow derived, microglia origin from the yolk-sac, and at a certain time window during embryogenesis, they populate the CNS before the BBB is formed and seals the CNS from the periphery [121, 122]. Microglia activation is instrumental in driving neurodegenerative disease and it has been suggested that their activation is involved in active tissue destruction [123]. Upon activation, microglia up regulate surface receptors like CD11b, ionized calcium-binding adapter molecule 1 (Iba1), and start expressing antigen presenting molecules major histocompatibility complex (MHC)-II, B7.1, and B7.2 (CD80/86) [124].

Activation of microglia has been associated with detrimental effects both in white and grey matter injuries in progressive patients [46, 125, 126]. Their pathogenic role in MS could be

mediated through mechanisms involving reactive oxygen species (ROS) production [127], phagocytic activity [128] and secretion of pro-inflammatory cytokines [129]. In addition, progressive MS is associated with widespread and diffuse microglia activation [47] and they are often found in close proximity to injured axons and their numbers correlate with the density of acute axonal transections [130, 131]. During EAE, the activation of Th1 or Th17 cells is associated with the expansion of microglia and infiltration of myeloid derived blood monocytes [132, 133]. In a recent study in mice, microglia and peritoneal macrophages were isolated in pair-wise manner and global transcriptome analysis were conducted by RNA sequencing [134] to compare these cell-types [135]. This study confirmed the large similarities between microglia and macrophages, but also identified novel genes and pathways unique for microglia. It was shown that microglia express a vast array of genes that were important in maintaining homeostasis and sensing their environment, which indicates that microglia are likely evolutionary better adapted to the environment in the CNS [135].

Moreover, also cellar studies have identified differences in the peripheral immune system of MS patients compared to healthy controls (HC). In one study, PBMCs from MS patients were enriched and treated with anti-CD3 antibodies *in vitro*. This resulted in increased secretion of interleukin 12 (IL-12) from SPMS cases, compared to RRMS and HC. IL-12 is a pro-inflammatory cytokine produced by DCs, a component of the innate immune system. In addition, IL-18 was increased in both SPMS and RRMS compared to HC, and a correlation to disease duration was identified in SPMS. In vitro studies such as this one are important, but they could also lead to artifacts and should thus be considered with caution. One major role of IL-12 is to skew naïve T cells into IFN-γ secreting Th1 cells, which highlights that adaptive immunity might be active, but to a lesser degree than the innate response. [104, 105].

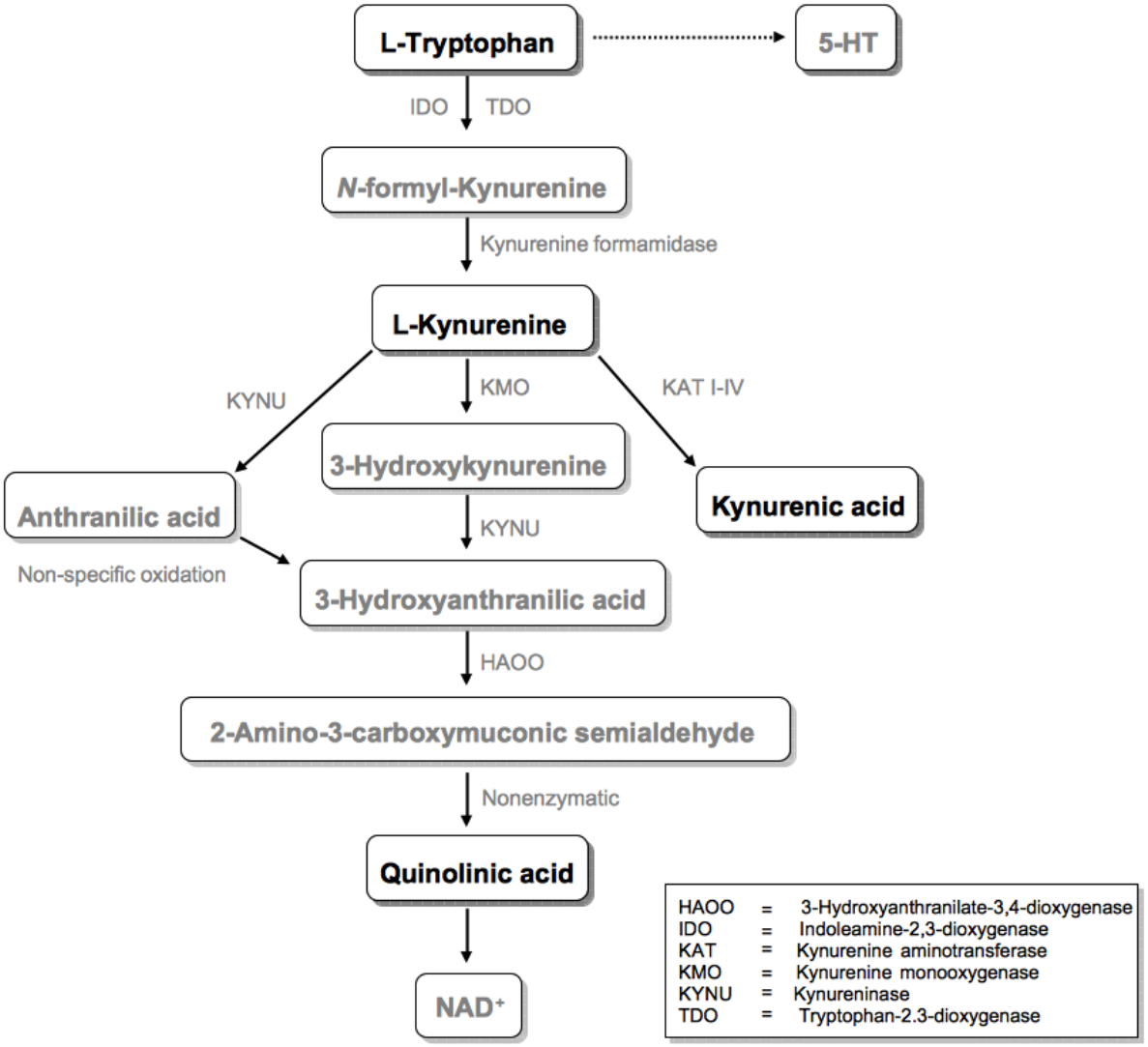
1.3 SYNAPTIC PLASTICITY

The neuronal networks of our brains represent immensely complex circuitries of connections between different nerve cells that is subject to continuous re-shaping in a process called synaptic plasticity. More than certain other neuronal functions, synaptic plasticity is involved in memory formation, where new connections are formed to store long term memories, which adapt us to the dynamic environment we experience. According to the theory postulated in 1949 by the psychologist Donald O. Hebb, "Neurons that fire together wire together"; meaning that when two neurons are in such proximity that they could form a synapse and fire together, the synaptic connection between them are strengthened, and thus they are more likely to fire again. If two neurons fire in uncoordinated manner, their connection weaken [136]. Thus, to maintain homeostasis, weak and redundant connections have to be cleared by some mechanism. Recently, the complement system has been suggested to be important in such re-shaping of synaptic networks in the CNS. In normal brain development, complement has a major role in the elimination of synaptic connections, by maintaining strong synapses and fine tuning the synaptic network by tagging weak synapses for subsequent elimination. These mechanisms could be involved in diseases with a neurodegenerative component [137].

1.4 THE KYNURENINE PATHWAY

L-Tryptophan (TRP) is one of the essential amino acids we obtain from dietary protein. TRP is widely recognized as the precursor to the neurotransmitters serotonin and melatonin, which convey important messenger molecules/transmitters for neuronal networks regulating physiological functions in the brain affecting mood, appetite, sleep and other behaviors. However, a large majority of the available tryptophan is catalyzed by the kynurenine pathway (KP) in the liver [138]. The KP in the CNS involves the enzymatic breakdown of tryptophan resulting in neuroactive metabolites that are either protective or toxic.

