Identifying novel soluble biomarkers in relapsing-remitting multiple sclerosis

by

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Abstract

Relapsing-remitting multiple sclerosis (RRMS) is a chronic immune-mediated inflammatory disease characterized by central nervous system (CNS) demyelination and axonal damage. Under current guidelines, a multiple sclerosis (MS) diagnosis most often occurs over the course of months and requires clinical assessment, magnetic resonance imaging (MRI), and a lumbar puncture. While clinically useful diagnostic, prognostic, and disease monitoring biomarkers do exist, they share commonalities with many other autoimmune and/or neurodegenerative disorders. As a result, this process leaves patients waiting for critical healthcare services. The objective of this thesis is to identify novel candidate biomarkers in blood plasma of MS patients and elucidate pathophysiological disease mechanisms in RRMS.

Blood plasma represents an accessible body fluid harboring many immune-related molecules that may inform on RRMS disease status and ongoing systemic pathological mechanisms. In this thesis, interleukin-1 receptor antagonist (IL-1RA) was identified as a plasma-based biomarker for increased disability in RRMS that is released from macrophages and microglia in active areas of lesions during activation of an inflammasome. Blood plasma of RRMS cases was also used to investigate the patterns of circulating extracellular vesicles (EVs). It was determined that RRMS cases have higher levels of immune cell derived EVs in circulation compared to healthy controls, and that this was unrelated to numbers of circulating parent cell populations. Finally, cerebrospinal fluid samples were analysed for 27 cytokines, and identified few differences in RRMS compared to non-inflammatory neurological disease controls. CXCL10 levels were significantly increased but were not associated with its most well-

known function of immune cell chemotaxis. Instead, an alternative pathological mechanism whereby CXCL10 leads to downregulation of glutamate transporters on astrocytes was identified.

This thesis highlights the wealth of information to be gained from studying body fluid-based biomarkers of ongoing inflammatory activity in RRMS and identifies three exploratory biomarkers for which future studies will be based on. These future works should focus on determining the sensitivity and specificity of these molecules in MS prospectively, longitudinally and across the disability and disease spectrum. Furthermore, future studies will work any functional mechanisms that are modulated by IL-1RA, immune cell derived EVs and CXCL10.

General Summary

Relapsing-remitting multiple sclerosis (RRMS) is a chronic neurological condition that is caused by abnormal targeting of the brain by immune cells. Right now, getting a confirmed MS diagnosis is a long and arduous process involving consultation with a neurologist, MRI scans and a lumbar puncture. While useful biomarkers to help diagnose and monitor MS disease do exist, none of them are specific to MS. As a result, the diagnostic process is lengthy, and often leaves patients waiting for critical healthcare services. The objective of this thesis is to identify new potential biomarkers in blood and spinal fluid samples of MS patients and determining whether they contribute to ongoing processes that lead to damage in RRMS.

Blood samples can be collected non-invasively and likely contain many immune-related molecules that can inform on RRMS disease. In this thesis, interleukin-1 receptor antagonist (IL-1RA) an anti-inflammatory molecule was found to be a marker of increased disability in RRMS that is released from immune cells in brain lesions in MS. Blood samples from RRMS cases were also used to investigate the patterns of circulating extracellular vesicles (EVs). It was determined that RRMS cases have higher levels of immune cell derived EVs in circulation compared to healthy controls, and that this was unrelated to numbers of circulating immune cells. Finally, 27 immune-related molecules were measured in cerebrospinal fluid samples. Levels of the molecule CXCL10 higher in RRMS but were not associated with its most well-known function of attracting immune cells to the brain. Instead, CXCL10 can alter the activation of astrocytes, the main support cell in the brain.

This thesis highlights the wealth of information to be gained from studying body fluid-based biomarkers of ongoing inflammation in RRMS and identifies three exploratory biomarkers for which countless future studies will be based on. These future works should focus on determining whether these markers are specific to RRMS, how they change over time with the evolution of the disease, and whether they can measure disability. As each molecule identified is related to immune mechanisms, future studies should also investigate how and if they contribute to destruction in RRMS.

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List of Abbreviations

ANOVA	Analysis of variance
APC	Antigen presenting cell
AQP-4	Aquaporin-4
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDWG	Biomarker Discovery Working Group
BMI	Body mass index
CCL	C-C motif chemokine ligand
CD	Cluster of differentiation
CIS	Clinically Isolated Syndrome
CLN	Cervical lymph nodes
CMSC	Consortium of MS Centers
CNS	Central Nervous System
CP	Choroid plexus
CSF	Cerebrospinal Fluid
CXC3	C-X-C motif chemokine receptor
CXCL	C-X-C motif chemokine ligand
Cy7	Cyanine 7
DIS	Dissemination in Space
DIT	Dissemination in Time
DMEM	Dulbecco's Modified Eagle Medium
DMT	Disease-modifying therapy
EAE	Experimental autoimmune encephalomyelitis
EBNA-1	Epstein Barr virus nuclear antigen 1
EBV	Epstein Barr Virus
EDSS	expanded disability status scale
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EV	Extracellular vesicle
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FLAIR	Fluid-attenuated inversion recovery
G-CSF	Granulocyte colony stimulating factor
Gad	Gadolinium
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein

GM-CSF	Granulocyte-monocyte colony stimulating factor
HC	Healthy control
HFA	Human fetal astrocytes
HFM	Human fetal microglia
HITMS	Health Research Innovation Team in Multiple Sclerosis
IB4	Isolectin B4
IFNg	Interferon gamma
IgG	Immunoglobulin
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IIH	Idiopathic intracranial hypertension
IL	Interleukin
IL	Interleukin
IL-1RA	Interleukin-1 receptor antagonist
IM	Infectious mononucleosis
IMSGC	International multiple sclerosis genetics consortium
IQR	Interquartile range
ISF	Interstitial fluid
IU	International units
LFB	Luxol fast blue
LP	Lumbar puncture
LPS	Lipopolysaccharide
M-CSF	Macrophage colony stimulating factor
MAGNIMS	Magnetic imaging in MS
MCP-1	Monocyte chemotactic protein-1
MDM	Monocyte-derived macrophages
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MIP-1b	Macrophage inflammatory protein
miRNA	microRNA
MoCA	Montreal cognitive assessment
MOG	Myelin oligodendrocyte glycoprotein
MOGAD	MOG-antibody associated disorders
MP	Microparticles
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis

MSFC	Multiple sclerosis functional composit
MSIF	Multiple Sclerosis International Federation
MVB	Multivesicular body
NAIMS	North American Imaging in MS Cooperative
NAWM	Normal appearing white matter
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NfL	Neurofilament light chain
NIND	Noninflammatory neurological disease
NK	Natural killer cell
NLR	Nucleotide-binding domain, leucine-rich repeat containing receptor
NLRP3	NLR family Pyrin containing 3
NMOSD	Neuromyelitis optica spectrum disorder
NTA	Nanotracking analysis
OCB	Oligoclonal bands
OIND	Other inflammatory neurological disease
PacBlue	Pacific Blue
PASAT	Paced auditory serial addition test
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF-bb	Platelet derived growth factor bb
PE	Phycoerythin
PerCP	Peridinin-chlorophyll-protein
PIRA	Progression independent of relapse activity
PPMS	Primary progressive multiple sclerosis
qPCR	Quantitative PCR
RANTES	Regulated on activation, normal T cell expressed and secreted
RIS	Radiologically isolated syndrome
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
RT-qPCR	Real-time qPCR
SC	Spinal cord
SCC	Side scatter
SCF	Stem cell factor
SD	Standard deviation
SDMT	Symbol digit modalities test

SEM	Standard error of the mean
Simoa	single molecule array
SLC	Solute carrier
SLC1A2	Solute carrier family 1 member 2
SLC1A3	Solute carrier family 1 member 3
sNfL	Serum NfL
SPMS	Secondary progressive multiple sclerosis
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
tRNA	Transfer RNA
V-SSC	violet side scatter
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor

List of Publications

1. Dylan A. Galloway, Samantha J. Carew, **Stephanie N. Blandford**, Rochelle Y. Benoit, Neva J. Fudge, Tangyne Berry, G.R. Wayne Moore, Jane Barron, Craig S. Moore. Investigating the NLRP3 inflammasome and its regulator miR-223-3p in multiple sclerosis and experimental demyelination. *Journal of Neurochemistry.* doi:10.1111/jnc.15650.

2. **Stephanie N. Blandford**, Neva J. Fudge, Christopher C. Corkum, Craig S. Moore. Analysis of plasma using flow cytometry reveals increased immune cell-derived extracellular vesicles in untreated relapsing-remitting multiple sclerosis (2022). *Frontiers in Immunology.* doi:10.3389/fimmu.2022.803921 ***Included as Chapter 3**

3. **Stephanie N Blandford**, Dylan A Galloway, John B Williams, Shane Arsenault, Janet Brown, Gregg MacLean, G.R. Wayne Moore, Jane Barron, Michelle Ploughman, Fraser Clift, Mark Stefanelli, Craig S Moore. Interleukin-1 receptor antagonist: An exploratory plasma biomarker that correlates with disability and provides pathophysiological insights in relapsing-remitting multiple sclerosis (2021). *Multiple Sclerosis and Related Disorders*. 52(5):103006. doi:10.1016/j.msard.2021.103006 ***Included as Chapter 2**

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5. Dylan A. Galloway**, Stephanie N. Blandford**, Tangyne Berry, Mark Stefanelli, Michelle Ploughman, Craig S. Moore. miR-223 promotes regenerative myeloid cell phenotype and function in demyelinated central nervous system (2019). *GLIA*. 67(5):857-869. doi:10.1002/glia.23576.

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Co-Authorship Statement

I, Stephanie N. Blandford, in collaboration with my supervisor, Craig S. Moore, designed, performed, and analyzed all experiments presented herein and wrote all portions of this thesis unless specified below. Essential contributions to this thesis were provided by collaborators as listed below:

Throughout this thesis, samples used were obtained from the HITMS patient biorepository, and a biorepository of CSF samples obtained from the Eastern Health Neurology Clinic (St. John's, NL) and Horizon Health (Saint John, NB) which was maintained by J. Bradley Williams and Neva J. Fudge. Drs. Mark Stefanelli, Fraser Clift, and Michelle Ploughman are essential collaborators for the HITMS biorepository along with Dr. Craig S. Moore. In Chapter 1, excerpts from a literature review revised by Dr. Dylan A. Galloway are included in section 1.3.3 were included and edited for continuity and updated content since time of publication with permission (Blandford et al., 2018). In Chapter 2, human fetal microglia and human monocyte-derived macrophages used for IL-1RA secretion experiments presented in Figure 2.4 were isolated, maintained and treated by Dr. Craig S. Moore, and clinical information was obtained with the assistance of Dr. Shane Arsenault. In Chapter 3, protocol for EV flow cytometry was developed alongside Christopher P. Corkum, and PBMC flow cytometry data presented in Figures 3.4 and 3.5 were collected by Neva J. Fudge. In Chapter 4, the BioPlex assay was performed by Elizabeth Belland and Aurelio Lobo in the Dr. George Roberston lab at Dalhousie University, and clinical information was obtained with the assistance of Dr. Shane Arsenault. In addition, CSF cell and PBMC flow cytometry data presented in Figures 4.3, 4. 5, and 4.6 were collected by Neva J. Fudge.

Chapter 1 – General Introduction and Overview

1.1 Multiple Sclerosis

1.1.1 General

For centuries, prior to the development of the medical system as we know it, people were suffering from progressive paralysis and psychiatric disturbances without diagnosis or explanation of their symptoms. With the evolution of medicine, and better technologies and investigations into pathologies, also came the progression to better classify syndromes that seemed similar in outward appearance. Medical historians describe documentations of individuals suffering from chronic progressive paralysis, believed to be the result of the condition we now know as multiple sclerosis (MS) in individuals as early as the late 1300's (Murray, 2005). MS was first fully characterised and described by Dr. Jean-Martin Charcot in a series of lectures in the late 1860s (Murray, 2005; Kumar et al., 2011). Once given a name and a recognizable set of symptoms and pathological hallmarks, recognition of the disease increased allowing better documentation and clinical investigation.

Landmark studies into the histopathology of MS were conducted by Dr. James Dawson and his mentor Dr. Alexander Bruce, and followed Charcot's initial clinical descriptions nearly 50 years later (Dawson, 1916). These studies were published in leading medical journals in the early 1900's and detailed the examination of nine subjects, including one deceased early in the disease process, which offered a unique picture of the disease rarely seen and virtually never documented (Dawson, 1916). While our view of MS has changed in the 100 years since Dawson's observations, his works remain a staple in medical literature.

We know now that MS is a chronic nervous system disorder, whereby an immune response is mounted against the axoglial apparatus in the central nervous system (CNS) leading to demyelination, neurodegeneration and the breakdown in communication between neurons. According to the most recently available statistics published by the Multiple Sclerosis International Foundation (MSIF) using data collected and displayed on the MS Atlas (www.atlasofms.org), approximately 12.8 Canadians are diagnosed with MS every day, leaving over 90,000 Canadians, living with MS, almost 3/4 of them being women (MSIF, 2020). These estimates rank Canada as having one of the highest prevalence of MS in the world (MSIF, 2020). Globally, it is estimated that 2.8 million people are living with MS (MSIF, 2020). Despite significant accruement of disability and disease burden, MS is not considered fatal, and individuals still retain a long lifespan leading to a significant economic burden to both those affected, their caregivers, and the healthcare system.

Symptoms of MS are highly variable between individuals and depend on the area of the CNS where the demyelination and inflammation is occurring. Nevertheless, common symptoms include loss of mobility, visual disturbances, intermittent weakness and clumsiness, fatigue, abnormal sensations, changes in mood and cognition, and pain. Clinically, MS is diagnosed according to the 2017 revised McDonald Criteria (Thompson et al., 2018). These criteria outline the necessity for evidence of two or more areas of demyelination, termed lesions, in distinct anatomical locations in the CNS (dissemination in space, DIS) and evidence that new lesions develop over time (dissemination in time, DIT). Evidence that these criteria have been met comes from using clinical observations and/or magnetic resonance imaging (MRI) and measuring immunoglobulin G (IgG)

synthesis in the cerebrospinal fluid (CSF). The 2017 McDonald criteria are the most recent iteration of the diagnostic guidelines originally proposed in 2001 (McDonald et al., 2001; Polman et al., 2011; Thompson et al., 2018). Recent evidence suggests that in its current form, the 2017 McDonald criteria identifies clinically definite MS cases with high sensitivity and similar accuracy, but significantly shortened time to diagnosis compared to earlier iterations (McDonald et al., 2001; Polman et al., 2011; Thompson et al., 2018; Filippi et al., 2022).

1.1.1.1 Clinical Course and Diagnosis

Typically, MS follows a clinical course whereby an individual seeking diagnosis has had one or more bouts of MS-like disease activity. These bouts of disease activity, termed "attacks" or "relapses" are defined as a single period of at least 24 hours of patient-reported symptoms accompanied with objective findings of an inflammatory demyelination event without evidence of fever or infection (Thompson et al., 2018). MRI and CSF analysis are subsequently used to confirm diagnosis. T2-weighted MRI scans identify CNS lesions; gadolinium (Gad) can be used during the scan to demonstrate active lesions by identifying areas of blood-brain barrier (BBB) breakdown (Housley et al., 2015). If a patient presents with scans indicating only one lesion, a diagnosis of clinically isolated syndrome (CIS) is appropriate until evidence of DIS and DIT are met radiologically following the appearance of a new lesion in a different area or clinically by the occurrence of a second distinct relapse (Fisniku et al., 2008). A diagnosis of MS can occur with a patient's first MRI scan if the scan shows evidence of two or more lesions separated anatomically (DIS), with both Gad-enhancing and non-enhancing lesions

present (DIT). Measuring IgG in CSF provides evidence that an active inflammatory event has occurred intrathecally (Thompson et al., 2018). The IgG index (comparison of the ratio of CSF IgG/albumin to serum IgG/albumin (Perkin et al., 1983)) or the presence of unique oligoclonal bands (OCBs) unique to the CSF are common metrics analysed. If OCBs are observed in the CSF, it can be used to fulfill the DIT criteria for MS diagnosis (Thompson et al., 2018).

Once a confirmed diagnosis is established, MS is characterized in distinct clinical phenotypes. Though these phenotypes are classified categorically, MS occurs on a continuum, whereby preclinical disease activity reaches clinical significance in the form of an initial attack that results in a diagnosis of CIS. If the preclinical asymptomatic disease activity is by chance observed by MRI scan ordered for another reason, an individual is diagnosed with a radiologically isolated syndrome (RIS, (Thompson et al., 2018)). Typically, those diagnosed with CIS progress to relapsing-remitting MS (RRMS), a condition characterized by bouts of clinical disability (attacks) followed by complete or partial remission of symptoms without permanent progression in disability between attacks (Lublin et al., 1996). Despite remission of symptoms following a relapse, ongoing pathological processes can continue asymptomatically, and as a result, ~50-80% of untreated RRMS patients progress to secondary progressive MS (SPMS) within 10-20 years of initial diagnosis (Scalfari et al., 2010). Patients with SPMS experience gradual and irreversible worsening of clinical symptoms resulting from progressive neurodegeneration in addition to insufficient recovery from relapses (Lublin et al., 1996). Diagnosis of progressive disease requires objective documentation of disability

progression over one year outside of the presence of clinical relapse (Thompson et al., 2018).

Finally, a subset of individuals diagnosed with MS are diagnosed with a progressive form at the time of diagnosis, termed primary progressive MS (PPMS). These individuals experience the development of symptoms and gradual worsening of neurological functioning with little to no recovery from the initial presentation of symptoms (Miller & Leary, 2007). Similar to a diagnosis of SPMS from RRMS, a diagnosis of PPMS requires objective documentation of progression over one year outside of evidence of relapse and requires MRI evidence of lesions in the brain and/or spinal cord and/or the presence of OCBs in the CSF (Thompson et al., 2018).

Although classifying the disease categorically makes logical sense and is useful from a theoretical perspective, the distinctions between clinical phenotypes is far from simple. Depending on the area of the CNS affected and the individual lesion load, symptom patterns between patients are highly heterogenous. In addition, more recent work proposed a new classification scheme that adds modifier terms to the subtype diagnosis, which adds information about the nature of disease activity in any given patient (Lublin et al., 2014). This method has been widely adopted in research literature and proposed adding the qualification of "active" vs. "non-active" to the diagnosis of CIS and RRMS disease phenotypes and separates progressive disease into combinations of "active" or "non-active" and "with progression" or "without progression" (Lublin et al., 2014).