The first step in the pathway is catalyzed by indoleamine 2,3-dioxygenase (IDO) [139], IDO-2 [140] or tryptophan 2,3 dioxygenase (TDO)[141], turning TRP to kynurenine (KYN). Interestingly, IDO is activated by an inflammatory environment, causing over activation of the KP [142-144]. The kynurenines easily enter the BBB [145], in contrast to kynurenic acid (KYNA) and quinolinic acid (QUIN) that are unable to pass the BBB and have to be synthesized locally in the CNS. Most of the pathway in the CNS, including QUIN formation, occurs in microglia, while only astrocytes express the KAT enzymes [146], required for the synthesis of KYNA. Macrophages can also express IDO and QUIN, upon induction with either IFN-α, IFN-β or IFN-γ [147, 148]. KYNA is an N-methyl-D-aspartate receptor (NMDAR) antagonist and a weak nicotinic acetylcholine receptor (nAChR) antagonist, and exerts neuroprotective properties. QUIN has opposite effects through its agonistic effect on NMDAR, which could cause neurotoxicity through prolonged glutamate signaling. Alterations of the KP has been found in many disorders in the CNS, including neurodegenerative and psychiatric conditions [149]. Generally, it is thought that diseases with microglia activation are associated with increased production of QUIN, while astrocyte activation would favor the balance toward KYNA production [149]. In Alzheimer's disease (AD), increased KYNA levels has been detected in the striatum and hippocampus [150], while decreased levels has been found in blood [151] and CSF [152]. Moreover, dysregulation of IDO and QUIN has been associated with amyloid-beta and tau production [153-155]. In Parkinson's disease (PD), decreased levels of KYNA has been found in several brain regions [156], while elevated IDO activity in serum and CSF has been correlated with disease severity [157]. Extensive research of the KP in Huntington's disease has revealed a myriad of alterations in KYNA, QUIN and important enzymes [152, 158-160]. Also in amyotrophic lateral sclerosis (ALS), increased levels of KYN and QUIN in CSF and serum has been detected, in association with increased IDO activity [161]. In psychiatry, a lot of attention has been given in understanding the role of KP in schizophrenia, since increased KYNA results in behaviors similar to those induced by administration of ketamine or Phencyclidine (PCP), also known as the street drug Angel Dust [162-167]. Dysregulation in the KP has indeed been reported in schizophrenia [168]. Moreover, dysregulation in the KP has been reported in depression [169, 170], bipolar disorder [171], and suicidality [172].


L-Tryptophan
5-HT
IDO
TDO
N-formyl-Kynurenine
Kynurenine formamidase
L-Kynurenine
KYNU
KMO
KAT I-IV
3-Hydroxykynurenine
Anthranilic acid
Kynurenic acid
KYNU
Non-specific oxidation
3-Hydroxyanthranilic acid
HAOO
2-Amino-3-carboxymuconic semialdehyde
Nonenzymatic
Quinolinic acid
NAD+
HAOO = 3-Hydroxyanthranilate-3,4-dioxygenase
IDO = Indoleamine-2,3-dioxygenase
KAT = Kynurenine aminotransferase
KMO = Kynurenine monooxygenase
KYNU = Kynureninase
TDO = Tryptophan-2.3-dioxygenase

1.5 CURRENT TREATMENTS IN MS

There are several approved drugs for the treatment of RRMS and it is likely that the list will grow. The first line treatments are the classical injectable drugs beta-interferons (Avonex; Betaferon, Rebib, Extavia, Plegridy) and glatiramer acetate (Copaxone). These are now accompanied by two oral treatments; teriflunomide (Aubagio) and dimethyl fumarate (DMF; Tecfidera). DMF had for long been used in Germany as treatment for chronic plaque psoriasis [173-175] and thus already had a well-established safety profile [176], before its efficacy in MS treatment were recognized. Its mechanism of action is not clearly understood, but one of its likely neuroprotective properties comes from its potent induction of the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), known to activate anti-oxidant pathways [177]. In addition, a number of studies have proposed immunomodulatory properties of DMF [178-180].

Teriflunomide is an anti-inflammatory drug which acts by inhibiting the mitochondrial enzyme dihydroorotate dehydrogenase (DHO-DH) and thereby preventing synthesis of pyrimidine, essential for rapidly proliferating cells including lymphocytes. The results of the clinical trial showed positive results over placebo [181] but teriflunomide was not better than (interferon beta 1-alpha) IFNb-1a [182].

Among second-line treatments are fingolimod (Gilenya) and the monoclonal antibodies natalizumab (Tysabri) and alemtuzumab (Lemtrada). In addition, in some countries including Sweden, rituximab (Rituxan; MabThera) to a variable extent is used off-label in MS. Fingolimod was the first orally-administered drug, which was approved as second-line treatment for MS [183]. It acts by modulating four out of the five spingosine-1-kinase receptors (S1P1, S1P3, S1P4 and S1P5), thereby preventing the egress of lymphocytes from secondary lymphoid organs, which in turn protects the CNS from their pathogenic actions [184].

Natalizumab targets the leukocyte integrin VLA-4 and prevents its interaction with VCAM-1 expressed by endothelial cells, thereby blocking leukocyte transmigration through the BBB and subsequent infiltration to the CNS.

Alemtuzumab targets the CD52 receptor, expressed on lymphocytes. It has been extensively used against leukemia and lymphomas, but has later been approved for the treatment of MS due to its superior efficacy over IFNb-1a (Rebif) [185].

The target of rituximab is the CD20 receptor expressed on B cells, causing their elimination while antibody plasma cells remain intact. Rituximab is approved for the treatment of B cell lymphomas and rheumatoid arthritis, but has also shown to be very efficacious for the treatment of MS [99], even though it is still not an approved MS drug due to lack of phase III studies and where the drug company has haltered all clinical development to instead focus on ocrelizumab, a modified anti-CD20 antagonist. Previously, mitoxantrone was used in severe cases of MS, including progressive forms. Mitoxantrone is a cytostatic drug used in cancer treatment but has also shown efficacy in MS [186]. However, its use in MS has been limited by cardiotoxicity and cases of leukemia.

Laquinimod, a quinoline 3-carboxamide derivative, is a new and interesting oral drug due to its potentially neuroprotective properties [187] as well as immunomodulatory effects mediated through nuclear factor kappa light-chain enhancer of activated B cell (NF-κB) pathways with capability to suppress antigen presentation [188]. Two phase III studies have already been conducted, where the effects of laquinimod were moderate [189], but not superior to the current first-line treatment IFNb-1a [190]. However, laquinimod indeed showed beneficial effects in reducing brain atrophy, compared to placebo [190]. Currently, a third phase III trial is ongoing, which is testing if a higher dose of laquinimod would improve efficacy outcomes.

2 METHODS

2.1 ENZYME-LINKED IMMUNOSORBENT ASSAY

Antibodies can be used as specific reagents to quantify the amount of certain antigens or other proteins. When using this technique called enzyme-linked immunosorbent assay (ELISA), an enzyme is covalently bound to a specific antibody that recognizes a target antigen. If the antigen is present, the complex will bind and the enzyme of the complex will catalyze a reaction generating a colored product. The Sandwich ELISA is a very sensitive assay, allowing both quantification and detection of a specific antigen. In this method, the well surface of a plate is coated with a known quantity of bound antibody in order the capture the antigen of desire. Thereafter, non-specific binding is blocked, and the antigen-containing sample is added to the wells, binding to the capture antibody. Further, a specific detection antibody is added, binding to the protein of interest. Finally, an enzyme-linked secondary antibody is linked to the whole complex and a certain substrate is added, allowing the enzyme to convert the substrate to a detectable form. This method were used to measure levels of NFL and C3 in **Study I** and in **Study II** we measured soluble CR2. As with most other biological samples, CSF containing proteins are sensitive to protein degradation due to freeze and thaw cycles. Therefore, it is important to always thaw samples on ice, and prior to the analyses look for information in the literature about the protein of interest, to see how sensitive it is for degradation. In some cases, it is extremely important to have fresh samples, while for other proteins that are stable, thawed samples could be used as well. To decrease handling bias, it is important to run samples in duplicates and make an average of the measured sample. The detection range of ELISA is limited, and due to this it is good to make pilot experiments to find out how the serial dilutions for the standard curve should be made, in order to cover the samples well. Since ELISA is an antibody-based method, its robustness and detection level is directly dependent on the quality of the antibodies used. The use of negative and positive controls is recommended, and in some cases, also validation with SDS-PAGE and western blot to assure correct specificity.

2.2 REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) is a method that "amplifies" small amounts of DNA and turns it into large amounts. The sequence of the "template" DNA is used to produce a complementary pair of nucleotide primers, that binds the 3' and 5' ends of the template respectively, and flanks the gene region of interest. Optimal primer length is around 20 base pairs (bp) and the amplified region named *amplicon*, is optimally 75-250 bp, but can be longer. In addition to the template DNA and the primer pair, the four building blocks of DNA, adenine, cytosine, guanine and thymine has to be added to the mix. The last component is DNA polymerase, which is the enzyme that elongates the DNA at the site where the primer resides, and actually creates the copies. There are three steps involved in PCR which requires different temperatures; dissociation of double stranded DNA into single strands at ~95°C, annealing of the primers at ~60°C followed by DNA polymerization and extension at ~72°C. Exact temperatures has to be explored empirically for every new experiment and primer specificity

has to be confirmed by melt curve analysis and gel electrophoresis of the amplicon. These three steps are cycled and new copies are created exponentially until a plateau phase has been reached. Regular PCR is a tool that amplifies DNA and quantitative real-time reverse transcriptase PCR (RT-PCR) takes it a step further and quantifies the relative number of transcripts created in each cycle. Therefore, this technique is suitable for the quantification of mRNA levels. First, mRNA is isolated from cells or tissues and then synthesized into double stranded complementary DNA (cDNA) using the enzyme reverse transcriptase. Quantification becomes possible through the SYBR green reagent, a cyanine dye included in the reaction mix that binds all double stranded DNA and emits green light at 520 nm which is detected by a machine. Lastly, since there could be inter-sample variations in mRNA levels, gene expression of each sample is normalized against one or several housekeeping genes, which are genes transcribed at high constitutive levels. Choosing good housekeeping genes is essential for accurate and reproducible results. Thus, before a new experiment, pilot studies or literature search have to be carried out for the certain cell-type or tissue, in order to find optimal and stably expressed house-keeping genes suitable to normalize against. In our studies, we used Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Hypoxanthine-guanine phosphoribosyltransferase (HPRT) to quantify the relative gene expression of certain genes in spinal cord of animals in **Study II**. The advantage of RT-PCR is that it is very sensitive and it is possible to acquire a lot of information within a short time. Since the SYBR green method detects all double-stranded DNA in the sample, it is extremely important to avoid contamination of genomic DNA, which could alter the measured signal and lead to false results. By using a melt-curve analysis at the end of the protocol, it is possible to evaluate the specificity of the primers, since only one peak represents the amplified target. To really confirm the specificity of the primers, one has to run the amplicon on a gel, and confirm a single band with correct length. One limitation with this method is that the data which is acquired, only gives information about the relative amounts of gene expression, and not absolute levels. Thus, it is difficult to compare results between experiments.

2.3 AFFYMETRIX GENE MICROARRAY

Gene expression microarray is a powerful tool that allows researchers to dissect the transcriptional events underlying a certain phenotype. The principle of microarray technology is to get an overview snapshot of the transcriptome in a high-throughput manner, based on the complementary properties of DNA. This is achieved by a chip, usually a glass slide, containing thousands of synthetic DNA probes with known sequences, each complementary to a certain gene in the transcriptome. The mRNA has to be prepared prior to analysis by conversion to cDNA and the addition of a fluorescent dye. The cDNA sample is then added to the chip and allowed to hybridize, followed by a wash that removes any unbound transcript. Transcript that are present in the sample, will hybridize to its corresponding probe. In the last step, the microarray chip is exposed to ultraviolet light and transcripts captured by a probe emits light that can be detected by a camera. The emitted light intensity of each probe is proportional to the abundance of its corresponding transcript, which makes quantification possible. Microarray data yields very large amounts of data. To dig out the relevant genes requires knowledge in

bioinformatics and data mining. Further validation studies are then required, which could be very time consuming. We used this method when we did the global genetic analysis of spinal cords in **Study II**.

2.4 IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

Immunohistochemistry (IHC) and immunofluorescence are antibody-based methods for labeling proteins on prepared tissue sections. Briefly, a monoclonal or polyclonal antibody specific for the protein of interested is incubated with a very thin sample section. If the target protein is present, the specific antibody binds to it, and excess antibodies are washed away. When a secondary antibody is added, it binds the primary antibody/protein complex. The secondary antibody is usually coupled with a fluorophore or a chemical moiety that gives a signal after addition of an appropriate detection system. Finally, the protein-antibody complex can be visualized with UV or light microscopy that are usually coupled to a camera and computer screen for recording. IHC allows staining with two antibodies, which makes it possible to co-localize an unknown protein with a known marker. IHC is a qualitative or semi-quantitative method, but appropriate controls and a blinded observer are a prerequisite. One of the major concerns with IHC is the high chance of finding false positives or false negatives. The sensitivity depends mostly on tissue integrity and the quality of the antibody, i.e. specificity. However, the method is not as sensitive as PCR. To make good sections, requires practice and patience, but could result in esthetic and informative images that helps both the researcher and the reader to get a clearer understanding the concept. This method was used extensively for **Study II**, where we had spinal cord tissue sections that had been snap frozen and cryosectioned on glass slides.

2.5 FLOW CYTOMETRY

Flow cytometry is a technique where cell-surface antigens are stained with fluorescently labeled antibodies and pushed through a laser that excited the fluorophore followed by the detection of its emission. Different color can be used which with the right setup allows simultaneous detection of multiple antigens. The emission intensity is proportional to the number of antibodies bound to cell surface antigens.

Analysis with flow cytometry can give information about cell size (forward scatter), granular density (side scatter) and quantification of surface receptors (fluorescent intensity). In addition, the method allows intracellular staining of cells and non-cellular staining not discussed herein. Flow cytometry is a very powerful method in the right hands, but could also easily introduce artifacts in the hands of someone lacking appropriate knowledge about the biological significance. Therefore, the use of appropriate controls is a prerequisite to get robust data, isotype controls for instance. **Study IV** is based on flow cytometry of whole-blood with prior lysis of erythrocytes and staining for monocyte surface markers.

2.6 ANIMAL MODELS

2.6.1 Ventral Root Avulsion

Ventral Root Avulsion (VRA), used in paper II, is a highly reproducible nerve injury model that causes neurodegeneration in context of local inflammatory activation in the surrounding tissue with very sparse infiltration of blood borne cells, and the subsequent death of motor neurons [191, 192].

2.6.2 Sciatic nerve transection (SNT)

Sciatic nerve transection (SNT) is an injury of the peripheral nervous system, where the sciatic nerve is transected below the obturator tendon on the thigh of the animal.

2.7 LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

Liquid chromatography-mass spectrometry (LC-MS) is a high sensitive and selective analytical method combining mass spectrometry (MS) with high performance liquid chromatography (HPLC). The chemicals of interest are separated by conventional chromatography by usage of a column. The metabolites bind to the column due to hydrophobic interactions in presence of a hydrophilic solvent, and subsequently eluted by another more hydrophobic solvent. Thereafter, the metabolites enter the mass detector where they are ionized and the solvent is eliminated. Finally, the molecules are separated by different masses. LC-MS were used to quantify KP metabolites in **Study III**.

2.8 CLINICAL DATA AND SAMPLING

In **Study I, II** and **III** CSF and clinical data were used, while peripheral blood were used in **Study IV**. Both were obtained from patients attending the Neurology Clinic, Karolinska University Hospital, Solna, Stockholm. Written informed consent were obtained from all patients and the study was approved by the regional ethical committee. Clinical examinations were performed by a board of certified specialist in neurology, and all patients diagnosed with MS fulfilled the McDonald criteria [26]. An Expanded Disability Status Scale (EDSS) score [193] was determined at the time of sampling by a certified rater. A control group consisting of patients with other non-inflammatory neurological/psychiatric conditions (OND) was also included. The patients in the OND group had normal MRI scans and no signs of inflammatory activity in CSF in terms of pleocytosis or intrathecal IgG production. In the MS group there were patients with RRMS, in both relapse and remission, PPMS and SPMS. CSF was drawn at the time of initial examination and diagnosis, centrifuged, aliquoted and stored in our local biobank until further analysis. Blood was collected and handled immediately prior to data acquisition.

3 RESULTS AND DISCUSSION

The scope of this thesis was to investigate the role and different aspects of innate immunity in the primary and secondary progressive stages of MS. The first two studies, were aimed to increase our understanding of how neurodegenerative conditions, in the context of inflammation, are regulated by the complement system. In **Study III**, we studied the kynurenine pathway (KP) in MS in order to find if dysregulation in this pathway were associated with the pathogenesis of MS, or if metabolites in the KP could predict neuropsychological comorbidity with MS. In **studies I-III** we looked for changes in CSF proteins and metabolites, while the focus in **Study IV** were on peripheral blood monocytes - here, we analyzed the expression levels of common monocyte surface markers, and the cell frequencies of monocyte subpopulations. The following sections will discuss and summarize these studies.

3.1 STUDY I: THE CENTRAL COMPLEMENT COMPONENT C3 IS ELEVATED IN PROGRESSIVE MS

3.1.1 Increased C3 correlates with the cholinergic enzyme BChE

In order to extend our previous studies on complement in animal models [194], we determined C3 in the CSF of MS patients compared to controls. The highest C3 levels were found in PPMS, followed by SPMS and then RRMS, as compared to controls. In our experimental study, we identified co-regulation of C3 and butyrylcholinesterase (BChE), an enzyme that hydrolyses choline esters, including acetylcholine. Except for being a neurotransmitter, acetylcholine exerts anti-inflammatory properties and regulates innate immunity through its action on the alpha-7 nicotinic acetylcholine receptor, a process known as “the cholinergic anti-inflammatory pathway” [195]. BChE activity was unaltered between the groups. However, at the individual level, there was a correlation between BChE and C3 which suggested a potential regulation of complement by a cholinergic tone.