While diagnosis of MS occurs after an individual presents with clinical symptoms suggestive of an MS attack, significant evidence indicates a prodromal period of ~5-15

years prior to disease onset (Makhani & Tremlett, 2021; Tremlett et al., 2022). This prodrome is defined as a period of time where signs of disease can be identified that predate and are not consistent the classical symptoms of the disease (Tremlett et al., 2022). Features of the MS prodrome include increased usage of healthcare services, poor cognitive performance, increased anxiety, depression, and symptoms of pain and fatigue (Makhani & Tremlett, 2021).

1.1.1.2 Clinical Monitoring of Relapses and/or Progression

Common measures to identify relapses and progression rely on physical symptoms and changes in physical disability, which fails to consider that many individuals who suffer from cognitive and behavioral symptoms that are harder to identify objectively. Critically, the differences in pathological processes between disease phenotypes is poorly understood. As it stands, it is generally accepted that RRMS and distinct clinical attacks are manifestations of ongoing immune-mediated demyelination, and that progression is a manifestation of chronic neurodegeneration that occurs as a result of the demyelination. Several lines of evidence suggest that this is true, as currently available disease modifying therapies (DMTs) successfully reduce relapse activity but fail to abrogate progression and lose their efficacy once progressive disease takes over. However, it is clear that ongoing neurodegeneration does occur between relapses in a phenomenon referred to as silent progression or progress independent of relapse activity (PIRA) suggesting neurodegenerative processes at play during RRMS independent of clinical disease activity (Cree et al., 2019; Kappos et al., 2020). In addition, the neurodegenerative processes involved in progressive disease are likely shared between

individuals with SPMS and those with PPMS, as once the individual reaches a moderate level of disability, the trajectory of progression between the two disease subtypes are very similar (Miller & Leary, 2007). Thus, the distinctions between stages of relapsing disease leading to progression, and distinctions between subtypes of progressive disease are far from clear. In addition, at present, the identification of individuals who are actively in transition, and those that have transitioned to progressive disease is exceedingly challenging.

There are many clinical tools used to measure the extent of disability in all forms of MS. The Kurtzke Expanded Disability Status Scale (EDSS; (Kurtzke, 1983) is used most often as a general measure of disability. The EDSS scale ranges from 0.0 to 10.0, where a score of 0 represents no neurological nor physical disability. The score then increases progressively with increased disability including use of a unilateral mobility aid (EDSS 6), bilateral mobility aids (EDSS 6.5), wheelchair bound most of the time (EDSS 7), bedridden (EDSS 8) and ending at death with a score of 10.0 (Kurtzke, 1983). Other commonly used measures include, but are not limited to, the timed 25-foot walk test to assess mobility and leg function, the nine-hole peg test to assess upper limb function, and the paced auditory serial addition test (PASAT) to assess auditory information processing speed and flexibility and calculation ability and the symbol digit modalities test (SDMT) to detect cognitive impairment (Ruet & Brochet, 2020). Together, the timed 25-foot walk test, the nine-hole peg test and the PASAT form the Multiple Sclerosis Functional Composite (MSFC). The MSFC was developed based on the recommendations made by the American National Multiple Sclerosis Society Clinical Outcomes Assessment Task Force in the late 1990's to include cognitive assessments which were not classically

measured in clinical assessments (Whitaker et al., 1995; Rudick et al., 1996). The MSFC has since become a staple assessment in most clinical trials in MS (Cutter et al., 1999). Numerous other tests have been adopted for use in MS research and combined to form various test batteries validated for use in MS (Kalb et al., 2018). Current recommendations suggest early screening during a period of clinical stability, annual reassessment using the same testing to monitor treatment efficacy, new relapses, and identify progression (Kalb et al., 2018). Unfortunately, due to the nature and limitations of clinic visits, these tests typically are not administered on a routine basis. As such, despite its limitation to assessing the physical symptoms of MS the Kurtske EDSS score remains the single most used measure of clinical disability due to its ease of use. However, when monitoring for progression, an increase in score at any given time can represent relapse activity and not PIRA. In addition, assessment on the need for mobility aids is somewhat subjective and involves a dialogue about what the patient and doctor feel is right for the given individual; as such, the inter-rater reliability is not ideal.

1.1.2 Epidemiology

While it is clear the disease processes leading to MS are autoimmune in nature, whether the condition arises initially as a defect in the immune system that results in the maladaptive targeting of oligodendrocytes and myelin, or whether initial CNS pathology triggers the immune reaction remains debated. As such, the etiology of the disease is largely unknown, but involves the complex interplay between multiple genetic and environmental factors, many of which are unknown (Compston & Coles, 2008).

1.1.2.1 Genetic Risk Factors

There is a small, but significant, familial risk for the development of MS. The concordance rate among monozygotic twins is roughly 30%, and there is a ~2-4% increased risk of MS in first-degree relatives of affected individuals (Ebers et al., 1986; Ebers et al., 1995). Currently, it is estimated that globally, approximately 12.6% of MS cases are familial (Harirchian et al., 2018). This proportion is possibly higher in Canadian populations, as familial MS in Saskatchewan has been reported as high as 32.7% (Hader & Yee, 2014). Family studies also suggest that there is some degree of inheritance associated with the particular disease phenotype, as affected individuals who are related tend to have more similar disease course compared to the general MS population (Kantarci et al., 2002).

Many large genome-wide association studies have identified a multitude of susceptibility genes for the development of MS, many of which are related to immune system functions and are shared susceptibility loci with other autoimmune disorders (Baranzini & Oksenberg, 2017; IMSGC, 2019). In fact, the strongest genetic susceptibility risk is associated with inheriting the HLA-DRB1*1501 haplotype on chromosome 6; this gene that corresponds to one isotype of the major histocompatibility complex (MHC) involved in antigen processing in the immune response (Barcellos et al., 2006; IMSGC, 2019). Taken together, these data suggest that genetic predisposition may cause many small-scale changes in molecular machinery and cellular pathways leading to defects in the immune system that renders an individual susceptible to the development of MS, given an environment with appropriate triggers.

1.1.2.2 Environmental Risk Factors

Many environmental and lifestyle risk factors that confer increased risk in developing MS have been identified. First, epidemiology studies have identified geography as a risk factor for the development of MS. Globally, the prevalence of MS increases as the distance from the equator increases, a phenomenon termed the "latitude effect" (Simpson et al., 2011). While this observation may represent a genetic gradient in populations in these areas, or an effect of a socioeconomic gradient resulting in better healthcare and higher incidence of diagnosis, one strong hypothesis suggests that this pattern is caused, at least in part, by differences in sun exposure resulting in differences in the availability of vitamin D. For example, there is clinical evidence that indicates that individuals who have higher systemic levels of vitamin D and its associated active metabolites, including higher levels induced by dietary supplementation of vitamin D ≥400 international units (IU)/day, have a lower risk of developing MS (Munger et al., 2004; Munger et al., 2006; Salzer et al., 2012). In addition, many studies have shown that low systemic levels of vitamin D and its active metabolites are associated with worse clinical outcomes and higher risk of progression in individuals diagnosed with CIS and MS (Simpson et al., 2011; Mowry et al., 2012; Runia et al., 2012; Ascherio et al., 2014; Fitzgerald et al., 2015; Wang et al., 2018). There is a possible genetic interaction with this environmental risk factor, as genetic variants of an enzyme that converts 25-hydroxyvitamin D into its active metabolites in the body have been documented and are associated with an increased risk of developing MS (Ramagopalan et al., 2011).

Another widely researched environmental risk factor for MS is infection with Epstein Barr virus (EBV). Within the MS population, EBV infection occurs in almost

100% of individuals documented in an early epidemiological study (Ascherio & Munch, 2000; Haahr et al., 2004), and a history of EBV-mediated infectious mononucleosis (IM) is associated with a significantly increased risk of developing MS (Ascherio & Munch, 2000; Handel et al., 2010). While the infection rate of EBV in the general population is also quite high, at >90% seropositivity (Smatti et al., 2018), the prevalent hypothesis is that EBV infection is an environmental trigger that cooperates with a genetically susceptible background to trigger MS (Hedström et al., 2015). A landmark paper published in early 2022 confirms this association with EBV infection and MS, suggesting a causal role of EBV in the development of MS harboring a 34-fold increased risk in infected individuals (Bjornevik et al., 2022). This study alludes to MS as a rare complication of EBV infection. EBV likely contributes to the generation of MS-relevant autoantigens through molecular mimicry, and through the continued persistence of EBV-infected B cells in the CNS.

Two potential sources of molecular mimicry have been identified. EBV releases a cytokine homologous to human interleukin (IL)-10 (Sin & Dittmer, 2012). The presence of circulating viral IL-10 can lead to the production of antibodies responsive to an epitope identical to one in the human homologue (Dreyfus et al., 2018). However, the immune response generated by these antibodies is believed to be extremely low (Dreyfus et al., 2018). In addition, antibodies generated against EBV nuclear antigen 1 (EBNA-1) can cross react with anoctamin 2, a neuronal calcium-gated chloride channel (Ayoglu et al., 2016; Tengvall et al., 2019). A significant gene-environment interaction has also been documented related to this; individuals carrying the HLA DRB1*15.01 allele who show high serum levels of antibodies against EBNA-1 or who have a history of IM have an

estimated 7-10-fold higher risk of developing MS (Nielsen et al., 2009; Sundström et al., 2009; Sundqvist et al., 2012). Direct and high affinity molecular mimicry between EBVNA-1 and GlialCAM, a cell adhesion molecule primarily found in oligodendrocytes, has recently been shown, suggesting that EBV infection plays a role in the development of MS (Lanz et al., 2022).

Another way EBV can contribute to the development of MS is through the survival of EBV-infected B cells and plasma cells that persist in the periphery and CNS of individuals with MS. These cells generate autoantibodies to CNS antigens and present an important source of antigen presentation and immunomodulation (Pender, 2011; Márquez & Horwitz, 2015). EBV infection is lifelong, establishing latent infection in memory B cells (Ressing et al., 2015). EBV infection has been documented in B cell and plasma cells in the CNS of individuals with MS (Serafini et al., 2007; Magliozzi et al., 2013; Hassani et al., 2018). While these cells are also seen in control populations, albeit to a lesser extent, there is evidence that suggests individuals with MS have a yet uncharacterized intrinsic dysregulation in the ability of $CD8^+$ cytotoxic T cells to clear infected B cells from the system (Pender et al., 2009; Angelini et al., 2013; Pender et al., 2017; Serafini et al., 2019). Clinical trial evidence supporting this hypothesis has been documented, in a small study, whereby individuals with progressive MS were treated with autologous transplants of T cells engineered to express an EBV-specific T cell receptor for 27 weeks, which resulted in minor improvements in EDSS and self-reported levels of fatigue with no adverse effects (Pender et al., 2018). B-cell depleting therapies such as rituximab, ocrelizumab, and ofatumumab have had success in treating MS; these therapies likely contribute to clearing the infected B cells from the CNS space, decreasing

the production of autoantibodies and drive for T cell entry into the brain (Dubey et al., 2017).

Finally, lifestyle factors that increase baseline systemic inflammation such as smoking and obesity contribute to MS by creating an environment conducive to heightened inflammatory responses (Olsson et al., 2017; Alfredsson & Olsson, 2019). Smoking has long been identified as a dose-dependent modifiable risk factor for the development of MS, where the higher cumulative dose of smoking results in a higher risk for developing MS (Handel et al., 2011; Hedström et al., 2013b). However, this risk is unrelated to the nicotine content in cigarettes and may instead be a result of lung irritation (Hedström et al., 2013a). Evidence indicating that exposure to second-hand smoke, as well as increased levels of air pollution, may also increase susceptibility to developing MS and thus provides some support to this hypothesis (Hedström et al., 2011; Heydarpour et al., 2014). Another lifestyle risk factor for the development of MS is adolescent obesity, whereby increased BMI is associated with increased risk for MS (Munger et al., 2013; Hedstrom et al., 2014). Interestingly, genetic risk studies have also shown that genetic determinants of increased BMI are also associated with MS risk (Mokry et al., 2016; Gianfrancesco et al., 2017). Low and steady levels of inflammation generated by both smoking and obesity can contribute to mechanisms involved in CNS damage of MS. The chronic inflammation generated by these factors is not restricted to the periphery. In the CNS, low but chronic inflammation can manifest as a constant low level of cellular stress in susceptible and metabolically active CNS cells, which leaves them vulnerable and unable to cope with subsequent inflammatory attacks. (Olsson et al., 2017).

1.1.3 Pathophysiology

1.1.3.1 Leukocytes in the CSF and CNS

Up until more recent years, the CNS had been considered an immunologically privileged site, referring to the ability of the tissue to tolerate the introduction of an antigen without eliciting an immune response to protect against the damaging effects of inflammation. Mechanistically, this manifests in low expression of MHC class I, increased expression of surface molecules known to inhibit complement activation, tonic production of immunosuppressive cytokines, and constitutive expression of Fas-L (Wraith & Nicholson, 2012). These mechanisms are present in the CNS, with the added protection provided by the highly selectively permeable BBB (Kadry et al., 2020).

The BBB is a highly specialized anatomical unit that compartmentalizes the CNS from the periphery. Neighboring capillary endothelial cells form tight junctions, and these endothelial cells are tightly wrapped with pericytes and astrocyte endfeet (Wraith & Nicholson, 2012; Kadry et al., 2020). The BBB forms a physical and chemical barrier that restricts the movement of pathogens, peptides, drugs, and hydrophilic molecules into the CNS. However, movement across the BBB is not impossible; the large surface area of endothelial cells provides valuable real estate for transport proteins, receptors, absorptive machinery, and diffusion thereby allowing the passage of molecules if the appropriate route is present (Kadry et al., 2020).

Immune surveillance of the CNS is critical to prevent damage from infectious agents. Lymphocyte transmigration across the BBB is driven by chemokines and requires lymphocyte binding to endothelial cells by selectin-mediated rolling, integrin activation

leading to firm adhesion, and transmigration across endothelial cells (Takeshita & Ransohoff, 2012). The growing use of potent immunomodulatory/immunosuppressive treatments for conditions like MS also reveals the dangers associated with blocking immune cell entry into CNS (Kleinschmidt-DeMasters & Tyler, 2005; Kappos et al., 2011; Ratchford et al., 2012). Evidence indicates that under physiological conditions, immune surveillance of the brain and spinal cord occurs mostly in three distinct immunological niches, the choroid plexus (CP), meninges, and CSF, therefore limiting access of immune infiltrates to the parenchyma (Croese et al., 2021). Together, these niches maintain surveillance to provide immunological support while protecting CNS tissue from the damaging effects of an activated immune system.

The CP is a specialized structure of highly vascularized tissue located in all four ventricles and is the site of CSF production (Ghersi-Egea et al., 2018). Capillaries located in the CP are fenestrated and surrounded by epithelial cells joined together by tight junctions and interconnected by gap junctions forming the blood-CSF barrier that regulates entry of soluble molecules and cells into the CSF (Solár et al., 2020). Critically, the CP also appears to be an important route of entry for cells to the CNS. Evidence indicates that CP epithelial cells constitutively express cell adhesion molecules necessary for leukocyte trafficking, and allow entry of small numbers of leukocytes, likely CD4+ effector memory T cells, into the CSF for immune surveillance (Steffen et al., 1996; Baruch et al., 2013).

Under inflammatory conditions, cell adhesion molecules are upregulated at barrier sites including the BBB and the CP, which facilitates leukocyte infiltration to the CNS (Kunis et al., 2013; Shechter et al., 2013; Baruch et al., 2015; Strazielle et al., 2016). On

the apical surface of the CP, in the CSF, tissue resident macrophages with high MHC Class II expression exist, serve as scavengers within the CSF space, and present antigen to T cells entering the CSF from the CP in an immune surveillance capacity (Nathanson & Chun, 1989; Serot et al., 1997; Coles et al., 2015). Within the capillaries of the CP, high densities of macrophages and dendritic cells present any detected antigen to T cells on the capillary surface (Bartholomäus et al., 2009; Lodygin et al., 2013; Coles et al., 2015). This local re-activation of T cells produces the necessary signalling to upregulate adhesion molecules and trigger the entry of immune cells, including T cells, macrophages, and B cells into the brain via the CP (Bartholomäus et al., 2009).

In a well-studied animal model of MS, experimental autoimmune encephalomyelitis (EAE), there are changes in CP tissue that precedes lesion development the establishment of brain/spinal cord neuroinflammation, including upregulation of adhesion molecules and mitochondrial alterations (Steffen et al., 1996; Engelhardt et al., 2001; Brown & Sawchenko, 2007; Schmitt et al., 2012). In addition, the CP has been identified as the preferential route of entry for encephalitogenic Th17 cells in the establishment of EAE. Similarly, in humans, MS cases show a 35% larger CP volume on T1-weighted MRI compared to healthy controls, and this difference is exacerbated in patients with active inflammation (Ricigliano et al., 2021). In addition, increases in numbers of MHC class II cells are found in the CP of MS cases, combined with an increase in VCAM-1 expression, T cells and plasma cells (Vercellino et al., 2008). Taken together, these results suggest that the CP represents an important route of entry for immune cells into the CSF compartment during neuroinflammation in MS.

The CSF represents the second immunological niche (Croese et al., 2021). CSF is generated by the CP and flows through the brain's ventricular system and into the subarachnoid space, where it is reabsorbed into the bloodstream in arachnoid granulations (Upton & Weller, 1985; Go et al., 1986; Raper et al., 2016). Comprehensive immunophenotyping studies using patients with varying neurological disorders (including MS) suggest that cells in the CSF are largely represented by T cells, with CD4+ cells outnumbering CD8+ cells by approximately a factor of 3 (Han et al., 2014). In particular, most cells circulating in the CSF represent CD4+ cells displaying an effector memory phenotype (de Graaf et al., 2011; Han et al., 2014). In addition to T cells, small numbers of B cells, natural killer (NK) cells and dendritic cells (DCs) are found in CSF (Han et al., 2014; Schafflick et al., 2020). More recently, single cell RNA sequencing of cells in blood and CSF of MS cases and cases of idiopathic intracranial hypertension (IIH) revealed a more nuanced heterogeneity of cells in the CSF (Schafflick et al., 2020). This study identified CSF enrichment of regulatory T cells (characterized by the expression of FOXP3 and CTLA4) and a population of myeloid derived dendritic cells in CSF compared to blood (Schafflick et al., 2020). In addition, increases in the numbers of a cytotoxic CD4+ T cell population and B cells in were observed in the CSF of MS compared to IIH, hence representative of a shift in CSF cell type diversity in MS (Patil et al., 2018; Schafflick et al., 2020).

CSF and parenchymal interstitial fluid (ISF) are continuously exchanged, providing a unidirectional flow through whereby neurons are provided nutrients and eliminate waste (Iliff et al., 2012; Iliff et al., 2013; Ringstad et al., 2018). In mice, cerebral arterial pulsation drives the inflow of CSF into the parenchyma by way of

paravascular spaces of penetrating arteries, and the outflow of ISF via paravenous spaces (Iliff et al., 2012; Iliff et al., 2013). This pathway is dependent on the presence of Aquaporin-4 (AQP-4) water channels on astrocytic endfeet and is hypothesized to be responsible for the clearance of molecular waste from parenchymal cells that is particularly effective during sleep (Iliff et al., 2013; Xie et al., 2013). Through the interconnection between CSF and ISF, macromolecules and proteins, including cytokines and antigenic peptides, secreted from parenchymal cells can enter the CSF space where they can exert their effects on any immune cells present.

The meninges and the meningeal lymphatic system represent the third immunological niche in the CNS important for immune surveillance (Croese et al., 2021). The meninges consist of three membranes, the dura mater, arachnoid mater, and the pia mater, that lay in between the brain and the skull, that contains the CSF as well as a rich source of immune cells (Alves de Lima et al., 2020; Croese et al., 2021). Immune cells move through the meninges by a network of lymphatic vessels in the perisinus spaces connected to deep cervical lymph nodes (Aspelund et al., 2015; Louveau et al., 2018). While not directly related to the studies presented in this thesis, the appreciation of all immunological niches in the CNS is critically important in biomarker discovery and the understanding of what they represent in a pathophysiological sense.

T cells in the meningeal space and in the CSF access cervical lymph nodes (CLNs) via meningeal lymphatic vessels (Louveau et al., 2018). Early evidence suggested that accumulation and proliferation of encephalitogenic T cells in the meningeal space during early stages of EAE interact with MHC Class II+ antigen presenting cells (APCs) in the meninges (Kivisäkk et al., 2009). More recently, it was determined that CNS

antigens circulating in CSF are captured by APCs in the dural sinuses and there, are presented to T cells (Rustenhoven et al., 2021). Following induction of EAE, increased accumulation of MOG-reactive T cells are observed in the dura primarily near dural sinuses, suggesting the perisinus region in the dura matter is also an important site for APC-T cell interaction in the CNS that may be particularly important in the onset of EAE (Rustenhoven et al., 2021).

The dura also contains many long-term resident B cells that appear to not be exchanged with circulation, and inflammation in the CNS results in increased antigen presentation capacity of these B cells (Schafflick et al., 2021). These cells may represent B cells born from bone marrow located at the top of the skull known as the calvaria (Brioschi et al., 2021). B cell maturation in the CNS has long been established, especially in the context of MS, where the development of ectopic B cell follicles is observed adjacent to cortical lesions (Serafini et al., 2004; Magliozzi et al., 2007).

1.1.3.2 Neuropathology and parenchymal distribution of immune cells in MS

A hallmark in MS pathology is the presence of demarcated areas of demyelination in the CNS termed lesions. The majority of what we know about MS lesions has been identified by histological examination of post-mortem and biopsied tissues. In general, lesions are classified based on inflammatory activity; active lesions displaying pronounced inflammation, mixed active/inactive lesions displaying an active rim around an area of demyelination that is devoid of inflammatory activity, and inactive lesions displaying no evidence of inflammation (Kuhlmann et al., 2017). While traditional classifications are valid for postmortem and in situ analyses, lesion formation is dynamic,

and each type likely represents different stages in the temporal evolution of MS pathology (Kuhlmann et al., 2017).

Active lesions represent the initial lesion phenotype and show up early in the disease course (Kuhlmann et al., 2017). These lesions are hypercellular with extensive infiltration of CD68+ and MHC class II+ microglia and macrophages throughout the lesion (Bö et al., 1994; Lucchinetti et al., 2000; Frischer et al., 2009). Active lesions display astrocytosis and loss of myelin (Kuhlmann et al., 2017). T cells are also present in active lesions, predominantly within the perivascular space of veins located in the lesion borders, with approximately 80% being CD8+ cytotoxic T cells (Machado-Santos et al., 2018). These CD8+ T cells often contain granzymeB+ granules (indicative of activation) and can proliferate in lesions (Machado-Santos et al., 2018). B cells are also often present within active lesions, however in variable numbers between patients and lesions, and are found focally in the perivascular space of larger blood vessels within the center of lesions (Machado-Santos et al., 2018). Curiously, evidence indicates that these CD8+ T cells display phenotypic characteristics of tissue resident memory T cells, including expression of CD69 and CD103 (Machado-Santos et al., 2018; Smolders et al., 2018).

Classically, active lesions are subdivided into four patterns based on the cellular players and level of oligodendrocyte destruction (Lucchinetti et al., 2000). All patterns of active lesions are defined by demyelination, irregular myelination, infiltrating macrophages and activated microglia with intracytoplasmic myelin debris, and T cell infiltration. Pattern I lesions are centered on small veins or venules and display clear edges (Lucchinetti et al., 2000). Pattern II lesions display all the same characteristics as Pattern I, but also display prominent immunoglobulin (IgG) and complement (C9neo)

antigen (Lucchinetti et al., 2000). Patterns III and IV show evidence of oligodendrocyte death. Evidence of oligodendrocyte apoptosis is observed in Pattern III lesions, where a rim of myelin is preserved around an inflamed blood vessel, but lesion borders are ill-defined (Lucchinetti et al., 2000). In contrast, while pattern IV lesions also display markedly reduced numbers of oligodendrocytes, no evidence of apoptosis is observed, and display a sharply demarcated lesion with radial expansion (Lucchinetti et al., 2000).

Mixed active/inactive lesions display characteristics similar to both active and inactive lesions. There is a clear area of demyelination, with a hypocellular center displaying loss of oligodendrocytes and axons, surrounded by an active rim of CD68+ cells at the lesion border (Lucchinetti et al., 2000). This border of CD68+ cells represents an area of active demyelination that may expand over time. For this reason, mixed active/inactive lesions are often described as slowly expanding or smoldering lesions within the literature. When considering the temporal aspect of lesion development, the consensus is that mixed active/inactive lesions develop from active lesions over time as the level of active T cell-mediated inflammation is reduced and the glial scar in the lesion core is formed (Machado-Santos et al., 2018). Nevertheless, in these lesions, demyelination and ongoing myelin breakdown still progresses (Prineas et al., 2001).

In contrast to active lesions, inactive lesions are hypocellular. These lesions are sharply demarcated, devoid of mature oligodendrocytes and loss of axons (Lucchinetti et al., 2000). Inactive lesions are filled by an astrocytic glial scar, and while microglia can be present, their density is much lower compared to other lesion types, and even lower than what is observed in normal appearing white matter (NAWM) (Kuhlmann et al., 2017). Evidence for the temporal development of lesions throughout the Kuhlmann et al.

classification system is supported by the overall distribution in lesion types by MS disease subtype diagnosis. Several studies have supported the theory that as an individual progresses from acute and relapsing-remitting subtypes to progressive disease, the dominance of active lesions wanes to a predominance of mixed active/inactive lesions and inactive lesions (Lucchinetti et al., 2000; Frischer et al., 2015; Kuhlmann et al., 2017).

In MS, lesions can occur throughout the CNS, including grey matter structures such as the cortex, brainstem, and deep brain nuclei (Lee et al., 1999; Vercellino et al., 2009; Kuhlmann et al., 2017). In the cortex, lesions can be subpial, intracortical, leukocortical and/or paracortical (Kuhlmann et al., 2017; Lassmann, 2018). Cortical lesions are likely grossly underestimated in living MS patients, as they are difficult to image using standard MRI sequences (Kuhlmann et al., 2017; Lassmann, 2018). Subpial lesions are thought to represent most cortical lesions and are likely unique to MS (Kuhlmann et al., 2017; Lassmann, 2018). These lesions are associated with meningeal inflammation and the presence of ectopic lymphoid-like structures reminiscent of tertiary follicles containing T cells, proliferating B cells, and plasma cells (Magliozzi et al., 2007; Howell et al., 2011; Kuhlmann et al., 2017). Ectopic lymphoid-like structures in the meninges likely appear early in the disease course and can be present in the brain and spinal cord and likely appear early in the disease course (Serafini et al., 2004; Howell et al., 2011; Choi et al., 2012). In the spinal cord, the presence of these follicles is associated with increased inflammation and neurodegeneration (Reali et al., 2020). Higher degrees of meningeal inflammation and the development of follicles are associated with more advanced lesion pathology with increased demyelination, glial activation, and

neurodegeneration and are associated with more severe clinical course of MS (Kutzelnigg et al., 2005; Magliozzi et al., 2007; Magliozzi et al., 2010; Choi et al., 2012). Cortical demyelination and neurodegeneration appear to occur in a radiating pattern, extending outward from the concentration of meningeal inflammation that suggests the contribution of soluble factors from these areas of inflammation (Lassmann, 2018). *In vitro* studies suggest that B cells in MS may play an active role in oligodendrocyte death and neurodegeneration via a soluble factor, possibly delivered by EVs (Lisak et al., 2012; Lisak et al., 2017; Benjamins et al., 2019).

The extent of axonal damage and neurodegeneration observed in a lesion is related to the lesion type rather than disease subtype of the patient (*ie* RRMS vs SPMS) (Kornek et al., 2000; Frischer et al., 2009). Similar significant correlation is observed between the extent of axon damage with the number of inflammatory cells present in both acute/RRMS cases and progressive cases (Frischer et al., 2009). Neurodegeneration in MS brains can occur by oxidative injury, as evidenced by cytoplasmic accumulation of oxidized phospholipids, by degeneration due to disrupted axonal transport, and by excitotoxic injury (Dziedzic et al., 2010; Haider et al., 2016; Singh et al., 2017).

Oxidative injury to neurons is observed in both active and inactive lesions and in areas of NAWM and is significantly correlated with meningeal inflammation (Vercellino et al., 2007; Haider et al., 2016). Oxidative stress in neurons is likely to trigger mitochondrial dysfunction and challenges to the cell's energy demand (Trapp & Stys, 2009; Haider et al., 2016). As a result of demyelination, increased metabolic demands are placed on neurons, presumably due to the loss of conductance provided by myelin and the redistribution of sodium channels that attempt to compensate for the change (Dutta et al.,

2006). Oxidative stress in neurons can also occur because of excitotoxicity, a neurodegenerative process whereby overstimulation by excitatory neurotransmitters, namely glutamate, triggers a cascade of damaging intracellular events (Wang & Qin, 2010). Glutamate homeostasis at the synapse is regulated by astrocytes expressing the glutamate transporters GLT-1 and GLAST (Rothstein et al., 1996). Respiratory deficient neurons are present in MS lesions and exhibit impaired activity in vital components in the mitochondrial respiratory chain, suggesting an important role of cellular bioenergetics in neurodegenerative processes occurring in MS (Dutta et al., 2006; Vercellino et al., 2007; Mahad et al., 2008).

Disruptions to axon transport can lead to stereotyped patterns of degeneration in an anterograde or retrograde direction. Evidence of retrograde degeneration as a secondary result of axon transection is observed most frequently in the cortex and deep cortical grey matter up- or downstream of areas of demyelination (Haider et al., 2016). Retrograde degeneration was not observed in subpial lesions, suggesting these lesions develop from the meningeal surface and do not result in early axon damage or transection due to demyelination (Haider et al., 2016). Anterograde, or Wallerian, degeneration is also observed in MS cases in the area surrounding demyelinated lesions in neurons that are not demyelinated themselves called periplaque white matter (Dziedzic et al., 2010; Singh et al., 2017). Important data presented in these works show that neuron damage and degeneration occur not only at lesions with active demyelination, but also in otherwise normal appearing CNS tissues, indicating widespread and generalized pathology induced by CNS inflammation.

1.2 Clinically Useful Biomarkers in MS

At the turn of the century, the definition of a biomarker was established as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention" (BDWG, 2001). In MS, categories of biomarkers identified by Comabella and Montalban in a seminal 2014 review include predictive biomarkers, diagnostic biomarkers, biomarkers to measure disease activity, and biomarkers to identify and monitor treatment responses (Comabella & Montalban, 2014). Each of these categories are important in the diagnosis and management of MS.

Predictive biomarkers allow the identification of individuals in the general population, or those with a family history of MS with increased likelihood of developing MS. These biomarkers are critical in the study and development of the MS prodrome, and testing interventions that prevent or delay the onset of disease activity (Comabella & Montalban, 2014; Makhani & Tremlett, 2021). In contrast, diagnostic biomarkers are those that identify positive MS cases from those with other neurological and autoimmune disorders presenting with similar symptoms (Comabella & Montalban, 2014). The third category includes biomarkers that inform on disease activity and/or related to ongoing pathophysiological mechanisms. Identification of disease activity markers can help to identify individuals susceptible to future increased disability and progression (Comabella & Montalban, 2014). Finally, biomarkers used to monitor treatment response are those that measure whether a particular disease modifying therapy (DMT) is working in an individual and help clinical decision making by identifying patients at risk of treatment failure or adverse reactions (Comabella & Montalban, 2014). Ideally, a perfect biomarker

is binary; it is present in MS cases and absent in other conditions and increases/decreases with disease worsening or amelioration with or without treatment (Comabella & Montalban, 2014).

At present, there are few established, validated, and clinically useful biomarkers in MS. MS is a disease diagnosed primarily based on exclusion of other autoimmune disorders that present similarity to MS (Thompson et al., 2018; Solomon et al., 2019; Wattjes et al., 2021). If specific self-antigens are identified MS can be ruled out in favor of the condition associated with the identified self-antigen (Wingerchuk et al., 2015; Jarius et al., 2018; Solomon et al., 2019). Two common MS mimics with known self-antigens are the neuromyelitis optica spectrum disorders (NMOSD; (Wingerchuk et al., 2015) and myelin oligodendrocyte glycoprotein antibody-associated disorders (MOGAD; (Jarius et al., 2018; Marignier et al., 2021). Each of these conditions are often associated with known self-antigens – NMOSD is caused by development of self-reactive immune cells targeting aquaporin-4 (AQP-4), a water channel abundant on astrocytes, and MOGAD is caused by autoimmune targeting of MOG, a protein found in myelin (Wingerchuk et al., 2015; Jarius et al., 2018; Marignier et al., 2021). Additional clinically useful biomarkers are limited to the identification of lesions on MRI, oligoclonal bands in the CSF.

1.2.1 Magnetic Resonance Imaging

MRI is a routinely performed paraclinical test that has proven critically useful in the diagnosis and monitoring of MS, since the adoption of the McDonald Criteria into the

diagnostic work up of individuals suspecting of having MS in 2001 (McDonald et al., 2001). CNS lesions are identified on scans and can satisfy both dissemination in time and space if proper sequences are implemented and often allow for an earlier and more accurate diagnosis of MS (Filippi et al., 2016; Thompson et al., 2018). The most recent iteration of the McDonald Criteria outlines the importance of MRI in the diagnostic toolbelt and recommends MRI be performed on any individual suspected of having MS (Thompson et al., 2018). MRI is so valuable to the diagnostic process for MS that several multi-national collaborative efforts (NAIMS – North American Imaging in MS Cooperative; MAGNIMS – Magnetic Imaging in MS, and CMSC – Consortium of MS Centers) have been established to combine expertise in neuroimaging in the aims to assess the relevant literature and develop reliable imaging-based biomarkers and provide consensus to the clinical field for the use of imaging markers for the diagnosis and monitoring of MS (Rovira et al., 2015; Wattjes et al., 2015; Filippi et al., 2016; Traboulsee et al., 2016; Wattjes et al., 2021). MRI-detected lesions remains a gold standard biomarker in MS and is routinely used in the diagnosis and monitoring of MS, and is incorporated into all clinical trials, often as a primary or secondary endpoint.

While MRI has been integrated in the diagnostic workup for MS for 20 years, the universal adoption of standardized approaches has been difficult due to the vast heterogenous landscape of healthcare services across the world (Wattjes et al., 2021). Nevertheless, the publication of recommendations from MAGNIMS and CMSC in 2015 and 2016 allowed the adoption of similar protocols and approaches by many societies across the world (Rovira et al., 2015; Wattjes et al., 2015; Filippi et al., 2016; Traboulsee et al., 2016; Wattjes et al., 2021). These guidelines represent the culmination of

substantial work among many collaborating fields summarizing the relevant literature and integrating the MRI guidelines within the clinical diagnostic criteria relevant at the time, the 2010 McDonald criteria, and subsequently updated with the 2017 revisions (Polman et al., 2011; Thompson et al., 2018; Wattjes et al., 2021). The recommended sequence to support the clinical assessment for diagnosis of MS includes a 25–30-minute protocol on a scanner with a field strength of ≥1.5T comprising axial density scans, T2-weighted and T2 fluid-attenuated inversion recovery (FLAIR), sagittal T2-FLAIR, and T1-weighted with and without the use of a Gad-containing contrasting agent (Rovira et al., 2015; Traboulsee et al., 2016; Wattjes et al., 2021). Additional sequences including dual inversion recovery and diffusion-weighted imaging are also recommended, but not included in the minimum necessary sequences outlined above (Rovira et al., 2015; Traboulsee et al., 2016; Wattjes et al., 2021). Recommendations state that these images should be acquired in the brain and SC imaging, and should only be ordered in special circumstances including clinical symptom presentation suggestive of SC involvement, non-specific or unclear brain MRI, or evidence of RIS in the brain MRI (Rovira et al., 2015; Filippi et al., 2016; Traboulsee et al., 2016; Wattjes et al., 2021). In addition, a follow-up MRI should be ordered in individuals with evidence of CIS or RIS within 12 months (3-6 months being ideal), to identify new lesions that allow satisfaction of DIS and DIT criteria as early in the disease course as possible (Rovira et al., 2015; Filippi et al., 2016; Traboulsee et al., 2016; Wattjes et al., 2021). Recommendation for these follow-up scans were initially recommended to be as identical to baseline scans as possible, however this has since been revised to simplify the protocol that is predicted to

have a higher level of adoption in centers around the world (Rovira et al., 2015; Traboulsee et al., 2016; Wattjes et al., 2021).