3.1.2 Increased levels of NFL, preferably in patients in relapse and high lesion load

The levels of CSF neurofilament light (NFL) were increased in MS, with the highest levels found in the RRMS group followed by PPMS and SPMS, as compared to OND. Interestingly, when the patients were stratified after number of lesions as detected by MRI, the patients that had nine or more cerebral lesions displayed increased levels of C3, over patients with less than nine MRI lesions. Similarly, a trend for increased NFL in patients with more relapses were identified. This data indicates that C3 and NFL could be potential biomarkers for patients with more severe disease.

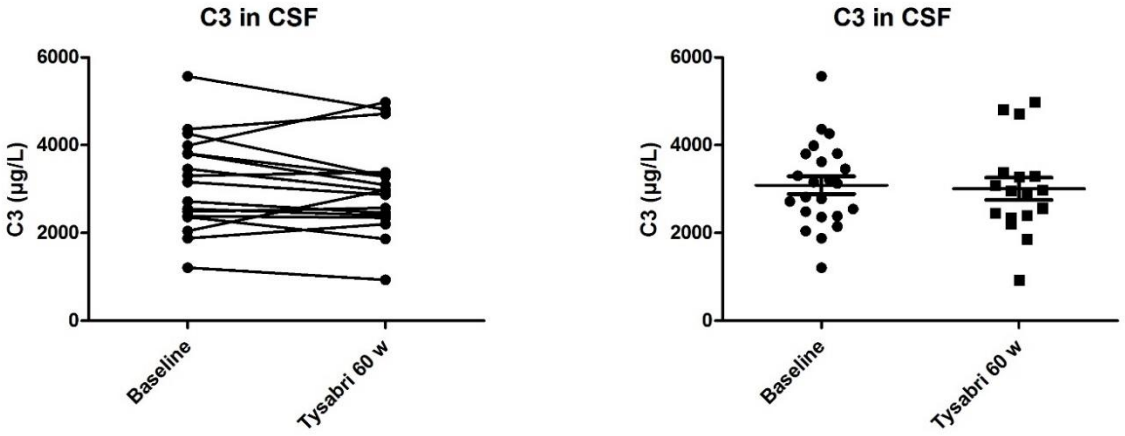
3.1.3 C3 correlates to EDSS and NFL

In line with the association between C3 and lesion load, there was a significant correlation between EDSS and C3. In addition, there was a small but significant correlation between NFL and C3 levels. Furthermore, we wanted to find out if C3, NFL and BChE would be

regulated by the inflammatory course of RRMS. Thus, we stratified the RRMS group after relapse and remission. Patients that were in relapse, both had similar C3 levels as well as BChE activity, compared to patients in remission.

The correlation of C3 to lesion load, EDSS and NFL were interesting since previous studies have shown that the number of lesions and the number of relapses are only weak predictors of the clinical outcome and time to progression[37], suggesting that active neurodegenerative processes, undetectable by MRI, occur in the brain parenchyma and normal appearing white matter.

The interpretation of this finding could be that C3 is a marker of a global and diffuse inflammation, perhaps due to low degree microglia activation rather than infiltrating adaptive immune cells from the periphery. We speculate that C3 modulates the CNS plasticity in long-term fashion, independent of the adaptive immunity. While NFL in contrast, could be a transient marker for active and ongoing injury, for instance due to inflammatory attacks. Another line of evidence in support of this hypothesis comes from a cohort (n=20) of PP/SPMS patients treated with the approved MS drug Natalizumab, a monoclonal antibody targeting the adaptive immune system. CSF were collected from these patients at baseline and after 12 months of treatment. In this cohort, there were no differences in C3 levels between baseline and treatment, suggesting that C3 activation in the CNS occurs independent of the adaptive immune system (Unpublished observation).



The exact mechanism by which the complement system could promote neurodegeneration is not completely understood. Dysregulation of the complement system in the mature CNS could be harmful in two ways, either by tagging axons and neurons, causing their elimination by activated microglia, or by creating a pro-inflammatory environment that could damage the neurons indirectly. Also, it should be stressed that we determined total C3 protein levels and not cleaved C3 fragments indicating complement activation. Whether C3 is playing an active role in promoting neurodegeneration in MS, or if the elevation is secondary to other mechanisms driving the pathogenesis of progressive MS is still an open question. In order to answer this, further studies with mechanistic approach are required. There is also a chance

that MAC formation could drive the pathogenesis, since it has been associated to other conditions with neurodegenerative components, such as Traumatic Brain Injury and spinal cord injury [196, 197]. However, complement activation does not always have to cause MAC formation. For instance, in our experimental studies using the VRA model, we did not find the presence of MAC [194]. A possible explanation for this could be the higher abundance of the MAC inhibitory molecule CD59 within the CNS compartment [198].

This is a correlation study, which does not provide any mechanistic insight to the role of complement in MS. Thus, when interpreting the results presented herein, it is important to consider that correlation does not implicate causality. This study came to life as a results of previously published data on inbred rats subjected to VRA, where we noticed an up regulation of C3 after nerve injury [194]. Even though VRA is not a model for MS, it provides a good tool to get a better understanding of the biological mechanisms underlying neurodegenerative processes, with certain aspects also found in MS. In the VRA study, we identified astrocytes as the main producers of C3 in rat glia cells [194].

The increase of BChE, which was also associated with the increase of C3, could be linked to the decrease of a transcription factor identified as a Forkhead Box Protein K2 (FoxK2) ortholog. However, this transcription factor does not have binding sites in the promoter region of C3, suggesting that this is regulated by upstream events.
Taken together, the data presented in this clinical study, nor in the previous animal studies are conclusive about the pathological mechanisms of complement activation in neuroinflammation. However, the agreement between the two studies suggest that the increase of complement factor C3 could be a general marker of neurodegeneration, rather than a specific marker for MS. Nevertheless, the data supports the notion of a dysregulated complement system, which is in line with previous studies in MS [199-203] as well as other neurodegenerative diseases like Alzheimer's and Parkinson's disease [204].

3.2 STUDY II: COMPLEMENT RECEPTOR 2 IS A NOVEL MARKER OF NEUROINFLAMMATION WITH NEUROPROTECTIVE PROPERTIES

The aim with this study was to use the data from the F2(DAxPVG) in order to characterize and genetically map potential differences in the local expression of complement receptors 1-4 by a F2 intercross of (Dark Agouti) DA and PVG (F2(DAxPVG)) and to validate findings in a clinical cohort.

Previous studies performed in our lab, using the VRA model, had shown strain-dependent differences in motor neuron survival between the inbred rat strain DA and PVG, with 23% higher survival in PVG, compared to DA. This suggested that neurodegeneration was genetically regulated and that DA rats were more vulnerable to motor neuron loss due to nerve injury [205]. In addition, following VRA, up regulation in gene expression of the early complement genes complement component 1 q beta chain (C1qb) and complement component 3 (C3) had been identified in the DA strain as compared to PVG. Moreover, the increase of C1q in DA spinal cords were correlated to the loss of neurons as a results of the injury, suggesting activation of the classical pathway in the complement system [206].

Prior to this study, we performed VRA on animals from a large intercross of DA and PVG (F2(DAxPVG), n=144), followed by a whole-genome expression and linkage analysis in order to increase our understanding in the pathways related to complement expression [194].

3.2.1 Cr2 is under strong regulation of the D13Rat49 loci

Here we identified a naturally occurring *cis*-regulating effect on the gene expression of complement receptor 2 (Cr2) in spinal cords, with a strong linkage to the genetic marker D13Rat49 (LOD score of 18, p-value <10-6) on chromosome 13. The expression of Cr3 (CD11b) and Cr4 (CD11c) were also *cis*-regulated, though not under such a strong genetic regulation as Cr2, while differences in the expression of Cr1 did not map anywhere in the genome. Since Cr2 expression was the most regulated, we focused our attention on this gene. To confirm the expression data, we performed RT-PCR for Cr2 and found that PVG D13Rat49 alleles were associated with increased Cr2 expression.

3.2.2 Cr2 correlates with anti-inflammatory genes

The D13Rat49 loci harbored 31 genes, which we used to create a complex genetic network analysis. Through this analysis, we found Cr2 to be directly co-regulated with several other transcripts, and its expression correlated well with anti-inflammatory genes like Sgpl1 [207] and Meis3 [208, 209] while it was inversely correlated with pro-inflammatory genes, for example Cd48, Cd244, F11r and Fcgr2a.