As per the 2010 and 2017 revisions of the McDonald Criteria, lesions identified on MRI can be used to satisfy both the DIS and DIT criteria. DIS is satisfied if lesions found by imaging are in two or more of the characteristic locations, and DIT can be satisfied using Gad-containing contrasting agents (Polman et al., 2011; Rovira et al., 2015; Traboulsee et al., 2016; Thompson et al., 2018). Gad-enhancing lesions are indicative of inflammatory activity and represent new active lesions, and their presence in addition to non-enhancing T2 lesions is sufficient to satisfy DIT, as these non-enhancing lesions represent older, more established lesions (Polman et al., 2011; Rovira et al., 2015; Thompson et al., 2018). However, the use of clinical assessment along with MRI is critical, as many non-MS conditions present similar radiological findings (Rovira et al., 2015). Certain sequences can be used to identify radiological anomalies that may be more specific to MS, including the central vein sign, the presence of a rim of iron deposition around the lesion (known as the paramagnetic rim), and the presence of cortical lesion (Rovira et al., 2015; Maggi et al., 2018; Absinta et al., 2019; Clarke et al., 2020; Oh et al., 2021). Addition of imaging sequences sufficient to identify these characteristics requires a scanner with field strength of ≥3T and add to the scan time making them unlikely to be adopted in routine clinical practice with ease, at least for the time being (Wattjes et al., 2015). At present, evidence supporting the use of these additional scan protocols in the diagnostic work up in MS is not sufficient to recommend adoption (Wattjes et al., 2015)

Criteria have also been developed for the recommended uses of MRI in prognostic assessment and monitoring of MS cases once confirmed diagnosis is established (Wattjes

et al., 2015). Research indicates a that high baseline T2 lesion load and/or the presence of cerebellar and/or brainstem lesions in CIS patients confers increased risk of transition to clinically definite MS (Fisniku et al., 2008; Tintore et al., 2015; Wattjes et al., 2015). Monitoring disease processes and progression using MRI relies on identifying changes between baseline scans and follow up images. However, assessing these changes is not as easy as it sounds. The recommendations outlined by MAGNIMS indicate follow up should be done at minimum once a year following diagnosis, and after clinical events, which may be beyond the capacity of many healthcare systems (Wattjes et al., 2015). It is also recommended that follow up scans be completed on the same machines with the same imaging protocols as done at baseline (Wattjes et al., 2015).

Critical to disease monitoring is identifying new and expanding lesions. This can be accomplished by Gad-enhanced T1 weighted scans to identify lesions with ongoing acute inflammation (Wattjes et al., 2015). New and expanding lesions can also be identified by subtracting T2 weighted images from baseline scans (Wattjes et al., 2015). Acute active lesions only uptake Gad for approximately 3 weeks following development, so the lack of new Gad+ lesions is not necessarily a sign that new lesions have not been formed in the period between scans (Cotton et al., 2003; Wattjes et al., 2015). Serial scans using Gad-containing contrasting agents is also cautioned as new evidence suggests it has the potential to accumulate (Guo et al., 2018; Zivadinov et al., 2019). T2 weighted images subtracted from baseline scans can also identify new lesions, and those that change in size (increasing or decreasing in size), as the T2 weighted lesion load identifies changes in brain tissue that persist following the resolution of inflammatory lesions in the time between scans (Moraal et al., 2010a; Moraal et al., 2010b; Erbayat Altay et al., 2013;

Wattjes et al., 2015). While safer than continuous administration of Gad containing contrasting agents, image subtraction must be done by a highly experienced reader, and while automated subtraction is available and improves the objective identification of new and changing T2 weighted lesions, as of 2015 it has yet to be integrated in many clinical centers (Battaglini et al., 2014; Wattjes et al., 2015).

1.2.2 Cerebrospinal Fluid Immunoglobulin G

B cells represent a critical cell type in the adaptive immune system in humans. Terminally differentiated B cells, or plasma cells, can be extremely long-lived and produce and secrete immunoglobulins (antibodies) that are essential for the development of immunological memory (Radbruch et al., 2006; LeBien & Tedder, 2008). Immunoglobulin G (IgG) is the most common class of antibody produced and secreted by plasma cells in humans and can be measured in CSF to inform on a variety of medical conditions, including MS (Link & Tibbling, 1977; Freedman et al., 2005; Link & Huang, 2006). The IgG index is a quantitative measure of amount of IgG in the CSF relative to serum, and an elevated IgG index can be indicative of an autoimmune disorder of the CNS (Link & Tibbling, 1977; Stangel et al., 2013; Simonsen et al., 2020; Zheng et al., 2020). The presence of clonally distinct IgG populations in the CSF but not serum is indicative of B cell clonal expansion and plasma cell secretion of antibody within the CNS and is suggestive of MS, but also other medical and neurological conditions (Freedman et al., 2005; Link & Huang, 2006).

CSF OCBs are identified by running CSF and matched serum samples from patients on an agarose gel, usually by isoelectric focusing, followed by immunoblotting for IgG (Freedman et al., 2005). Comparisons are made between CSF and serum banding patterns; if the banding pattern is identical, it indicates systemic IgG production and is present in the healthy normal populations, but if bands are present uniquely in CSF, it is interpreted as an indication of intrathecal IgG synthesis (Freedman et al., 2005; Link & Huang, 2006). Unique CSF OCBs are present in >90% of MS cases, and their identification is useful in the differential diagnosis of MS, as lack of banding or patterns indicative of widespread damage to the blood CSF barrier can steer investigations in favour of alternate diagnoses (Freedman et al., 2005; Link & Huang, 2006). However, CSF OCBs are not unique to MS and occur in numerous other neurological conditions including neurosyphilis, epilepsy and migraine (Kabat et al., 1942; Laterre et al., 1970; Link & Kostulas, 1983). In addition, a lack of OCBs does not rule out MS; current evidence suggests that measuring CSF OCBs are not perfect, is not consistent between medical centres, and false negatives do occur (Link & Huang, 2006). Moreover, even when more rigorous protocols are applied, some MS patients show no evidence of CSF OCB, and no evidence of intrathecal plasma cells when paired with post-mortem analysis of brain tissue, suggesting that a minority of MS patients truly exhibit no local IgG production in the brain (Farrell et al., 1985; Link & Huang, 2006). Nevertheless, based on the 2017 McDonald criteria, the presence of CSF OCBs can be used to satisfy the DIT criteria when paired with MRI satisfying DIS, as the establishment of plasma cells in the CSF is suggestive of ongoing inflammatory processes persisting for longer periods of time (Thompson et al., 2018).

Much like MRI based biomarkers, CSF OCBs offer some utility for prognostication and monitoring of clinically definite MS. Evidence indicates that the presence of OCBs in the CSF of CIS cases confers increased risk of developing clinically definite MS in the future and may be associated with greater risk of disability accrual in shorter amounts of time than OCB-negative MS cases (Dobson et al., 2013; Tintore et al., 2015; Gasperi et al., 2019). Recent evidence also suggests that while CSF IgG is not associated with the number and severity of white matter lesions, there is a significant association with greater cortical lesion load and consequently greater disability and cognitive decline at 10 years post disease onset (Farina et al., 2017). In support of this association, several studies have documented milder disease course in OCB-negative MS patients, even suggesting that lack of OCBs in the CSF reflects a high likelihood of a benign MS disease defined as functional in all neurological areas >15 years after disease onset (Stendahl-Brodin & Link, 1980; Link & Huang, 2006; Ferreira et al., 2014). Furthermore, several recent studies point to the utility of identifying and quantifying intrathecal IgM production as having prognostic value in MS, though these results have yet to be validated and adopted in clinical practice (Magliozzi et al., 2020; Capuano et al., 2021; Oechtering et al., 2022).

In summary, the use of OCBs in clinical practice remains limited to support a clinically definite diagnosis of MS when MRI is not conclusive to satisfy DIT. On its own, however, the presence of OCBs offers little else in routine assessments and have little, if any, impact on clinical decision making (Comabella & Montalban, 2014). As OCBs are present in many other neurological conditions that mimic MS, both MRI and clinical evidence is required in addition to OCBs measurements.

1.3 Novel Body Fluid Biomarkers in MS under investigation

The lack of biomarkers specific for MS necessitates a continuing search for novel candidates. Of specific interest are biomarkers in accessible body fluids (e.g. blood). While MRI-based biomarkers, and those derived from the CSF offer the advantage of providing information directly within the compartment and environment whereby pathological events are taking place in MS, the expense of MRI and the invasiveness of lumbar punctures leads to limited utility for continued longitudinal sampling in clinical practice, thus limiting their potential (Comabella & Montalban, 2014; Khalil et al., 2018). A summary of desired characteristics for a molecular biomarker in MS was published by Comabella and Montalban (2014) and is summarized in Figure 1.1. As such, recent advances have identified potential new biomarkers for monitoring disease processes in MS that are not well captured by MRI, including measures of neurofilament light chain (NfL), extracellular vesicle cargo and patterns, and various cytokines within circulation.

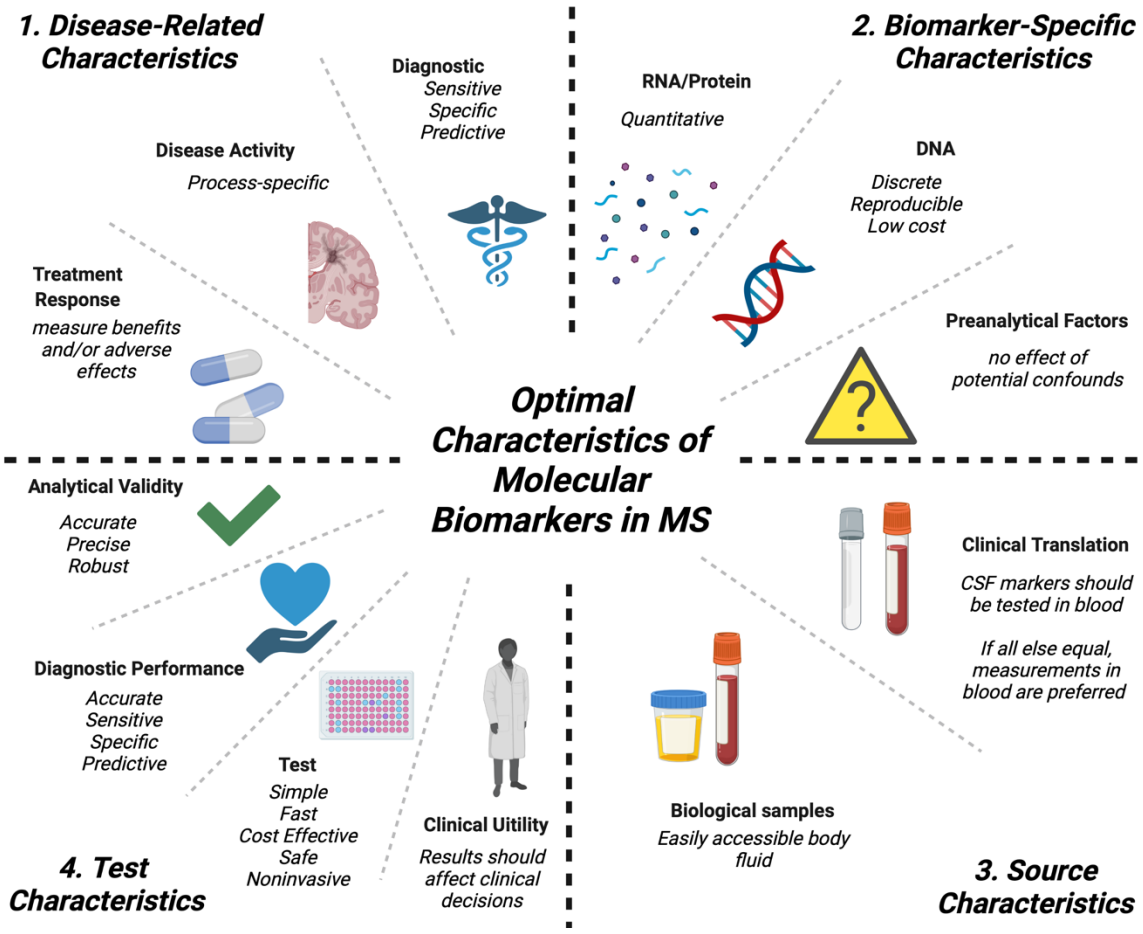

Figure 1.1 Optimal Characteristics of a molecular biomarker in MS. Original figure created using BioRender based on information presented in Panel 2 of Comabella and Montalban, 2014.

1.3.1 Neurofilament Light Chain

Neurofilaments are intermediate cytoskeletal filaments approximately 10nm in diameter and expressed exclusively in neurons. Under normal conditions, neurofilaments are stable with low turnover and are involved in axon growth and stability (Yuan et al., 2017; Khalil et al., 2018). Neurofilaments interact with many other proteins and organelles, and thus likely have other important functions that are not completely understood at this time (Yuan et al., 2017). In the context of injury, neurofilaments are released from neurons during axonal damage, and can be measured in body fluids, including CSF and blood (Norgren et al., 2002; Petzold et al., 2010; Rissin et al., 2010; Yuan et al., 2017; Khalil et al., 2018). There are five types of neurofilaments, including neurofilament heavy chain, medium chain, light chain (NfL), α-internexin and perpherin; NfL in particular was identified early as being an important marker of axon injury in MS and other neurological conditions (Yuan et al., 2017; Khalil et al., 2018; Ferreira-Atuesta et al., 2021).

Long term disability in MS is determined by the extent of axon injury in lesions, a feature that is not well measured by current MRI sequences. As such, the potential use of NfL as a biomarker of neurodegeneration has garnered a lot of attention over the last decades (Trapp et al., 1998; Kuhlmann et al., 2002; Tallantyre et al., 2010; Rocca et al., 2017; Khalil et al., 2018; Ferreira-Atuesta et al., 2021). Initial studies focused on measuring NfL in CSF as the initial methods/assays developed were not sensitive enough to reliably measure NfL in blood products (Khalil et al., 2018). These early studies established strong evidence that NfL is elevated in the CSF of MS cases compared to controls with non-inflammatory neurological diseases (NIND; (Lycke et al., 1998;

Malmeström et al., 2003; Norgren et al., 2004; Teunissen et al., 2009; Khalil et al., 2013). In addition, several studies demonstrated an association between increased NfL levels and recent relapse activity (Lycke et al., 1998; Malmeström et al., 2003; Norgren et al., 2004), T2 and Gad+ lesion counts, EDSS, and CSF cell counts (Norgren et al., 2004; Teunissen et al., 2009; Khalil et al., 2013). The development of the single molecule array (Simoa) technology revolutionized the study of NfL as a biomarker as its ultrasensitive methods allowed the detection of small concentrations of NfL in blood products, including plasma and serum (Rissin et al., 2010; Gisslén et al., 2016; Kuhle et al., 2016; Disanto et al., 2017).

As mentioned previously, blood analysis allows more accessible and less invasive repeated sampling and can permit a more optimal monitoring of systemic levels over time within an individual patient. Critical to this is the establishment that in both CSF and blood (plasma and serum), NfL levels are tightly correlated in matched samples (Gisslén et al., 2016; Kuhle et al., 2016). Seminal work by Disanto and the Swiss Multiple Sclerosis Cohort Study Group further validated this strong correlation between CSF and serum NfL and extended these findings to show significant associations between serum NfL (sNfL) levels and brain T2 lesion load, and Gad+ lesion load (Disanto et al., 2017). In addition, they found that high levels of sNfL compared to the healthy control group were associated with increased risk of relapse and EDSS worsening (Disanto et al., 2017). These results have subsequently been validated by other studies, suggesting NfL is an important indicator of disease activity and a potential prognostic indicator of future lesion load and disability accumulation (Barro et al., 2018; Chitnis et al., 2018; Kuhle et al., 2019; Kuhle et al., 2020; Thebault et al., 2020; Benkert et al., 2022; Lokhande et al.,

2022). In MS, measurements of NfL are also useful in monitoring responses to DMTs. Several studies on CSF and blood NfL have shown that NfL levels decrease with the use of DMTs (Gunnarsson et al., 2011; Novakova et al., 2017; Piehl et al., 2018; Kuhle et al., 2019; Sormani et al., 2019).

Despite this wealth of information, the use of NfL as a biomarker in clinical practice is not without limitations. For example, in both CSF and blood, NfL is significantly associated with normal ageing. Early studies using Simoa investigated CSF and plasma NfL levels in healthy populations and found each were significantly correlated with age (Gisslén et al., 2016; Yilmaz et al., 2017). In addition, NfL levels measured in both healthy and pathologic populations are subject to confounds including body mass index (BMI), and other conditions that cause variability in blood volume (Manouchehrinia et al., 2020a). Furthermore, as NfL measured in body fluids is a marker of axon damage, it's not surprising that significantly increased levels are found in almost all neurodegenerative disease investigated. Studies have identified elevated levels in amyotrophic lateral sclerosis, various dementias, normal pressure hydrocephalus, concussion, and trauma (Rosengren et al., 1996; Rosengren et al., 1999; Sjögren et al., 2000; Agren-Wilsson et al., 2007; Bridel et al., 2019; Mattsson et al., 2019). In addition, NfL is associated with several non-CNS conditions, including peripheral nervous system damage, cardiovascular and kidney function, and pregnancy (Barro et al., 2020). For the reasons mentioned above, the clinical validity of NfL is restricted to monitoring disease activity, treatment response and predicting future disability. In addition, while the widespread adoption of Simoa certainly aids in the accessibility of routine monitoring of NfL levels, the technique, until very recently, still requires specialized equipment and

trained users and at present is inaccessible to smaller centers. An ELISA-based method for the analysis of sNfL levels only became commercially available in late 2021 (Uman Diagnostics, cat# 20-8002 RUO) and a new microfluidics-based assay called Ella™ (ProteinSimple) has been developed, both of which will likely provide accessible NfL monitoring for research purposes in smaller centers (Gauthier et al., 2021). However, the use of multiple platforms leads to concerns over analytical validity as it requires additional considerations over preanalytical variables, reliability and reproducibility and assay and analytical standardization across multiple platforms (Thebault et al., 2021).

1.3.2 Serum Glial Fibrillary Acidic Protein and Chitinase 3-Like Protein-1

Glial fibrillary acidic protein (GFAP) is an intermediate filament present predominantly in astrocytes that is involved in maintaining synapse stability and plays a role in astrocyte-mediated injury control (McCall et al., 1996; Brenner, 2014). GFAP is upregulated and overexpressed in astrocytes with a reactive phenotype, and astrocyte damage results in the release of GFAP that can be measured in the blood and CSF (Brunkhorst et al., 2010; Liddelow & Barres, 2017). While still a relatively new field of research, considerable advances have been made in the investigation of GFAP as a biomarker in MS.

Several recent systematic review and meta-analyses have summarized strong evidence supporting elevated serum and CSF GFAP in MS cases compared to controls (Sun et al., 2021; Heimfarth et al., 2022). Evidence indicates that serum GFAP rises in MS cases compared to controls and is further elevated in individuals in relapse compared

to those in remission (Abdelhak et al., 2018; Högel et al., 2020; Saraste et al., 2021; Sun et al., 2021). Similarly, serum GFAP levels are correlated with disability as measured by EDSS, disease duration, age, and MRI measures including T1 and T2 lesion loads (Abdelhak et al., 2018; Abdelhak et al., 2019; Högel et al., 2020). Interestingly, recent evidence also suggests that serum GFAP levels are significantly associated with MRI measures of diffuse neuroaxonal damage in NAWM in MS cases (Saraste et al., 2021).

Chitinase-like proteins (CLPs) are structural homologues to true chitinases that lack enzymatic activity. True chitinases are required for the metabolism of chitin, an abundant polysaccharide in nature that is absent in mammals (Pinteac et al., 2021). CLPs are expressed in mammals, the most well characterised being Chitinase 3-like protein-1 (CHI3L1, also known as YKL-40). The physiological function of CHI3L1 has yet to be fully described, and is without a doubt complex and cell- and tissue-specific; it is expressed in myeloid cells, chondrocytes, synovial cells, osteoclasts and astrocytes (Bonneh-Barkay et al., 2010; Bonneh-Barkay et al., 2012; Hinsinger et al., 2015; Pinteac et al., 2021).

Despite the poor understanding of the physiological role of CHI3L1, it has been implicated as a potential biomarker in many inflammatory conditions, including MS (Pinteac et al., 2021). Evidence indicates inflammatory activation results in the upregulation and secretion of CHI3L1 from macrophages and astrocytes and can be measured in the CSF and blood from humans (Johansen et al., 1999; Koutroubakis et al., 2003; Rehli et al., 2003; Bonneh-Barkay et al., 2010; Comabella et al., 2010; Bonneh-Barkay et al., 2012; Cantó et al., 2012). Studies have documented significant differences between CSF levels of CHI3L1 between MS cases and controls (Hinsinger et al., 2015).

Increased CHI3L1 may provide prognostic value, as higher levels in CIS are associated with greater risk of developing clinically definite MS, and CHI3L1 levels may predict progression from RRMS to SPMS (Cantó et al., 2015; Modvig et al., 2015; Gil-Perotin et al., 2019). Similarly, progressive forms of MS show further elevation of CSF CHI3L1 in progressive subtypes of MS, and are associated with worse clinical and radiological outcomes including cognitive decline, Gad-enhancing lesions, EDSS worsening, and brain volume loss (Cantó et al., 2012; Cantó et al., 2015; Martínez et al., 2015; Modvig et al., 2015; Burman et al., 2016; Håkansson et al., 2018; Gil-Perotin et al., 2019)

However, much like NfL and the clinically useful biomarkers used in MS, serum GFAP and CSF CHI3L1 are also significantly associated with many neurodegenerative and systemic diseases including Alzheimer's disease, Parkinsons's disease, and CNS trauma (Pinteac et al., 2021; Heimfarth et al., 2022). As such, the widespread adoption of serum GFAP biomarker in MS remains limited.

1.3.3 Extracellular Vesicles

Extracellular vesicles (EVs) are small biological compartments secreted from cells under homeostatic and pathological conditions. Depending on their biogenesis pathway, EVs are generally separated into categories (Figure 1.2). First, exosomes are small (~30-200nm) lipid bilayer enclosed vesicles originating by budding of late endosomal membranes, creating intracellular multivesicular bodies (MVBs) that are secreted from cells by exocytosis (Pan & Johnstone, 1983; Johnstone et al., 1987; Wollert & Hurley, 2010). In contrast, microvesicles (or ectosomes) are typically considered larger vesicles

that bud directly from the plasma membrane (Cocucci et al., 2009; Mathieu et al., 2021). The third EV subtype are apoptotic bodies that are shed from the cell during the process of apoptosis. Once extracellular, distinguishing between exosomes and microvesicles has proven to be extremely difficult and an accepted consensus on the definition of each has yet to be achieved (Shen et al., 2011; Gould & Raposo, 2013; Kowal et al., 2016). In this thesis, exosomes and microvesicles collectively will hereby be referred to collectively as EVs.

All EVs contain several common protein markers (major histocompatibility complex, flotillin, and heat-shock 70kDa protein), and display several surface tetraspanin molecules, including CD9, CD63, CD81 (Keerthikumar et al., 2016; Kowal et al., 2016). More recent evidence suggests that under experimental conditions microvesicles are released in higher abundance than exosomes and display higher levels of CD9 compared to CD63 (Mathieu et al., 2021). In contrast exosomes display higher levels of CD63 compared to CD9 suggesting relative levels of CD9 and CD63 may be used to distinguish EV subtypes (Mathieu et al., 2021). The highly heterogeneous extracellular EV population can be broadly categorized based on size, density, tetraspanin expression, and cargo composition (Kowal et al., 2016). Biologically active components of EVs (termed "cargo") can include proteins, lipids, and various RNA species (*e.g.* mRNAs, miRNAs, tRNAs) (Yáñez-Mó et al., 2015). Virtually all cells are capable of releasing EVs, which can contain cargo that is either unique to the cell-of-origin (*e.g.* GFAP is present within EVs derived from astrocytes) or non-specific (*i.e*. found within all or most EVs independent of the cell of origin (Keerthikumar et al., 2016; Kowal et al., 2016; Willis et al., 2017; Théry et al., 2018). EV cargo may not always be representative of the cellular

contents of the secreting cell, suggesting that its cargo may be selectively sorted and packaged for deliberate secretion; however, the potential contribution of culture additive contaminations make this a matter of current debate (Valadi et al., 2007; Nolte-'t Hoen et al., 2012; Colombo et al., 2013; Wei et al., 2016; Jovičić & Gitler, 2017; Tosar et al., 2017; Théry et al., 2018).

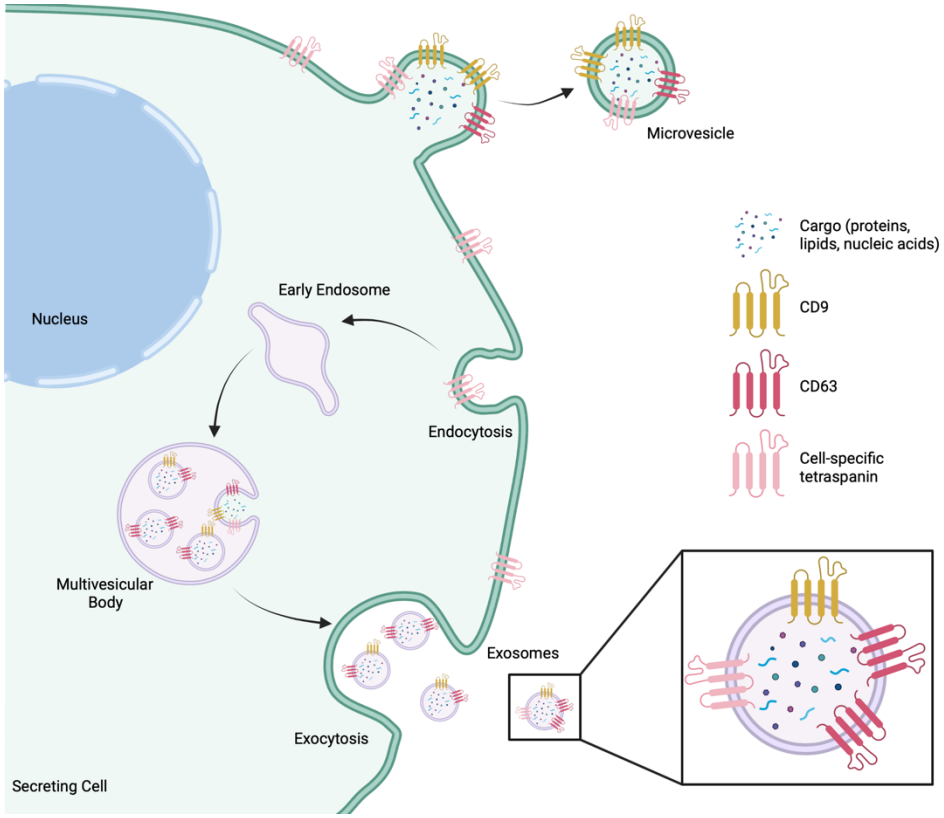

Figure 1.2 Extracellular Vesicle Biogenesis. Two varieties of extracellular vesicles of particular relevance to this thesis are exosomes and microvesicles. Microvesicles are secreted by direct outward budding from the plasma membrane, and display characteristic EV markers CD9 and CD63, with a higher abundance of CD9, and display cell-specific tetraspanins and cargo. Exosomes are formed by inward invaginations in endosomal membranes forming multivesicular bodies, which are secreted from cells by exocytosis. Exosomes display both CD9 and CD63, with a higher abundance of CD63, and also display cell-specific tetraspanins and cargo. Original figure created using BioRender.

In mammalian systems, secreted EVs have numerous biological functions (Yáñez-Mó et al., 2015). Of particular interest is their role as mediators of intercellular communication (Tkach & Théry, 2016). Once extracellular, EVs can either act locally on surrounding cells or be transported systemically *via* body fluids. EVs can be found in all biological fluids, including blood and CSF (Raposo & Stoorvogel, 2013). In the CSF, EVs of various cellular origins have been isolated and identified as putative important signaling entities within the CNS (Street et al., 2012; Yáñez-Mó et al., 2015).

In the last few years, significant advances have been made in using EVs and their cargo patterns in the search for biomarkers for MS. In EAE, differential microRNA (miRNA; short non-coding RNA molecules that post-transcriptionally regulate protein expression and most biological processes) profiles for each phase of disease (pre-onset, onset, disease peak) may provide valuable insight into putative biomarkers for MS diagnosis (Singh et al., 2016; Cuomo-Haymour et al., 2022). Several miRNAs identified were also differentially regulated with glatiramer acetate (GA) treatment, suggesting that changes in EV-derived miRNAs may present a useful and non-invasive method to monitor treatment efficacy. In the clinical MS population, two recent studies profiled serum derived EVs by next generation sequencing and revealed different RNA profiles between RRMS patients and healthy controls (Ebrahimkhani et al., 2017; Selmaj et al., 2017). Curiously, these two studies did not identify the same miRNAs. Nevertheless, these investigations provide important insights into the utility of profiling the miRNA content of body fluid-derived EVs to provide useful biomarkers for MS in general, as well as for differentiating between MS subtypes, and treatment-responders. They also

allude to the possibility that this approach can be used to identify unique biomarkers for a multitude of other CNS disorders.

Additional progress has been made in identifying patterns of EVs secreted from MS-relevant cell subsets in the search for identifying new biomarkers in MS. EVs can cross the BBB and enact inflammatory changes in recipient cells (Verderio et al., 2012; Dickens et al., 2017). Therefore, identifying dysregulated EV populations in MS have the potential to not only serve diagnostically, but also could inform on undetected inflammatory activity. Early investigations focused on platelet-derived and endothelial-derived particles in blood, which is unsurprising as they reflect the majority of EVs present in blood samples; these studies found a significant increase in these particles in MS cases compared to controls, with the highest levels observed in cases with active relapse and Gad+ lesion activity on MRI, and identified changes with the use of DMTs (Minagar et al., 2001; Jy et al., 2004; Sheremata et al., 2006; Lowery-Nordberg et al., 2011; Marcos-Ramiro et al., 2014; Sáenz-Cuesta et al., 2014; Alexander et al., 2015; Zinger et al., 2016; Ebrahimkhani et al., 2020). Two more recent studies extended these findings by profiling immune cell derived EVs in the blood of MS cases. Zinger and colleagues identified a significant decrease in CD19+ B cell derived EVs, but no change in any other marker investigated, including CD3 and CD14 (Zinger et al., 2016). A separate study found significant decreases in immune cell derived EVs, including CD4+ T cell derived during relapse compared to healthy controls and stable RRMS cases (Groen et al., 2020). However, this study did not observe any differences in other relevant populations including CD8+, CD20+ and CD14+, nor did they observe any difference between HC and stable RRMS or SPMS (Groen et al., 2020).

Within CSF, similar results have been demonstrated in EV populations, although these studies are primarily focused on myeloid cell derived EVs marked by the presence of isolectin B4 (IB4; (Dalla Costa et al., 2021; Gelibter et al., 2021). Evidence indicates that IB4+ EVs are observed in the CSF of patients with CIS compared to control non-MS cases and increases in these EVs are associated with shorter time to clinical manifestation of MS compared to CIS cases with lower IB4+ EVs (Gelibter et al., 2021). IB4+ EVs are also increased in RRMS cases, compared to CIS, RIS and progressive MS, and are significantly associated with the presence of Gad+ lesions, in a dose dependent manner with more lesions observed in the individuals with highest IB4+ particle counts (Dalla Costa et al., 2021). Furthermore, this study also demonstrated that IB4+ EVs are a predictor for disease activity in RRMS cases, and enhanced disability progression in progressive cases (Dalla Costa et al., 2021). Based on nanotracking analysis, an earlier study also showed that there are elevated numbers of particles in the CSF of RRMS with active relapses compared to stable cases. These cases, however, exhibited lower levels of IB4+ EVs, and an increase in EVs displaying both CD19 and CD200 (Geraci et al., 2018). No changes in apoptotic bodies in CSF of MS cases has been observed (Masvekar et al., 2019). However, the marker used in this study, AnnexinV, has been used in other studies as a general EV marker, which highlights the lack of standardized protocols across the field of EV research, especially using flow cytometry to quantify circulating EV populations (Théry et al., 2018; Welsh et al., 2020).

As this is the case, with the technology used to quantify EV populations in body fluids is relatively new, and the lack of instrument-specific standardized protocols, the use of EVs in clinical practice for diagnosis and monitoring of MS is only in its infancy.

Significant advances have been made in the attempt to standardize the field. Guidelines for the minimal information required for reporting EV studies are in its second iteration and makes critical recommendations to improve standardization and reproducibility in the field (Lötvall et al., 2014; Théry et al., 2018). Specific guidelines for EV flow cytometry have also been developed and will prove valuable in the field moving forward (Welsh et al., 2020). Nevertheless, studies currently available consist of moderate sample sizes and have yet to be reproduced in sufficiently large populations to know the full scale of applicability as a biomarker in MS. Future studies are needed before strong conclusions can be made.

1.3.4 Circulating Cytokines and Chemokines

As RRMS is inflammatory in nature, early studies initially investigated the possibility of using circulating cytokines/chemokines as biomarkers in MS; research is still actively ongoing in this area. Cytokines and chemokines have potential to also provide information about ongoing systemic inflammation that may represent disease activity both during and outside of periods of clinical exacerbation. A recent comprehensive meta-analysis analyzed 226 studies published between 1990 and 2018, combining datasets resulting in the analysis of 13,256 MS cases and 8428 controls and identified blood and/or CSF cytokines differentially regulated in MS compared to controls (Bai et al., 2019). Cytokines readily discussed in the literature as possible biomarkers in MS include CXCL13, CXCL10, IL-17, IL-23, and IL-1RA.

Chemokine C-X-C motif ligand 13 (CXCL13) is a chemokine that mediates B cell recruitment, and several studies have documented increases in CSF concentration of CXCL13 during active disease (Sellebjerg et al., 2009; Haas et al., 2011; Khademi et al., 2011; Ragheb et al., 2011). CSF CXCL13 is a widely studied cytokine in the search for new biomarkers in MS (Bai et al., 2019). Furthermore, evidence indicates that elevated CSF CXCL13 identifies CIS cases at higher risk for converting to clinically definite MS (Brettschneider et al., 2010; Khademi et al., 2011). However, CXCL13 was also identified to be highly specific to ongoing pathology, as levels were shown to decrease with normal ageing. It is noteworthy that this increase is not specific to MS and occurs in other CNS inflammatory conditions such as viral and bacterial infections and NMOSD (Khademi et al., 2011; Alvarez et al., 2013). Preliminary data also suggest levels of CXCL13 in the CSF are altered by treatment with DMTs, including fingolimod and rituximab (Alvarez et al., 2015; Karaaslan et al., 2022). More recently, a study identified that calculating a CXCL13 index indicative of intrathecally produced CXCL13, by calculating CSF levels relative to serum, corrected for diffusion across the blood-CSF barrier, significantly predicted disease activity better than IgG index and NfL levels (DiSano et al., 2020). In addition, the meta-analysis performed by Bai and colleagues identified CSF CXCL13 as being significantly elevated in MS compared to controls, with an overall average CSF CXCL13 in MS cases sitting 11 standard deviations above the mean of controls with very low heterogeneity between samples studied (Bai et al., 2019). Taken together these results suggest the utility of CSF CXCL13, particularly the CXCL13 index proposed by DiSano and colleagues, may have clinical utility in predicting conversion from CIS to MS, increased disability accrual and treatment response.

Additional studies are required before CXCL13 is completely validated and adopted into clinical practice.

CXCL10 is another cytokine that research suggests is dysregulated in MS. CXCL10 is a chemokine involved in the recruitment of immune cells to sites of injury. CXCL10 binds to its receptor CXCR3, commonly expressed on T cells, NK cells, and monocytes/macrophages and downstream signaling results in chemotaxis and other cellular processes (Vazirinejad et al., 2014). In the CNS, CXCL10 is released from astrocytes upon inflammatory stimulation (Ransohoff et al., 1993). CXCR3 is expressed on all CNS resident cells and regulates microglia chemotaxis and impairs oligodendrocyte progenitor cell differentiation (Xia et al., 2000; Biber et al., 2002; Nelson & Gruol, 2004; Rappert et al., 2004; Omari et al., 2005; Moore et al., 2015). Several studies have documented increases in CXCL10 in the CSF of MS cases compared to controls, and have documented associations with CXCR3+ T cell infiltration to the CNS (Balashov et al., 1999; Simpson et al., 2000; Sorensen et al., 2001; Mahad et al., 2002; Scarpini et al., 2002; Roberts et al., 2015; Khaibullin et al., 2017). Furthermore, increases in CSF CXCL10 have been suggested to indicate increased disease activity (Franciotta et al., 2001; Scarpini et al., 2002; Donninelli et al., 2021).

Another cytokine that appears to be dysregulated in MS is IL-17. IL-17 is produced by a subset of pro-inflammatory T cells implicated in pathogenesis in EAE and are highly prevalent in active and chronic active/inactive MS lesions in humans (Langrish et al., 2005; Tzartos et al., 2008). In addition, it is argued that Th17 responses are critical to the development of MS (Luchtman et al., 2014). Serum IL-17 was identified as being moderately dysregulated in MS cases compared to controls, theoretically due to the

expansion of Th17 cells as a result of ongoing CNS pathology (Tzartos et al., 2008; Bai et al., 2019). IL-17 levels were measured on average 4.7 standard deviations higher than controls. The inter-study variability observed was quite high, however, as is evident when reviewing the literature (Bai et al., 2019). While several studies documented significant increases in MS cases compared to controls, these studies noted only limited biomarker potential, as no associations with disease subtype, disability, progression, or the presence of enhancing lesions have been documented (Wen et al., 2012; Kallaur et al., 2013; Babaloo et al., 2015; Khaiboullina et al., 2015; Lebrun et al., 2016; Kallaur et al., 2017; Trenova et al., 2017). In addition, there are several studies reporting no differences in serum IL-17 compared to controls (Esendagli et al., 2013; Kallaur et al., 2013). Clinical trials investigating the use of monoclonal antibodies directed against IL-17 in MS have shown moderate favorable results, but understanding the full scope of the efficacy of targeting IL-17 in MS has been hindered by the development of better antibodies leading to termination of follow-up studies (clinicaltrials.gov, NCT01051817 and NCT01874340)(Havrdová et al., 2016).