3.2.3 Confirmation of *cis*-regulation

In order to confirm the findings from the F2(DAxPVG) intercross. We subjected additional 161 animals from a G12(DAxPVG) to VRA and performed RT-PCR, followed by a more narrow microsatellite mapping around the peak marker from the F2 intercross. This study

could both confirm the *cis*-regulatory effect of D13Rat49 on Cr2 as well as the higher expression of Cr2 in PVG rats with D13Rat49 alleles.

3.2.4 Kinetics of CR1-2 expression in association to glial response following VRA and SNT

We used RT-PCR on DA and PVG rats subjected to VRA to dissect the kinetics of Cr2 expression following nerve injury. We found generally higher Cr2 expression in the PVG strain on most time points, with peak expression detected 7 days post-VRA. There is a large literature about Cr2 expression and that if forms a complex together with CD19 on B cells [210-212]. To investigate the potential role of B cells, we also performed RT-PCR on the CD19 marker. The results showed barely measurable CD19 expression with the lack of evidence for strain differences, which lead us to the conclusion to rule out B cells as the potential source of Cr2 expression in spinal cord.

In order to assess glial activation in spinal cord following VRA, we stained spinal cords for glial fibrillary acidic protein (Gfap) for astrocytes and CD11b, Mrf-1 and Iba1 as markers for microglia activation. The expression of both Gfap and Mrf-1 increased initially after 1-3 days and secondly 7 days post-injury, and on both time points higher Gfap expression was evident in PVG spinal cord. No strain differences were found in Mrf-1 expression, which suggested that the general microglia activation were unchanged between the strains. However, there were an overall increase of CD11b in DA compared to PVG, which suggested higher activation in the Cd11b+ microglia subset of DA rats.

The Sciatic nerve transection (SNT) is a model similar to VRA, though it induces a milder inflammatory response around the site of injury [213]. We used the SNT model in the Cr2 knockout mice, since VRA is very challenging in mice due to the size difference. For comparison purpose, we also performed SNT in DA and PVG rats. Here, we found astrocyte activation in both strains, but more distinct in PVG. We also looked for CD19 expression, but only found very low levels, without strain influences, speaking against B cell infiltration.

Previous studies have shown that CR2 binds C3 fragments [214]. In addition, we had found up regulation of C1q and C3 following injury [194]. To investigate the role CR2 in nerve injury, we used Balb/c wildtype (WT) and Cr2-/- mice on Balb/c background in the SNT model. Our analyses of Gfap expression did not reveal any differences while staining for GFAP protein showed increased levels in WT compared to knockout. Both strains showed signs of increased microglia activation. In addition, C1q was also up regulated in both groups, while C3 expression was increased in Cr2-/-. Cr2 was up regulated in WT and not detectable in the knockout mice. The knockout mice also had a higher degree of synaptic loss, assessed by synaptophysin staining.

To localize the expression of CR2, we co-stained CR2 with astrocyte or microglia/macrophage markers, and found co-localization with astrocytes. The findings were also confirmed when we used primary glial cultures from DA and PVG rats, to pinpoint the source of Cr2 expression.

3.2.5 Soluble CR2

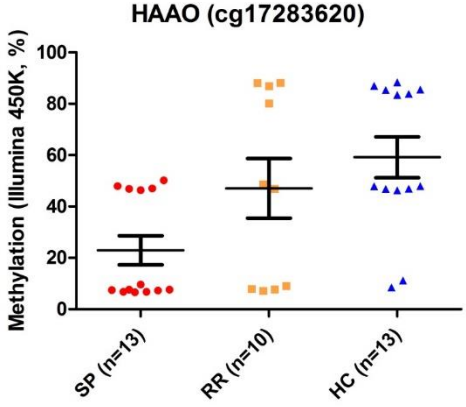
Several studies have reported the presence of a soluble form of CR2 (sCR2) [215-218]. To investigate this in our models, we used ELISA to measure sCR2 in CSF from PVG rats following VRA, and found a significant increase compared to naïve PVG. To translate these findings in a clinical setting, we used ELISA to measure sCR2 levels in 32 patients with RRMS, 9 patients with SPMS and a control group consisting of 18 patients with other non-inflammatory neurological/psychiatric diseases (OND; other neurological diseases). Interestingly, elevated levels were found in MS compared to controls. In addition, these levels also correlated to CSF C3 levels reported in **Study I**.

Taken together, we suggest a functional role for CR2 by the observation of an increased elimination of synaptic elements in the injured area of transgenic mice lacking functional CR1/2, which are transcribed from the same gene in mice but not rats or humans, compared to wild type. We also provide novel evidence that sCR2 affects the cleavage of C3b into active metabolites suggesting that it has a modulatory function in situations of complement activation. The finding of a correlation between sCR2 and CSF C3 is interesting since C3 is a large and complex molecule with multiple active breakdown products, and in this context, elevated sCR2 could reflect an intrinsic regulatory mechanism in the CNS, where up regulation and/or increased shedding of CR2 serves to modulate increased C3 activity. Further work is needed to explore if CR2/sCR2 treatment is feasible and beneficial in conditions characterized by loss of nerve terminals and dysregulated expression of complement, for example chronic neurodegenerative disorders.

3.3 STUDY III: CEREBROSPINAL FLUID KYNURENINES IN MULTIPLE SCLEROSIS; RELATION TO DISEASE COURSE AND NEUROCOGNITIVE SYMPTOMS

3.3.1 The kynurenine pathway in MS

The KP has been implicated in several psychiatric and neurological diseases. Due to the neuroprotective and neurotoxic balance in the KP, it was of high interest to study these metabolites, in particular since some of the involved KP enzymes are regulated by inflammatory cytokines known to be up regulated in MS. We also had preliminary data that were suggesting a potential role for the KP in MS. First, recent genetic data had identified a risk gene, rs2163226, in close association with the gene encoding 3-Hydroxyanthranilate 3,4-Dioxygenase (HAAO) [13]. HAAO is an enzyme expressed at low levels in the CNS, and it is responsible for catalyzing formation of the neurotoxic QUIN from 3-hydroxyanthranilic acid. Second, we had preliminary findings from our genome-wide methylation analysis in monocytes that HAAO was differentially methylated in MS patients, compared to healthy controls. Hypothetically, this change could cause differential gene expression toward a neurotoxic balance, which was something we wanted to investigate. Moreover, the KP was a good candidate to potentially provide a link between chronic inflammation and neuropsychological conditions such as fatigue and depression, which are common among MS patients [6, 219].



Credits to Dr. Lara Kular for this figure

Thus, the aims here was to address both changes in the KP during different stages of the disease (cohort 1, n=71), but also in relation to neurocognitive symptoms (cohort 2, n=48). For collection of the second cohort, we initiated prospective sampling of patients with recent onset and active disease (RRMS) and no prior psychiatric condition or treatment for such symptoms in order to address any possible relation between MS related CNS inflammation, alterations in the KP and neurocognitive symptoms.

In this study, we quantified the levels of tryptophan (TRP), kynurenine (KYN), kynurenic acid (KYNA) and quinolinic acid (QUIN) in both cohorts. The first step in the KP is catalyzed by IDO or TDO, and results in the synthesis of KYN from TRP. Therefore, the ratio of TRP/KYN gives a hint about the degree of KP activation. The levels of IDO increases by

pro-inflammatory cytokines, which could result in over activation of the KP in inflammatory conditions. Dysregulation of the KP has been implicated in neurological diseases [149] such as Alzheimer's, amyotrophic lateral sclerosis (ALS), Parkinson's and in psychiatric conditions such as depression [169, 170], bipolar disorder [171] and schizophrenia [220]. Only two smaller studies have previously attempted to describe KP in MS, however, limited only to examine KYNA. Thus, they found lower CSF KYNA compared to controls in the first [221] and higher levels in patients that were in a relapse in the subsequent study [222].

In addition, in the second cohort we quantified KP metabolites in CSF of RRMS patients that had undergone a thorough psychiatric evaluation prior to our analyses and multivariate analysis (MVA) was used to find potential associations between psychiatric and KP status. The evaluations included The Montgomery-Asberg Depression Rating Scale (MADRS-S) [223] and the Modified Fatigue Impact Scale (MFIS) [224], both administered as self-rating instruments, and a structured diagnostic interview (The Mini International Neuropsychiatric Interview (M.I.N.I.) [225]), performed by a psychiatrist, for diagnosis of major depressive disorder (MDD).

3.3.2 The KP in different MS courses

In the first cohort, we found that levels of different KP metabolites were unchanged between MS and the control group. However, when the patients were stratified into different disease courses, we found interesting differences. Hence, QUIN levels and the ratio of QUIN/KYN was increased in the RRMS patients in relapse. Patients with SPMS had a trend for lower tryptophan and KYNA, while PPMS patients had increased levels of all KP metabolites. There are several lines of evidence that shows how inflammatory stimuli can activate the KP [226-229]. Generally RRMS is considered to be an inflammation driven process and progressive MS is characterized by neurodegenerative features, where inflammation do not play a major role. Therefore, one of the most interesting findings here was that PPMS, considered as less inflammatory than RRMS, displayed a more altered KP, which in turn suggests specific regulatory mechanisms not solely depending on the general level of inflammation. This "paradox" was also evident by the large and variable alterations in the inflammatory other neurological disease (iOND) group. Notably, this group consisted of several patients with systemic lupus erythematosus (SLE), an autoimmune disease affecting several organs including the CNS, resulting in neurocognitive symptoms.