The Th17 cell phenotype is induced by the cytokine IL-23, and a few studies investigating IL-17 as a biomarker also evaluate the potential of IL-23 for the same purpose (Langrish et al., 2005; Wen et al., 2012; Esendagli et al., 2013; Khaiboullina et al., 2015). Two studies similarly found increased IL-23 in the serum and CSF of MS cases compared to controls, and one study in particular found that serum IL-23 may be correlated with EDSS (Wen et al., 2012; Khaiboullina et al., 2015). Meta-analysis results also suggest a strong association of IL-23 levels with MS diagnosis with average levels measuring 8.1 standard deviations above the mean of control levels (Bai et al., 2019). A

third study, however, failed to find an increase in IL-23 in MS cases, and meta-analysis suggests high variability between studies; taken together these results make it difficult to infer a relationship between MS disease activity and IL-17 and IL-23 (Esendagli et al., 2013; Bai et al., 2019). However, IL-23 still seems to represent the most promising blood-based cytokine identified to date, and may represent an important clinical assessment in the future (Bai et al., 2019).

Finally, IL-1RA is an endogenous soluble cytokine that blocks the effects of IL-1β at the IL-1 receptor that is thought to be secreted in conjunction with IL-1β at sites of injury in order to limit its damaging effects (Nicoletti et al., 1996; Corr et al., 2011; Dosh et al., 2019; Nitta et al., 2019). In a limited number of studies, serum levels of IL-1RA have been documented to be increased in MS and associated with active disease (Nicoletti et al., 1996; Heesen et al., 2000; Al-Nashmi et al., 2017). However, serum IL-1RA appears to be further elevated with treatment with IFNβ -1a, suggesting that elevated levels of IL-1RA beyond physiological levels in MS may be beneficial and play a role in response to IFN treatments (Nicoletti et al., 1996; Comabella et al., 2008). At present, there is an ongoing phase I/II clinical trial investigating effects of a recombinant IL-1RA, anakinra, on inflammatory lesions in RRMS with an estimated completion date of December 2022 (clinicaltrials.gov, NCT04025554). Results from this study have the potential to shed light on the biology of IL-1RA in the context of inflammatory lesion activity and development in MS.

It is clear that circulating cytokines have important potential to act as an accessible measure of local and systemic inflammation in MS. However, the publication

of the meta-analysis conducted by Bai and colleagues highlights the poor comparability between studies which significantly hinders the validation of identified markers (Bai et al., 2019). Many of the desired characteristics of a molecular biomarker in MS (Figure 1.1) include specificity, sensitivity, and accuracy; all which contribute to the clinical and analytical validation of the markers and their associated tests (Comabella & Montalban, 2014; Thebault et al., 2021). Ideally, the marker is present in MS and not in other conditions, and increases/decreases because of increasing or decreasing disease activity and is measured in an accessible body fluid by a simple, safe, and cost-effective assay (Comabella & Montalban, 2014). As of yet, no circulating marker identified throughout the literature has sufficiently satisfied these criteria.

1.4 Thesis Objectives and Overview

While clinically useful biomarkers for diagnosis, prognosis and treatment monitoring of MS exist, a significant limitation is the lack of specificity for MS. As it currently stands, a diagnosis of MS requires several lines of evidence and often takes time, leaving patients waiting for access to healthcare services, including DMTs. Therefore, the active search for novel biomarkers that may improve time to diagnosis, diagnostic accuracy, and provide prognostic information is an active and ongoing area of research. In this thesis, I investigate blood plasma and CSF with the objective of identifying novel immune-relevant candidate biomarkers that can inform on pathophysiological disease mechanisms in RRMS. I hypothesize that blood plasma and CSF of MS cases contain inflammatory mediators that: 1) contribute to ongoing pathological mechanisms in the CNS, 2) help in the diagnosis and disease monitoring of RRMS, and 3) potentially guide novel therapeutic development.

In chapter 2, I investigated the anti-inflammatory cytokine IL-1RA as a biomarker in plasma. I found that IL-1RA is associated with disability in RRMS independent of other clinical and demographic variables and identified its cellular source *in vitro* and in *in situ* MS lesions. In chapter 3, I developed and optimized a flow cytometry protocol to immunophenotype EVs in plasma samples and investigated the contribution of individual immune cell subsets to the circulating EV pool. In this study, I determined that levels of immune cell derived EVs were elevated in untreated RRMS cases compared to healthy controls and was independent of levels of circulating parent cell populations. Finally, in chapter 4, I immunophenotyped CSF samples from RRMS and NIND cases and

investigated the pathophysiological relevance of elevated CXCL10 on immune cell infiltration into the CNS and astrocyte phenotype.

Chapter 2 – Interleukin-1 receptor antagonist: A novel soluble biomarker that correlates with disability and neurofilament light in relapsing-remitting multiple sclerosis.

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2.1 Introduction

Multiple sclerosis (MS) is a chronic demyelinating immune-mediated neurodegenerative disease, whereby an immune response is mounted against myelin-forming oligodendrocytes in the central nervous system (CNS). In the majority of patients, the most common clinical presentation begins as relapsing-remitting MS (RRMS) followed by an eventual progression to secondary progressive MS (SPMS). While not mutually exclusive, these unique clinical presentations are thought to differ in their primary pathophysiological mechanisms; RRMS is largely driven by inflammatory-mediated demyelination, while SPMS is accompanied by significant neurodegeneration (Peterson & Trapp, 2005).

In MS, the most common and routine method to measure disability is the Kurtzke Expanded Disability Status Scale (EDSS; (Kurtzke, 1983). A significant limitation of the EDSS is that it relies on physical symptoms and changes in physical disability but fails to account for cognitive and behavioural processes that are difficult to assess objectively. Furthermore, changes in EDSS can either be indicative of a recent relapse or reflect disability progression but does not distinguish between the two. The identification of a biologically relevant biomarker that can objectively distinguish disability progression over relapse activity would provide clinicians with a valuable tool when considering appropriate disease management strategies.

In recent years, numerous studies have demonstrated an association between increased plasma and/or CSF levels of neurofilament light chain (NfL) and markers of clinical disease activity and progression in people living with MS (Lycke et al., 1998; Malmeström et al., 2003; Disanto et al., 2017; Piehl et al., 2018; Kuhle et al., 2019;

Thebault et al., 2020). NfL is easily measured in CSF, however, obtaining CSF from patients requires an invasive and undesirable lumbar puncture procedure. While NfL can be measured in blood plasma or serum, reliable measurements require specialized equipment that is not routinely available for most clinics (Kuhle et al., 2016). Reported increases from baseline NfL are also not unique to MS and has been documented in normal ageing and most other neurodegenerative diseases (Khalil et al., 2018; Bridel et al., 2019).

Interleukin-1 receptor antagonist (IL-1RA) is a cytokine readily detected in blood and an endogenous soluble antagonist of the IL-1 receptor that blocks IL-1β-mediated pro-inflammatory signaling (Dinarello, 1994). Following activation of the NOD-Like Receptor Family Pyrin Domain Containing 3 (NLRP3) inflammasome, an inflammatory process linked to oligodendroglial cell death in MS, IL-1β is released predominantly by macrophages and microglia (McKenzie et al., 2018). In serum, increased levels of IL-1RA have been previously reported in MS; individuals with active disease have reported elevated levels (Nicoletti et al., 1996; Heesen et al., 2000; Al-Nashmi et al., 2017). In MS and other inflammatory-mediated diseases, it has been suggested that increased IL-1RA functions as an endogenous anti-inflammatory mechanism against elevated IL-1β (Nicoletti et al., 1996; Corr et al., 2011; Dosh et al., 2019; Nitta et al., 2019).

The objectives of this study were to determine whether IL-1RA levels are associated with clinical measures of disease activity and disability. Herein, we demonstrate that IL-1RA levels correlate with EDSS and NfL, which suggests its utility as a potential novel biomarker of disability.

2.2 Methods

2.2.1 Study Participants

All experiments involving human participation were approved by the Newfoundland (NL) Health Research Ethics Board, which follows the Canadian government's ethical conduct for research involving humans and the world medical association declaration of Helsinki. Written informed consent was obtained from all participants prior to study initiation. For plasma analyses, 96 RRMS patients were recruited through the Health Research Innovation Team in Multiple Sclerosis (HITMS) or Neurology clinic at Memorial University of Newfoundland, St. John's NL, Canada between June 2015 and September 2018. All clinical parameters were determined based on neurological assessment by a MS neurologist. For CSF analyses, 64 patients were recruited from the Eastern Health neurology clinic (St. John's, NL) or Horizon Health (Saint John, NB) between February 2015 and December 2019. For both cohorts, in order to demonstrate real-world relevance, all RRMS patients whose samples were collected during the time frames outlined were eligible for inclusion in the study. Patients were only excluded on the basis of inadequate sample availability, missing demographic or clinical variables or undetectable IL-1RA levels (cut-off value 2pg/mL). There were no other exclusion criteria. Patient demographics are presented in Table 2.1.

Table 2.1: Patient Demographics

Plasma Cohort (n=96)	
Age (years; mean±SD)	43.6 ± 10.1
Sex (%)	
Males	25.0%
Females	75.0%
Diagnosis at Blood Draw (%)	
RRMS	100%
EDSS	
Range	0-6.5
Median (IQR)	2(2)
Disease Duration (years; mean±SD)	11.1 ± 7.5
Last Relapse (months; mean±SD)	58.7 ± 59.4
DMT use (%)	
None	16.6%
GA	20.8%
Natalizumab	3.1%
Fingolimod	11.5%
DMF	27.1%
Teriflunomide	13.5%
IFNβ-1a	7.29%
CSF Cohort (n=64)	
Age (years; mean±SD)	44.6 ± 15.6
Sex (%)	
Males	32.8%
Females	67.2%
Diagnosis at CSF Draw (%)	
RRMS	54.7%
NIND	28.1%
OIND	17.2%

Abbreviations: RRMS = relapse remitting multiple sclerosis, y = years, DD = Disease Duration, EDSS = expanded disability status scale, IQR = interquartile range, DMT = disease modifying therapy, GA = glatiramer acetate, DMF = dimethyl fumarate, IFN = interferon, CIS = Clinically isolated syndrome, NIND = non-inflammatory neurological disease, OIND = other inflammatory neurological disease.

2.2.2 Biological Sample Collection, Processing and Storage

Venous blood was drawn and plasma was obtained following centrifugation (300xg) of EDTA-coated vacutainers. Plasma was immediately frozen at -80°C and transferred to liquid nitrogen. CSF was drawn by lumbar puncture, centrifuged (300xg), aliquoted, frozen at -80°C, and transferred to liquid nitrogen.

2.2.3 Human Cell Isolation, Culture, and Inflammasome Activation

Human primary microglia were derived from fetal CNS tissue (gestational age 10–20 weeks) following maternal informed consent and approved by the Newfoundland Health Research Ethics Board. CNS tissue was digested with DNase/trypsin and passed through a nylon mesh to obtain a single cell suspension. Cells were then plated in DMEM containing 5% FBS, penicillin/streptomycin and glutamine. After 10-14 days, supernatants containing floating microglia were removed, centrifuged (300xg, 10 minutes), and seeded at 10^5cells/mL in DMEM containing 10% FBS, penicillin/streptomycin, glutamine and macrophage colony-stimulating factor (M-CSF; 25ng/mL).

Primary human monocyte derived macrophages (MDMs) were obtained as previously described (Galloway et al., 2019). MDMs were derived from $CD14^+$ monocytes, seeded at $5x10^5$cells/mL in RPMI media containing 10% FBS, penicillin/streptomycin, glutamine, and M-CSF (25ng/mL).

Inflammasome activation protocols were initiated 3-5 days following cell plating. Cells were treated with lipopolysaccharide (LPS; 100ng/mL) in media for 4hrs, followed

by the addition of adenosine triphosphate (ATP; 5mM) or nigericin (10μM) for 1hr. Supernatants were collected and stored at -80℃. Inflammasome activation of all primary cultures was confirmed by measuring IL-1β in supernatants by ELISA (BD Biosciences).

2.2.4 Cytokine Analyses

All assays were performed according to manufacturer's instructions. Cytokine measurements were obtained for IL-1RA and IL-18 (R&D Systems) by ELISAs. Plasma levels of IL-1β were measured using ProQuantum™ immunoassay (ThermoFisher). CSF levels of IL-1RA were measured as part of the Bio-Plex Pro™ Human Cytokine 27-plex Assay and analyzed on a Bio-Plex® 200 system. Cytokine measurements below the detection limit of the assay were excluded from analysis.

2.2.5 Neurofilament Light Chain Analysis

CSF NfL was measured by a commercially available ELISA (R&D quality, Uman Diagnostics) according to manufacturer's instructions.

2.2.6 Histology and Immunohistochemistry

Formalin-fixed paraffin-embedded human brain sections from mixed active/inactive MS lesions used for *in situ* studies with a next-of-kin consented autopsy and was approved by the Newfoundland Health Research Ethics. The patient was a 42-year-old female, with an MS disease duration of 5 years. Post-mortem delay was noted at 22hrs. Cause of death was hypersensitivity and myocarditis. Sections were subjected to

heat-induced antigen retrieval using sodium citrate buffer (pH = 6.0) and blocked and permeabilized in PBS containing 10% normal goat serum, 2% horse serum, 0.1% Triton-X) for 1hr. For CD68 immunostaining, following the overnight incubation of primary antibody (1:40; Dako), sections were washed in PBS and blocked in 0.6% hydrogen peroxide (Sigma) for 10mins at RT. Sections were then washed and incubated in biotinylated secondary antibody from the VECTASTAIN® Elite® ABC kit according to manufacturer's instructions. For immunofluorescence, following primary antibody incubation, sections were washed and incubated with secondary antibodies for 1hr. Primary antibodies included: CD68 (1:40; Dako) and IL-1RA (1:100; Abcam); secondary antibodies were used at 1:500.

2.2.7 Statistical Analysis

Data analysis was performed using Prism 8 software (GraphPad) and SPSS (IBM). For all statistical tests, $p<0.05$ was considered significant. Data are presented as mean±SEM unless otherwise indicated. To analyze the relationship between IL-1RA and EDSS when controlling for demographic and clinical variables, the data were analyzed using multiple regression. We fit two multiple regression models with EDSS as the dependent variable. The associations between the dependent and independent variables were assessed using R^2, and the unique contribution of IL-1RA was assessed using the R^2 change between the two models. IL-1RA levels in males and females were compared using an unpaired t-test, and DMT use was compared using a one-way ANOVA. Levels of IL-1RA in plasma were also correlated with levels of IL-1β and IL-18 in the plasma

using Pearson's Correlation test. IL-1RA, IL-1β and IL-18 were correlated to EDSS using Spearman's correlation test. For these analyses a majority subset of the cohort was included due to sample availability and exclusion of values below the limit of detection of the assays (IL-1β n=82/96; IL-18 n=93/96). Pearson's correlation test was also used to assess the correlation between CSF IL-1RA and NfL. Levels of IL-1RA in CSF was compared by diagnosis using a one-way ANOVA. *In vitro* cytokine secretion from MDMs and HFMs were analyzed using a paired t-test.

2.3 Results

2.3.1 Plasma IL-1RA is correlated with EDSS and is independent of age, sex, disease duration, and time since most recent relapse activity.

Plasma levels of IL-1RA were investigated in relation to EDSS and various demographic and clinical variables, including age, sex, disease duration, and time since last relapse, first by simple linear regression. IL-1RA in MS patient plasma (495.0 ± 70.9pg/mL) was significantly correlated with EDSS (Figure 2.1A; r=0.210; p=0.040). In contrast, plasma IL-1RA was not correlated with age (Figure 2.1B; r=-0.088; p=0.394), disease duration (Figure 2.1C; r=-0.008; p=0.941), nor time since most recent clinical relapse (Figure 2.1D; r=-0.064; p=0.534). No significant differences in IL-1RA levels were detected between males and females (Figure 2.1E) and did not differ between patients on various DMTs (Figure 2.1F).

To confirm that the correlation between EDSS and IL-1RA persisted when controlling for multiple demographic and clinical variables, the data were analyzed by multiple regression. We fit two multiple regression models with EDSS as the dependent variable. The associations between the dependent and independent variables were assessed using R^2, and the unique contribution of IL-1RA was assessed using the R^2 change between the two models. The first model contained the following set of independent variables: age, sex, disease duration, and time since most recent relapse. This model accounted for 6.5% of the variability in EDSS (R^2=0.065, p=0.184), and its predictive capacity was not significant ($F_{(4,91)}$=1.588, p=0.184). The second model was identical as the first, except with the addition of plasma IL-1RA concentration as an independent variable. Adding IL-1RA into the model explained an additional 9.8% of the

variability in EDSS (R^2 = 0.163, R^2 Change=0.098) and this change in the R^2 value was statistically significant (p=0.002). Additionally, the predictive capacity of the final model was statistically significant ($F_{(5, 90)}$=3.510; p=0.006).

2.3.2 NLRP3 Inflammasome-related cytokines are not correlated with EDSS.

Given IL-1RA is a soluble IL-1β receptor antagonist, we next sought to determine whether levels IL-1β and IL-18 (cytokines released following inflammasome activation) were also correlated with EDSS in our cohort. IL-18 was not correlated with EDSS scores (r=0.078, p=0.458, Figure 2.2A), nor with plasma IL-1RA levels (r=-0.064, p=0.541, Figure 2.2B). Similarly, plasma levels of IL-1β were not correlated with EDSS (r=-0.144, p=0.198, Figure 2.2C) nor with IL-1RA (r=0.0289, p=0.798, Figure 2.2D). Furthermore, levels of both cytokines released following inflammasome activation, IL-1β and IL-18, measured in plasma were not correlated with one another (r=0.051, p=0.657, Figure 2.2E).