3.3.3 The KP in relation to neurocognitive symptoms

The outcome of the psychiatric evaluation were that we identified 12 depressed patients and 7 that displayed clinically relevant fatigue. These numbers were relatively small to make good predictions, but they represented real life frequencies and could at least provide indications. Psychiatric comorbidity could not be predicted by KP the KP status with MVA, which suggested that KP were not involved in neuropsychological dysfunction in MS. Future studies with enrichment of patients with psychiatric co-morbidities are needed to rule out any association with psychiatric symptoms with higher sensitivity.

The KP is active both in the periphery and in the CNS, though the concentrations of TRP and KP metabolites is 2-3 orders of magnitude higher in the periphery compared to CSF. We did not measure KP metabolites in plasma or serum, instead the main focus here was to report levels in the intrathecal compartment. We hypothesized that if the KP would be involved in the pathogenesis of MS, then alterations would be better represented in the CSF, since it better mirrors changes in the CNS. To get a better understanding in the role of KP in MS, future studies could measure serum levels of KP metabolites and determine their association to levels found in CSF. However, a large overlap would not be expected due to the biology of tryptophan transportation. The initial step of KP in the CNS involves the transportation of L-TRP from the blood and across the BBB into the CNS. This is facilitated by a transporter that recognizes TRP, but also other amino acids like phenylalanine, leucine and methionine, causing competition between TRP and the other amino acids for the carrier. Thus, the brain levels of TRP is not a direct function of its blood concentration, but also depends on the concentration of the other amino acids. In addition, different cell types produce KP metabolites in the CNS compared to the periphery, e.g. astrocytes and microglia in the CNS. In line with this, a previous study reported correlation between plasma and CSF levels of KYN as well as QUIN, while no correlation was evident for TRP [230].

3.4 STUDY IV: MONOCYTE SUBSET FREQUENCIES AND CHEMOKINE EXPRESSION IN MULTIPLE SCLEROSIS

MS is a heterogeneous disease with many components. Inflammatory attacks by the adaptive immune system is the hallmark in the early phases of the disease. In addition, our lab previously reported decreased gene expression of vascular endothelial growth factor A (VEGF-A), peripheral blood cells of SPMS patients in relation to RRMS and controls [231]. Monocytes were identified as the main source of VEGF, which were suggesting that monocytes potentially had an altered phenotype in SPMS patients. These data motivated the initiation of a cross-sectional study where we collected blood samples and isolated CD14+ monocytes from RRMS, SPMS and healthy controls (HC) that were carefully selected based on clinical history, age, sex and disease course. The goal was to make a global gene expression analysis of monocytes from MS patients and controls, in order to get a better understanding of potentially altered phenotypes of monocytes. Extracted mRNA of CD14+ blood monocytes from a cohort that consisted of 48 patients and controls (RRMS n=15, SPMS n=17, HC n=16) were analyzed with an Affymetrix genome-wide expression microarray. Due to unexplained large batch variations, it proved impossible to extract useful data from the experiment. However, DNA from the same MS cohort were used for whole genome methylation analysis which revealed several changes, with the most interesting being a prominent alteration in methylation pattern of the most common MS risk allele, HLA-DRB1*15:01. The gene of MS patients were significantly hypomethylated compared to the healthy controls. Decreased methylation pattern had a dose-dependent inverse effect on gene expression in patients and controls (Submitted manuscript, Jagodic et al.). To validate findings from genome-wide studies is very time consuming since it could require sample collection and new experiments with different methods. However, these studies did not make it to the thesis, but encouraged further investigations in the subject.

Several experimental studies showing that monocytes and macrophages could indeed have a role in neuroinflammatory conditions. In the most common animal model of MS, experimental autoimmune encephalitis (EAE), it has been shown that blood monocytes are required for initiation and progression of disease [117]. Monocytes are produced in the bone marrow, where they mature before they enter the blood circulation. During inflammation, monocytes enter the CNS and differentiate into macrophages.

Experimental and post-mortem studies have often found macrophages in close proximity to MS lesions [232-234], where they have are thought to be involved in the breakdown of myelin. Since it is difficult to study the phenotype of monocytes and macrophages in the CNS of patients, we chose to analyze blood monocytes instead.

It would also be interesting to look for phenotypic changes in CNS infiltrating monocytes isolated from the CSF. However, the CSF in general, as well as in MS patients constitute much fewer cells compared to the periphery, and cell frequencies are heavily skewed towards T cells, with low frequency of monocytes [235-237]. Due to this, it has been technically

challenging to isolate enough cells for analysis and has therefore been outside the scope of this thesis.

Since previous studies have shown alterations in the monocyte phenotype in progressive MS, the aims of this study was to investigate if blood monocytes from MS patients had an altered phenotype, or if any of the subpopulations would be expanded compared to healthy controls.

Blood from MS patients with different disease course, and healthy controls, were collected in cohort 1 (n=69). In cohort 2 (n=21), patients that were about to start the new MS-approved oral treatment dimethyl fumarate (DMF/Tecfidera) were followed from baseline and follow-up samples were collected after 2 weeks, 3 months and 6 months of treatment.

Briefly, after collection, the blood was stained with antibodies specific for the monocyte markers CD14, CD16, chemokine receptor 2 (CCR2) and chemokine (C-X3-C motif) receptor 1 (CX3CR1). Flow cytometry was used to acquire information about the cell frequencies for the classical, intermediary and non-classical monocyte subsets, and to measure the cell surface levels of CCR2 and CX3CR1 for each subpopulation.

Initially, we did pilot experiments where we screened monocytes from MS cases and controls for a spectrum of surface receptor in order to find potential candidates that had differences distinguishing the SPMS group from RRMS and healthy controls. In these pilot experiments, the only markers that were showing interesting trends were CCR2 and CX3CR1, which is why we chose to focus our studies on these surface receptors. The CCR2 receptor is essential for monocytes to egress from the bone marrow and is attracted to the site of injury in response to chemokine (C-C motif) ligand 2 (CCL2) [238, 239]. CX3CR1 is the receptor for chemokine (C-X3-C motif) ligand 1 which is also known as fractalkine. CX3CR1 is expressed on lymphocytes, NK cells, monocytes and is involved in diverse functions like trafficking of monocytes to the lungs, regulation of autoimmune inflammation in the CNS, modulate microglia neurotoxicity, homeostasis and cell survival of monocytes [239, 240].

Surprisingly, we did not detect any major quantitative or qualitative differences in MS patients, independent of disease course, compared to healthy controls. In addition, the patients treated with DMF, which is generally considered to exert anti-inflammatory properties, were associated with a heterogeneous response. There were three types of effects found among the DMF patients; one group that did not result in any differences, a second group that had expanded classical monocytes in conjunction with decreased non-classical monocytes, and a third group that had difference opposite to those in the second group. These changes could not be explained by patient-experience treatment effects, or other clinical parameters such as EDSS, age and sex.

There are some possible reasons that could explain the lack of major changes. First and foremost, there might not be major changes in the peripheral monocytes, and if there would be any alterations, they would be found in the CNS. One way to study this would be to overcome the technical challenges and study monocytes in the CSF. There are presently

techniques that would allow analyses on single cell level, and we made some efforts to do this, but that project did not make it due to time limitations. A second option could clearly be that we looked at wrong receptors, or phenotypic changes are present in intracellular process, for instance production of reactive oxygen species and nitric oxide. The last option could be that there are subtle changes in many of the receptors analyzed, but the techniques and laboratory handlings are lacking enough sensitivity. However, it can be argued whether changes found with more sensitive methods would have any biological significance.

4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In this thesis work I have looked at different biomarkers related to later stages of MS. The finding of a dysregulation in the complement system that correlates with neurological disability, but with relatively lower NFL level - a marker of neuroaxonal degeneration, suggests more of a synaptic pathology. Thus, there is now a growing body of evidence showing that complement is involved in synaptic remodeling.