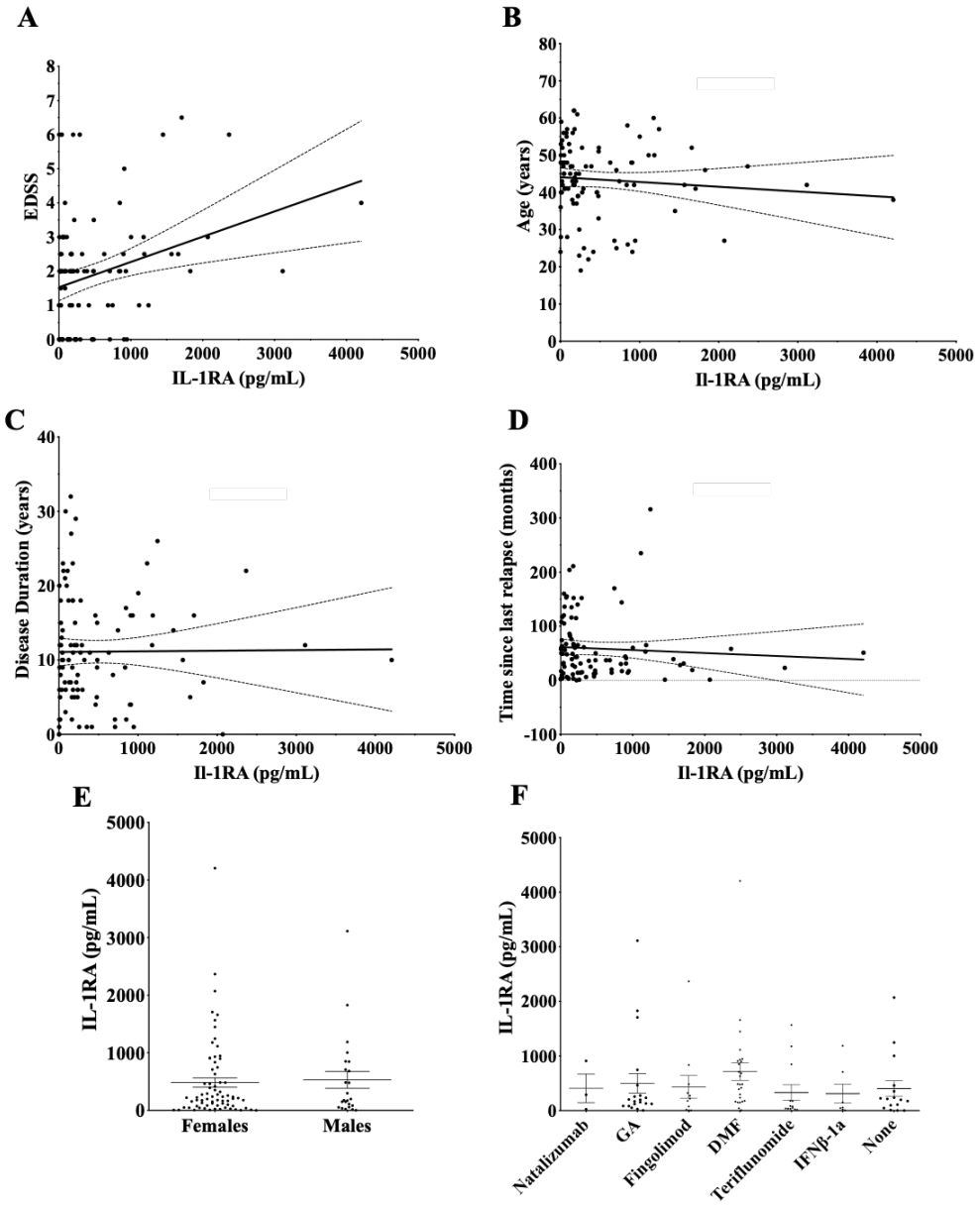

Figure 2.1: Plasma IL-1RA is correlated with EDSS, but not with other demographic variables. Plasma levels of IL-1RA is correlated with A) EDSS scores, but not with B) age, C) disease duration, or D) time since last relapse. E) Plasma levels of IL-1RA did not differ between males and females. E) Use of different DMTs did not affect plasma IL-1RA. Data were analysed by A) linear regression and Spearman's correlation B-D) linear regression and Pearson's correlation E) unpaired t-test or F) one-way ANOVA.

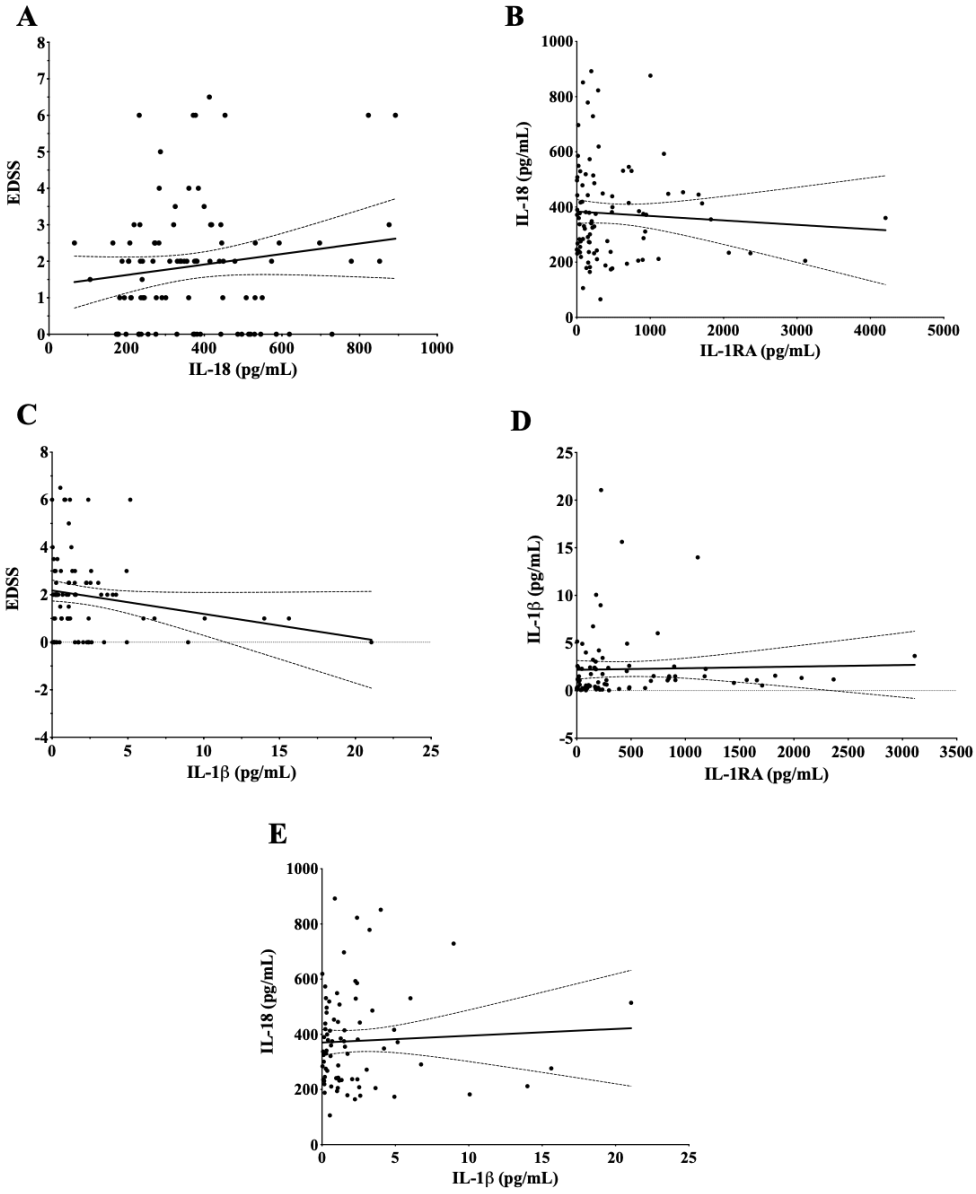

Figure 2.2: Other inflammasome-relevant cytokines are not correlated with EDSS or IL-1RA. A) Plasma levels of IL-18 were not correlated with EDSS or B) with plasma IL-1RA (n=102). C) Levels of IL-1β measured in plasma from were not significantly correlated with EDSS or D) with plasma IL-1RA (n=89). E) Levels of IL-18 and IL-1β (n=84) are not correlated with one another. Data were analyzed by linear regression and Spearman's (A and D) or Pearson's (B and C) correlation.

2.3.3 IL-1RA is correlated with NfL levels in CSF.

We measured levels of IL-1RA and NfL in the CSF of patients with various neurological conditions and categorized patients as either relapsing-remitting MS (RRMS), non-inflammatory neurological diseases (NIND), and other inflammatory neurological diseases (OIND). Overall, CSF levels of IL-1RA did differ between groups with the exception of elevated levels in the OIND group (Figure 2.3A). When considering all samples, levels of IL-1RA in CSF were significantly correlated with NfL (Figure 2.3B, r=0.284; p=0.023). Importantly, this correlation also remained significant when non-MS cases were excluded (Figure 2.3C; r=0.292, p=0.045). Similar to the analysis performed in the plasma, levels of IL-1β in the CSF (as measured by the Bio-Plex) were examined in relation to IL-1RA and NfL. No correlation between IL-1β and NfL were noted (not shown), however IL-1RA levels were significantly higher in CSF samples that had measurable levels of IL-1β compared to samples that had undetectable levels (i.e. below the lowest standard, (Figure 2.3D, p=0.0009). When only considering non-MS cases, CSF IL-1RA was also significantly correlated with CSF NfL (data not shown, r=0.486, p=0.003).

2.3.4 Activation of the NLRP3 inflammasome induces IL-1RA release from primary human cells.

To determine the possible contribution of inflammatory circulating and brain-resident immune cells on the plasma levels of IL-1RA, supernatants from inflammasome-activated primary human MDMs and human fetal microglia (HFM) were assayed (Figure

2.4). Under normal culturing conditions, both MDMs and HFM secrete no detectable level of IL-1β; following activation of the NLRP3 inflammasome, IL-1β secretion was increased in both MDMs (Figure 2.4A; 1340±294.8pg/mL; p=0.003 treated vs. untreated) and HFM (Figure 2.4B; 1016±300.0pg/mL; p=0.028 treated vs. untreated), confirming activation of the NLRP3 inflammasome. When considering IL-1RA, under normal culture conditions MDMs produced 1336 ± 547.1pg/mL, whereas inflammasome stimulated MDMs produced 1779 ± 629.1pg/mL (Figure 2.4C; p=0.024, untreated vs treated). Basally, HFMs secreted 153.0±59.47pg/mL, however following activation, HFMs secreted 439.0 ± 135.2pg/mL (Figure 2.4D).

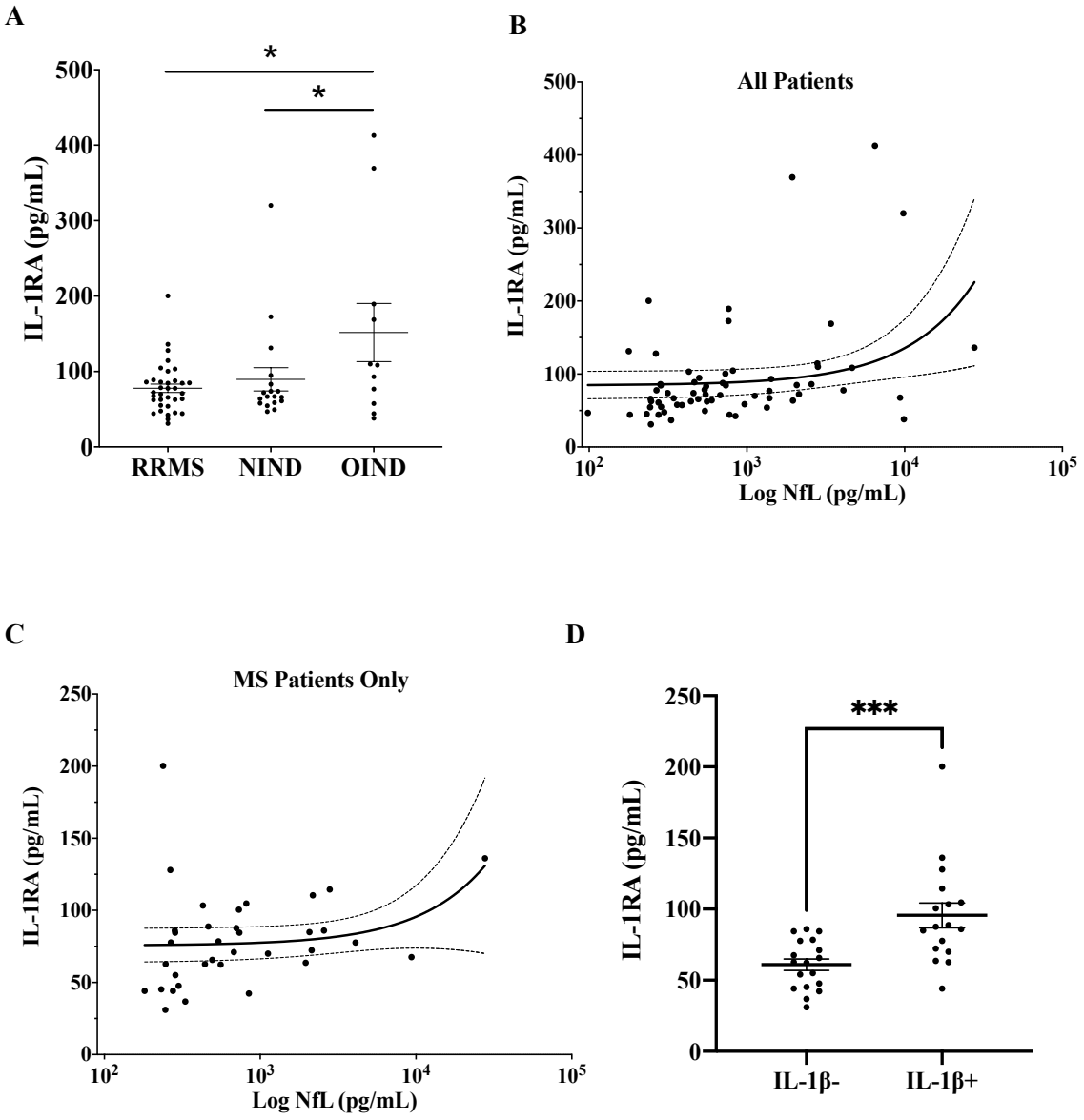

Figure 2.3: IL-1RA in CSF is correlated with NfL. A) CSF levels of IL-1RA were comparable between groups of diagnoses; OIND patients had significantly higher levels compared to RRMS patients. B) CSF levels of IL-1RA were correlated with NfL in all patients (n=73). C) The correlation between IL-1RA and NfL persisted when only considering patients diagnosed with MS (n=46). Data were analysed by A) one-way ANOVA, followed by Tukey's multiple comparisons, B) linear regression, and two-tailed Pearson's correlation or C) linear regression and one-tailed Pearson's correlation, D) CSF levels of IL-1RA were significantly higher in samples with detectable IL-1β levels compared to samples that had undetectable IL-1β levels (i.e. below the lowest standard) *p<0.05, ***p<0.001

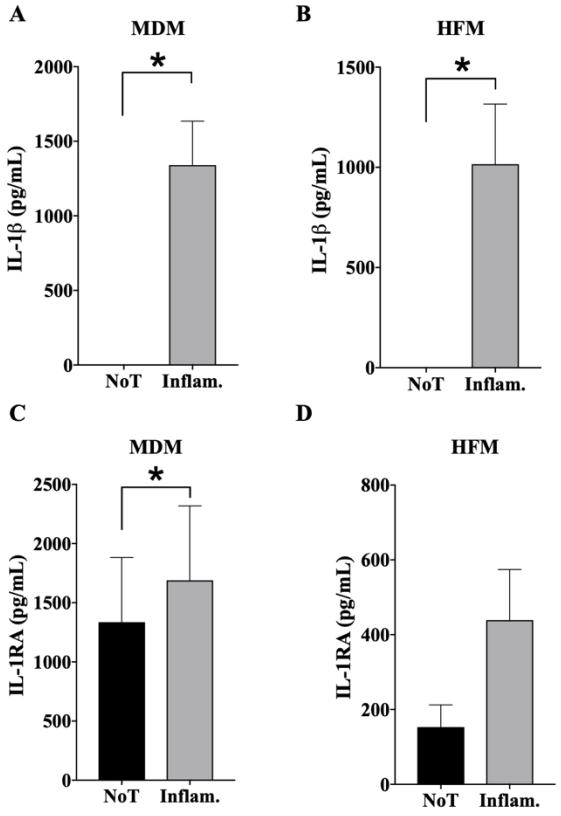

Figure 2.4: *In vitro* activation of the NLRP3 inflammasome in primary cells stimulates IL-1RA release. Stimulation of primary MDMs (n=8) and HFM (n=5) with LPS (100ng/mL; 3 hours) and ATP/Nigericin (5mM/10μM, 1 hour) to induce the NLRP3 inflammasome resulted in increased A-B) IL-1β and C-D) IL-1RA secretion compared to unstimulated cells. NoT: no treatment. Data were analyzed by paired t-test. *$p<0.05$.

2.3.5 IL-1RA is expressed in myeloid-derived cells in mixed active/inactive MS lesions.

LFB stained sections were used to identify demyelinated lesions and to perform a general histological examination. CD68+ DAB immunohistochemistry was used to identify lesion subtype according to (Kuhlmann et al., 2017) (Figure 2.5, red outlines). The lesions studied were of the mixed active/inactive type and may also be classified as chronic active plaques (Bö et al., 1994). Immunofluorescence analysis was subsequently conducted on adjacent tissue sections stained with primary antibodies against CD68 and IL-1RA. This analysis revealed that IL-1RA^{+}/CD68^{+} cells with morphologies consistent with microglia and macrophages were present at the hypercellular edge of mixed active/inactive lesions (Figure 2.5).