Synapse formation is a dynamic process, essential for memory consolidation and reformation of synapses occur through life. We are born with a huge excess of synapses that are stripped away during embryogenesis and the first years in life. Synaptic elimination decreases during later stages of life. However, to maintain the homeostasis in the brain, redundant synapses that are no longer in use are stripped away. Synapses are to some degree to be likened to muscles that have to be used in order to prevent atrophy. A life without enrichment for instance will decrease the number of synapses. While the synapses responsible for walking, moving and balance might become redundant if they are not used. We hypothesize that this could be the outcome in progressive patients with physical disabilities. Synaptic elimination or pruning is a normal process to shape the CNS and make it more effective. However excess elimination could lead result cause neurological deficits such as cognitive decline and might also be the reason for brain volume shrinkage evident in MS patients.

NFL is a marker for axonal damage and correlates to the number of injured axons. When the levels of NFL are measured, absolute levels cannot be compared without accounting for the source. That is, NFL is not an absolute marker for how great the injury is. For instance, the spinal cord consist of long nerve bundles of axons. A small injury could thus result in very high levels of NFL, while a similar cerebral focal lesion not affecting long tract nerve trajectories might results in much lower levels of released NFL even if the number of affected neurons would be the same.

Inflammatory biomarkers, but also NFL decreases in patients with progressive disease [241]. At the same time atrophy continues in spite of the fact that focal inflammatory lesions decrease in frequency. A continued enigma is what are the mechanisms driving the neurodegenerative processes leading to atrophy and decrease in brain volume in progressive MS?

NMDAR encephalitis (NMDARe) is a disease where the brain volume decreases substantially in severe cases [242]. The disease is mediated by autoantibodies that targets the NMDA receptors. In our own preliminary data patients with NMDARe display clearly elevated levels of NFL, but not extreme levels needed to explain the rapid atrophy (un-published observation). This indicated that the NMDARe patients have a more diffuse low degree inflammation that causes atrophy. It may be speculated that NMDARe is more of an attack on dendritic arborizations rather than on cell bodies and axons, which would fit with the location of NMDARs. Extensive loss of synapses, but not cell bodies, would then explain the brain atrophy seen in these patients. It would therefore be interesting to extend studies of complement

proteins to this patient group in order to understand if upstream complement components could serve as markers of synaptic pathology and diffuse neurodegeneration. It could then be that the relative ratio of NFL, being a marker of neuroaxonal injury mainly in RRMS, to C3 could shift as a patient enters a secondary progressive disease state. The respective molecule reflecting different disease processes both resulting in brain atrophy. Another interesting aspect would be to study if physical activity would impact on complement levels. Based in part on the findings presented here, our group is currently making efforts towards these questions. In a recent pilot study, 20 stable RRMS patients participated in a high intensity muscle strength exercise program over 12 weeks under supervision of physiotherapists (Kierkegaard, submitted). Apart from improved muscle strength, the patients reported significant reductions in fatigue, anxiety and MS related mental and physical symptoms. Interestingly, cytokine analyses also revealed significantly lowered levels of tumor necrosis factor (TNF) in blood, indicating a decreased systemic inflammation in the periphery. In future studies, we will measure C3 levels in this cohort, before and after exercise. A lowering in C3 levels could indicate positive effects if excess C3 is detrimental as our hypothesis suggests. In addition another ongoing study that includes healthy individuals participating in an aerobic exercise program, CSF and blood collected before and after interventions will be examined. Results from these two studies may give an indication if the hypothesis of a role for C3 in activity dependent synaptic plasticity is correct. If so, this would strengthen the role for continued physical training and rehabilitation, in combination with the indicated disease modulatory drugs, in patients with MS.

Animal studies are important tools to dissect genetic mechanisms in complex disease and the starting point of the present thesis work was an experimental model a local inflammation reaction in the CNS, in the context of degenerating neurons with little influx of blood borne cells, which could provide insights into certain aspects also evident in MS. For instance inflammation and neurodegeneration are the major hallmarks of MS. By having a local injury and an experimental model that is highly reproducible, it is possible to do mechanistic studies, which can be difficult in a clinical cohort. Both first studies strengthen the notion of a role for complement in MS and also provide interesting insights into possible disease mechanisms that operates in MS. Such knowledge is important to be able to devise novel treatment strategies that may prove more useful in later stages of MS.

In the second part of the thesis I studied alterations in a metabolic pathway downstream of TRP. The KP is interesting as an increasing number of studies suggest that metabolites of this pathway could be neurotoxic and thus highly interesting for progressive disease. KP has also been implicated in many neurodegenerative and psychiatric conditions. We did not find any striking differences, but interestingly the PPMS group displayed a very different pattern compared to SPMS and other groups. It has been suggested that PPMS are SPMS patients amputated from the RR phase. The explanation for this could be that the inflammatory attacks occur but never reaches the clinical threshold which would give rise to the symptoms common for RRMS. Due to a relatively low number of patients in the PPMS group, results have to be interpreted with caution until further studies with larger groups have been conducted. There have been several reports of dysregulation of the KP in psychiatric conditions, including

depression, suicidality, bipolar disorder and schizophrenia. There is an increased risk for depression among MS patients. In our study, we used MVA to investigate if dysregulation in KP also had a role in psychiatric conditions among MS patients. We found that patients with depression were clustered in the MVA, but also together with non-depressed patients, which suggests a low predictive value. Nevertheless, the results argues against a role for KP as a causative feature of mental symptoms in MS. Thus, other causes such as damage to the actual wiring of the brain or the psychological impact of having a chronic disease, acting through other biochemical processes, may prove more important. It would be interesting to evaluate in a larger training study if depression could be treated by exercise among MS patients, which may also help to identify the putative processes. Although outside of the scope of this thesis, exercise is an interesting way of treating inflammatory and neurodegenerative conditions. It would be naïve to believe that this would be the main treatment that would cure progression in MS. However, the benefits of exercise could be many, both physiological as well as psychological. Exercise has beneficial effects in several ways, it stimulates the production of neurotrophic factors and promotes the release of hormones that increases general well-being and mood. Group exercise is also a form of enrichment and help patients to get outside and meet other people. It is not only the exercise in itself, but the social atmosphere of working in a group with other people and have the feeling of being involved in something. Partial physical dysfunction could become worsened by immobility, due to loss of synaptic connections. We hypothesize that exercise could not only help to maintain healthy synapses but also promote regeneration and thus prevent brain shrinkage, cognitive decline and excessive physical disability.

We here could not detect alterations in the phenotype of blood derived monocytes. However, this does not exclude that differences in cellular innate immune activation actually exist, for example with regard to the possible presence of a low grade wide spread chronic microglia activation. Future studies should further investigate the pathogenic and inflammatory phenotypes monocytes, macrophages and microglia, causing progression in MS.

What is the importance of these findings?

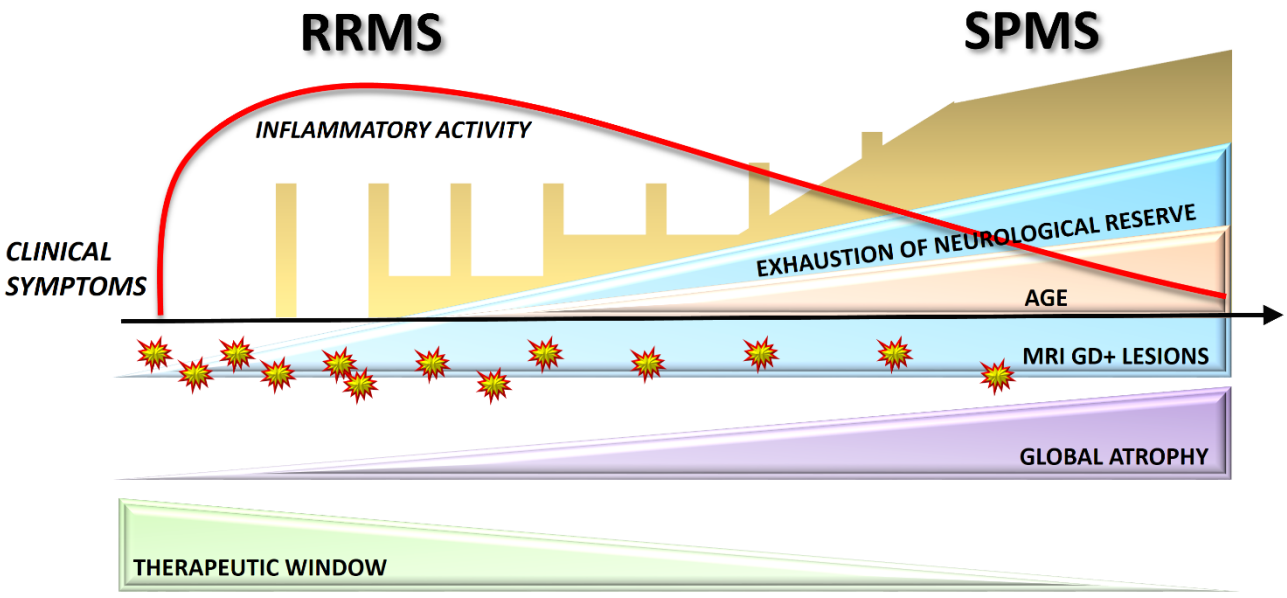
Complement dysregulation is established in many neurodegenerative conditions, and now also demonstrated here and other studies in MS. The animal studies have been important since these things are difficult to investigate in human subjects. For example, the genetic mapping of expression differences in context of a standardized nerve injury has been the first such effort performed to date. In contrast, many studies have used post-mortem brain materials. We found alterations in the complement system, which indicates a direct or indirect role for complement in MS. This is also supported by evidence from other studies. Though our studies can neither confirm, nor deny a direct role, I believe that over-activation of the complement system is detrimental to the CNS, since it would create an environment reinforcing deconstruction of synaptic connections and also reinforce inflammation, both having a negative impact in the long run. Local complement expression, especially C1q, is up regulated in the ageing brain,

and the combined effect of disease induced complement expression and ageing could prove important components of the progressive MS phenotype.

I also studied KP, which showed to be differently regulated in different MS disease stages. The most interesting observation was that PPMS and SPMS differed so much, indicating that true differences in the pathogenic processes may be at hand, with more pronounced metabolic differences in the former group. However, this finding needs to be verified in larger materials. In contrast, no clear relation between KP patterns and mental symptoms were identified, thus speaking against KP as a potential drug target for such symptoms.

Finally, although there have been indications that progressive MS entails a shift towards innate immune activation detectable also in the periphery, I could not find evidence for a shifted monocyte phenotype in blood from progressive MS patients as compared to RRMS and controls.

In summary, current therapies in MS are directed mainly at modulating the adaptive immune defense, important mainly in initial phases of MS. My findings of altered complement expression and metabolic changes involving the KP provides two examples of pathways deserving more attention as potential therapeutic targets in later stages of MS.



5 ACKNOWLEDGEMENTS

During the six years I have been at CMM, I have been surrounded by so many talented and awesome people that made it really fun for me to work here. There are many people I would like to thank, in particular:

My main supervisor Professor **Fredrik Piehl.** Thank you for allowing me to join your group and for taking me under your wings. You have always been available and given me support whenever needed, even though you're also very busy with the great work you're doing in the clinic. It has been inspiring to have you as a supervisor and I am really impressed of how you manage to combine research, clinic, and family yet still be so good at what you do! I appreciate that you always included me in every meeting with our collaborators, both in our own research but also within psychiatry, it has indeed been very rewarding. With these things said, you are a very humble boss and a true role model!

My co-supervisor Dr. **Mohsen Khademi.** You have been a tremendous support for me through the years in the lab. It is very easy to talk to you and we always have interesting discussions, not only about science but also other things, which made me feel like you're not only my colleague but also a friend. You are very helpful and never too busy to give me samples from the biobank with short notice, or to answer my questions, or to give me motivation whenever needed. You're a great person and you keep this lab together!

My other co-supervisor Dr. **Maja Jagodic**. Thank you for the journal clubs we used to have at your place, those gatherings were really nice times. I think that you're really great, both for the humble person that you are, but also for being a very intelligent researcher. Also thank you for the nice collaborations with the microarray and methylation projects, I hope that the work gets accepted in Nature Genetics - that would be awesome! Professor **Tomas Olsson**, the head of the Neuroimmunology Unit. Thank you for giving me the opportunity to work in your lab. There are few professors that are as generous and humble as you are. You believe in giving your students freedom to work under responsibility, which I think creates a nice atmosphere. And in return, I am really happy that I have been able to help you with IT-related questions. **Ingrid** for providing genetic data.

Professor **Bob Harris**, thank you for being such as nice and relaxed person. It is always interesting and inspiring to discuss things with you. Also, it feels good to have a person like you in the lab, because you are so keen about the well-being of people surrounding you. **Rickard**, I started here as your student when I did my master's thesis. So you have been one of my closest colleagues during these years. I have a lot to thank you for, since it was probably thanks to you I got this position. You have taught me a lot of things and involved me in your projects, which gave me lots of practice in various laboratory techniques, experience in working with animal models and great publications. Thank you for being such a nice and humble person, I know you will soon become a specialist doctor and continue saving even more lives! **Faiez**, I am really happy that I got to know you. You are such a fun person, but also deep and philosophical, intelligent, caring and humble. Also thank you for our collaborations! **Karl, Harald, Rasmus,** for creating a really nice atmosphere in the lab and all the fun we have had on the slopes at our ski conferences. **Hannes**, for your kindness, it's soon your turn to finish your PhD. **Andreas**, thank you for being such a nice person and for sharing your knowledge in western blot and immunology. **Roham** for being a fun person, for interesting discussions and for sharing your knowledge in FACS. I am happy that I didn't get us killed. **André** for always being such a nice person, for the fun times we had both on the slopes and on conferences abroad. **Nada**, you're a wonderful person and you always felt like a big sister to me. **Sabrina** for being so kind and for the collaborations we had during the first years, it was really fun! **Marie** for clocking in my raps and your positive attitude. You have proven that it is possible to be messy and still be a good scientist. **Sevi**, for your friendship both inside and outside the lab. And for introducing me to **Dimitris**, that is now like a brother to me. **Eliane** for your positive and caring personality, good luck with your PhD! **Mathias G**, for all the interesting discussions that we have had. Good luck with your PhD! **Lara** for being such a nice person and for the collaborations with the methylation data. **Xing-Mei**, thank you for teaching me about how to culture glial cells and for spreading so much positive energy with your laughter. **Brinda** for your friendship and being such a nice and caring person. **Tojo** for being so friendly and all the nice chats about everything from science to real estate.

Venus, Rux, Sreeni, Alexandra, Izaura, Magdalena, Pernilla, Nånis for our nice little chats and for creating a good atmosphere to work in.

I would also like to thank some of the previous lab members: **Petra** for teaching me your tricks in Excel and how to ski. **Cecilia** for being such a nice person and a good friend. **Mélanie** for being so nice and friendly. **Ame** and **Alan** for making me feel welcome in the lab, teaching me many things and all the times in the gym or playing football. **Mikael**, for making me feel welcome in the lab when I was new and for your nice home brew beer. **Milena** and **Manuel**, for our collaborations. Looking forward in finalizing the paper. **Sohel** for being such a nice and humble person and for our interesting discussions. **Peter** and **Alex** for being great colleagues and the times we played in CMM locomotive. **Agnes** and **Joan** for your warm and friendly personalities. Other people in CMM, **Anton**, **Albert**, **Sahl**, **Timmy,** you´re great friends and I'm happy I got to know you. And please forgive me if I forgot anyone!

Outside our lab, I would also like to thank my collaborators:

Taher, for being such a nice and helpful person, and for our great collaborations, they were very valuable and resulted in several publications! Also thanks to **Swetha** for helping out with the experiments.

Sophie Erhardt, Maria Bhat, and Sara Ståhl: Thank you for our great collaborations, without you I wouldn't have had **Study III** in my thesis.

Jussi Jokinen, Josef Isung, Philip Brenner, Karin Collste and Simon Cervenka: Thank you for the fruitful collaborations and also for allowing me to be involved in your projects within psychiatry. Our collaborators in Uppsala, **Kerstin Sandholm, Bo Nilsson and Kristina Nilsson Ekdahl**, thank you for a fantastic job!

To the **nurses** at the Neurology clinic, you're doing a fantastic job, not only for the patients but also for research. Thanks a lot for always being so nice and helpful!

On the private side, I would like to thank all my friends, you would be too many to name, but I appreciate you all.
Keisha and **Diesel**, you're in my heart forever and always.
Apollo, Dev and **Pesi** for all the love and joy you bring!

All my **cousin and relatives**, even though I don't see you very often, I want to stress how much you mean to me and that I appreciate you a lot! Thank you for all the love and support!

The persons I need to thank the most is my **Family**. **Mamma** och **Pappa**, jag är så tacksam över att ha så bra föräldrar. Tack för att ni alltid har stöttat mig i det jag gör. För att ni bryr er så mycket och alltid försöker underlätta för mig. Tack för all den goda maten som ni har lagat åt mig under alla år. Min **syster Shabnam**, tack för att du är världens bästa och finaste syster med ett så varmt och fint hjärta. Önskar dig all lycka i livet!

Tore och **Tilla**, ni är så fina människor och jag är himla tacksam över att få ha er som mina svärföräldrar! Tack för att ni bryr er så mycket om hur det går för mig!

Till min älskade **Katrin**, ord kan inte beskriva vad jag känner för dig. Jag är oändligt tacksam för att du finns i mitt liv, för att du kompletterar mig, alltid ger mig stöd när jag behöver och för all kärlek jag får av dig. Älskar dig massor!

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