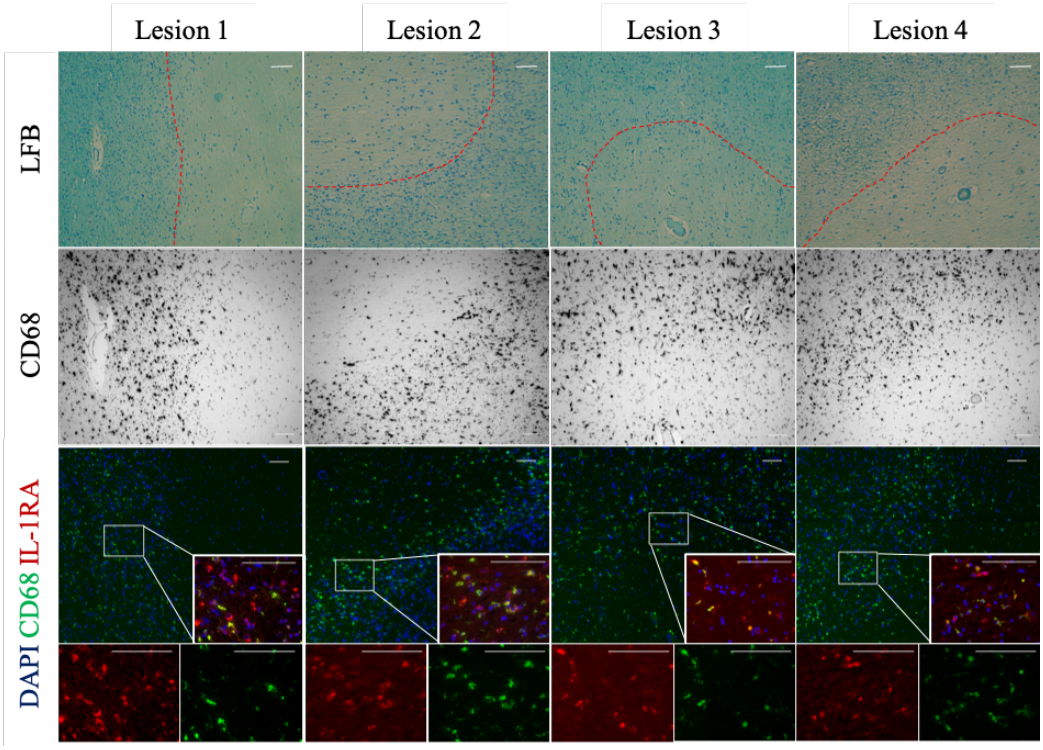

Figure 2.5: IL-1RA$^+$/CD68$^+$ cells are present at the edge of mixed active/inactive MS lesions. Bright-field images of 4 chronic active lesions from post-mortem human brain tissue stained with LFB show areas of demyelination (lesions) that are outlined with a red dashed line. Bright field images of the same lesions using near-adjacent sections were immunostained with CD68 using the DAB method to identify mixed active/inactive lesions showing numerous CD68$^+$ microglia/macrophages at the lesion edges. Immunofluorescent images of near-adjacent sections that were labeled with anti-IL-1RA (green), anti-CD68 (red) and DAPI (blue). Note that many of the IL-1RA$^+$ cells at the lesion edges are also CD68$^+$, producing yellow immunofluorescence, consistent with microglia/macrophages expressing IL-1RA. Scale bar = 100μm.

2.4 Discussion

While the search for biomarkers to diagnose MS and predict disease disability and/or progression has been an active area of research, very few candidates have been validated and are routinely used in a clinical setting. In fact, no blood-derived biomarkers have proven adequate for diagnosis and monitoring progression, keeping the push for new candidates at the forefront of MS research (Reich et al., 2018). The results of the current study demonstrate that IL-1RA is a novel exploratory biomarker that correlates with disability and NfL, suggesting its utility in terms of monitoring MS disability independent of recent relapse activity.

In MS, using multiple regression analysis, we have demonstrated that plasma IL-1RA is significantly correlated with EDSS (Figure 2.1A). To date, finding biomarkers in MS that correlate with EDSS scores has been an unanticipated challenge. Even validated biomarkers used routinely in the clinic (for example, T2 weighted white matter lesion load) are not well correlated to EDSS score (Paul et al., 2019). While the use of EDSS has several limitations, since it does not assess the wide range of physical and cognitive disability experienced by people with MS, it remains the most widely used clinical scale used to measure clinical disability. Therefore, novel exploratory biomarkers proposed to be associated with clinical disability should also be investigated in addition to EDSS (Uitdehaag, 2018). Increased EDSS scores and subsequent disability progression have been previously shown to be associated with age and disease duration, which makes it less effective for measuring the nuanced changes in disability that are independent of a person's age and/or how long an individual has been diagnosed with MS (Cree et al., 2016; Jokubaitis et al., 2016). Herein, we demonstrate that plasma IL-1RA levels

correlate with EDSS independent of age or disease duration (Figure 2.1), suggesting that this putative biomarker can potentially detect these subtle changes in disability.

Previous studies investigating IL-1RA in MS have yielded variable results and conclusions. While serum IL-1RA has been shown to be elevated in patients diagnosed with MS compared to healthy controls (Heesen et al., 2000; Al-Nashmi et al., 2017), these findings are not supported by other studies using serum, plasma, or CSF (Nicoletti et al., 1996; Pasquali et al., 2015; Hornig et al., 2016). Furthermore, elevated IL-1RA has been noted in patients actively undergoing relapses, suggesting that IL-1RA may be an endogenous mechanism to counterbalance the elevated IL-1β levels that accompanies a relapse (Nicoletti et al., 1996). In this study, we have demonstrated that IL-1RA is not associated with time since a clinically definite relapse (i.e. disease activity; Figure 2.1D). While this report did not include actively relapsing patients, our results suggest that heightened inflammatory disease activity associated with a relapse does not result in elevated IL-1RA and hence is not associated with inflammatory activity.

Unlike several other proposed biomarkers in MS, plasma IL-1RA was not associated with sex or DMT use (Figure 2.1E-F), suggesting IL-1RA could be used to predict disability in diverse groups of patients. Previously, some smaller studies have found associations between increased circulating IL-1RA levels and various DMTs. Elevated serum IL-1RA was measured in 11 RRMS patients following 6 months of IFNβ-1a treatment compared to pre-treatment baseline (Nicoletti et al., 1996). This result was also replicated in a larger cohort of patients with progressive MS, whereby IFNβ-1b treatment increased serum IL-1RA levels as early as 3 months after treatment initiation

and was sustained 21 months later (Comabella et al., 2008). Similar to results found with IFNβ treatments, in experimental autoimmune encephalomyelitis (EAE), a common animal model of MS, glatiramer acetate (GA) treatment was associated with elevated serum IL-1RA. A small number of patients treated with GA (9) also had increased serum IL-1RA compared to controls and untreated RRMS patients (Burger et al., 2009). Our results did not show this trend, whereby neither IFNβ-1a nor GA resulted in elevated plasma IL-1RA compared to untreated patients (Figure 2.1F).

IL-1RA is a member of the IL-1 family of cytokines and an endogenous antagonist to the pro-inflammatory-mediated effects of IL-1β signalling at the IL-1R1 receptor. At the site of an MS lesion, the maintenance of balance between pro- and anti-inflammatory mediators within the injury environment is critical for limiting lesion size, clearing debris, and initiating repair mechanisms. IL-1RA can be synthesized and released in response to elevated IL-1β (or in tandem with IL-1β release) in areas of inflammation, suggesting its key role in maintaining balance in the tissue/injury microenvironment (Palin et al., 2004; Dujmovic et al., 2009). Specifically, the activation of inflammasomes results in a particularly heightened IL-1β release. Inflammasomes are intracellular signaling platforms that become activated in response to a variety of danger signals. The NLRP3 inflammasome is the most well-characterized and is highly expressed in myeloid-derived innate immune cells, including macrophages and microglia. Activation of the NLRP3 inflammasome leads to the release of the pro-inflammatory cytokines IL-1β and IL-18, and has been documented in oligodendrocytes, microglia, and

infiltrating macrophages in lesions from *post mortem* brain samples from MS patients, as well as in the CNS of EAE mice (Latz et al., 2013; McKenzie et al., 2018).

In contrast to results obtained with IL-1RA, neither IL-1β or IL-18 significantly correlated with EDSS in our study (Figure 2.2A and C). We also examined the relationships between levels of IL-1β and IL-18 with levels of IL-1RA to determine whether the increase in IL-1RA measured in patient plasma was correlated with increases in cytokines associated with inflammasome activation. Our results demonstrated that plasma IL-1RA did not correlate with plasma levels of IL-18 (Figure 2.2B) or IL-1β (Figure 2.2D). Furthermore, IL-1β and IL-18 were not correlated with each other (Figure 2.2E). Considering that IL-1RA rises as an endogenous inhibitor to IL-1β, and IL-18 and IL-1β are released simultaneously as a result of inflammasome activation, one would expect levels of IL-18 and IL-1β to rise together. These results suggest that inflammasome activation and the potential pyroptotic mechanisms that are occurring in the CNS of MS patients cannot be easily measured in the periphery using these specific cytokines.

Investigation of IL-1RA in the CSF of individuals with varying neurological diseases revealed that IL-1RA was significantly correlated with NfL (Figure 2.3B). In addition, when only MS patients were investigated, this correlation remained significant (Figure 2.3C). Elevated NfL levels in the CSF and/or blood has been broadly used as a biomarker of neurodegeneration that has received significant attention in research fields for various neurological diseases (Khalil et al., 2018; Bridel et al., 2019). In MS, increased NfL in CSF and/or blood is associated with enhancing MRI lesions, increased

lesion load, increased relapse activity, decreased brain volume, worsening disability, and worse prognosis (Lycke et al., 1998; Malmeström et al., 2003; Disanto et al., 2017; Piehl et al., 2018; Kuhle et al., 2019; Thebault et al., 2020). Since measurements of NfL in blood plasma/serum requires specialized equipment not available in most clinics, it's widespread use as a biomarker remains restricted. Herein, we demonstrate that IL-1RA can be readily measured in both plasma and CSF, is correlated with NfL in MS, and may serve as a novel method to measure disability. Future studies investigating an MS cohort with highly active disease will be needed to confirm any association of IL-1RA in the context of highly active disease.

IL-1β levels in the CSF have also been linked to measures of disease progression. CSF IL-1β has been shown to be associated with early cortical pathology in MS (Seppi et al., 2014). More recent work has also suggested that the increase of IL-1β and IL-18 in the CSF of MS patients compared to controls may be indicative of ongoing inflammasome activity within the CNS (Voet et al., 2018). Interestingly, research also suggests that during periods of remission, the presence of CNS inflammation as measured by the detection of IL-1β in the CSF was associated with increased disability progression at longitudinal follow-up (Rossi et al., 2014). The authors of this study suggest that higher levels of IL-1β that persist during periods of low disease activity are indicative of inflammatory mechanisms that drive progression independently of relapse activity. This suggests that CSF IL-1β may serve as a marker of disability independent of active relapse activity. In support of this study, here we shown that RRMS patients that have measurable

IL-1β in their CSF have significantly higher CSF levels of IL-1RA compared to patients without measurable CSF IL-1β (Figure 2.3D).

IL-1β has long been known as a pathogenic factor in MS and its commonly used animal model EAE. In fact, animals lacking either functional IL-1 receptors or IL-1β are resistant to developing symptoms in EAE (Schiffenbauer et al., 2000; Lévesque et al., 2016; Ronchi et al., 2016). However, as of yet, a causal role for IL-1β in the development and/or maintenance of EAE and MS has yet to be established.

To further elucidate MS-relevant pathological mechanisms and implicate biological relevance, we sought to determine whether induction of the NLRP3 inflammasome was able to stimulate the release of IL-1RA as an endogenous modulator of IL-1β activity. Following activation of the inflammasome *in vitro*, IL-1RA was measured in the supernatants from primary human macrophages and microglia (Figure 2.4). Though the cellular mechanism by which IL-1RA secretion increases as a result of NLRP3 inflammasome activation has yet to be elucidated, it supports the hypothesis that cells affected by inflammasome activation may simultaneously release IL-1RA as a protective mechanism to prevent secondary damage. Whether this mechanism results in the release of sufficient levels of IL-1RA to be successful remains to be seen, however, treatment with exogenous IL-1RA may prove to be an effective therapy. Many early studies have shown that treatment with exogenous IL-1RA provides protection in murine EAE (Martin & Near, 1995; Badovinac et al., 1998; Aubé et al., 2014). However, reports also indicate that IL-1 signaling is critical for CNS remyelination which begs the question of how useful IL-1RA will be as potential new DMT for MS (Mason et al., 2001; Vela et

al., 2002). An open-label clinical trial for the use of Anakinra, a recombinant and modified IL-1RA biopharmaceutical, for the treatment of inflamed white matter lesions in MS is ongoing (clinicaltrials.gov, NCT04025554) and results from this trial will provide data to significantly enhance our understanding of IL-1RA and its utility in treating MS.

Our *in situ* analysis of active/inactive lesions in MS revealed $CD68^+$ myeloid cells that positively stained for IL-1RA (Figure 2.5). These double-stained positive cells were observed in abundance at the hypercellular edge of mixed active/inactive lesions. These lesions were characterized as areas of extensive demyelination with a hypocellular center void of myeloid cells and an active rim of $CD68^+$ macrophages and/or microglia at the border of the lesion (Kuhlmann et al., 2017). Our findings demonstrate that IL-1RA is expressed in myeloid cells at lesion borders (Boven et al., 2006). Similar findings have also been documented in various animal models of MS, suggesting that our results are consistent between species (Prins et al., 2013). Future studies are needed to determine the specific pathological conditions required to induce IL-1RA expression in MS, and whether IL-1RA production is sufficient to limit the downstream damage caused by IL-1β-mediated signaling.

In conclusion, we have demonstrated that IL-1RA is correlated with EDSS, independent of other demographic variables, and is a pathologically relevant molecule released from human microglia and macrophages in response to CNS inflammation. We also show that $IL\text{-}1RA^+/CD68^+$ cells are present the edge of mixed active/inactive lesions. Together with our *in vitro* data, these results provide evidence that myeloid cells are a cellular source of IL-1RA and links this cytokine to MS pathology. Additionally, we show that in IL-1RA levels in the CSF are correlated with NfL. Taken together, these

results demonstrate that IL-1RA is a promising novel exploratory soluble biomarker in MS that can monitor disability independent of age, sex, disease duration, DMT use, and previous relapse activity.

Chapter 3 – Analysis of plasma using flow cytometry reveals increased immune cell-derived extracellular vesicles in untreated relapsing-remitting multiple sclerosis

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3.1 Introduction

Extracellular vesicles (EVs) are small (~30-200nm) biological vesicles continuously secreted from cells under both physiological and pathological conditions (Yáñez-Mó et al., 2015; Blandford et al., 2018). Secreted EVs can be detected within all biological fluids and have numerous functions, however, they have only recently emerged as an important mechanism of intercellular communication (Yáñez-Mó et al., 2015; Marostica et al., 2020). EVs contain biologically active cargo, which can be either non-specific or unique to the cell of origin (Keerthikumar et al., 2016; Kowal et al., 2016). The presence of cell-specific markers on the surface of EVs allows for the identification of the EV's cell type of origin and permits their quantification using specialized instrumentation and standardized assays (Brittain et al., 2019). EVs of various cellular origins have emerged as important signaling entities that may help to drive pathophysiological conditions and could serve as putative biomarkers for various neurological conditions (Yáñez-Mó et al., 2015; Blandford et al., 2018).

Multiple sclerosis (MS) is a chronic inflammatory neurological disease characterized by the immune-mediated destruction of myelin that results in impaired neurotransmission and subsequent neurodegeneration (Compston & Coles, 2008). The search for easily accessible, non-invasive, and cost-effective biomarkers for complex CNS conditions, including MS, have proved difficult to clinically validate. In MS, current clinically useful biomarkers require expensive equipment (e.g. MRI, Simoa™) and/or CSF analyses obtained from an invasive lumbar puncture (Paul et al., 2019). Despite decades of investigation, an easily detectable blood-based biomarker for MS has yet to be fully adopted by the field; blood-derived EVs have the potential to bridge this gap.

Unique immune cell-derived EV signatures (and their associated cargo) may provide valuable insights into the phenotype and activity/function of immune cells and help to further elucidate how they are contributing to ongoing pathological processes.

In MS, early studies focused on the secretion of EVs from endothelial cells and platelets due to their relatively high abundance within human blood. These studies demonstrated significantly increased levels of total EV particles (including exosomes, microparticles, and apoptotic bodies), particularly in clinically active cases (Minagar et al., 2001; Jy et al., 2004; Sheremata et al., 2006; Lowery-Nordberg et al., 2011; Marcos-Ramiro et al., 2014; Sáenz-Cuesta et al., 2014; Alexander et al., 2015). In more recent years, a focus has shifted to investigate particles secreted from major effectors of MS pathology, including T cells, B cells, and monocytes (Sáenz-Cuesta et al., 2014; Zinger et al., 2016; Groen et al., 2020). When investigating these specific populations, the published literature has been inconsistent due to small sample sizes, and differences in staining and flow cytometry parameters, assays, and instruments.

The objectives of this study were to provide a comprehensive evaluation of circulating EVs secreted from cells that are of interest to MS pathophysiology, namely T cells, monocytes, and B cells, and compare them to healthy controls. We also aimed to determine whether any changes observed in EV populations were related to any overall changes in circulating lymphocyte populations within an individual.

3.2 Materials and Methods

3.2.1 Human Sample Collection and Preparation

Protocols and experiments involving human participants were approved by the Newfoundland Health Research Ethics Board. Written informed consent was obtained from all participants prior to study initiation. MS patients were diagnosed according to 2017 McDonald criteria and recruited through the Health Research Innovation Team in Multiple Sclerosis (HITMS), an MS patient registry and biorepository at Memorial University of Newfoundland, St. John's NL, Canada between February 2016, and January 2021 (Thompson et al., 2018). Patient plasma samples were chosen based on sample availability; the only exclusion criteria considered was use of DMT (including steroids) within 12 months of sample collection. This study consisted of 33 disease modifying treatment (DMT) free relapse remitting MS (RRMS) patients and 22 age- and sex-matched healthy controls (see Table 3.1). All healthy control samples used in this study were obtained from healthy donors with no prior history of systemic diseases or administration of any immunosuppressing or immunomodulatory drugs. In the MS cohort, 20 patients had no previous history of DMT use, 6 had previously been on interferon beta-1a and discontinued 6-12 years prior to sample collection, 3 had previously been on dimethyl fumarate and discontinued 1-5 years prior to sample collection, 2 had previously been on teriflunomide and discontinued 1-3 years prior to sample collection, and 2 had previously been on glatiramer acetate and discontinued 4-6 years prior to sample collection.

Venous blood was drawn into BD Vacutainer® K2 EDTA tubes and plasma was obtained following 300xg centrifugation for 10 minutes. Plasma was aliquoted, immediately frozen at -80℃ for ~24 hours, and then transferred to liquid nitrogen for long-term storage. All samples were processed within 6 hours of collection. Blood was also drawn to obtain PBMCs using Ficoll density gradient centrifugation (ThermoFisher) and SepMate™ Tubes (StemCell Technologies) as per manufacturer's instructions. Once isolated, PBMCs were cryopreserved and stored in liquid nitrogen. Prior to experimentation, cells were thawed quickly and immediately fixed and stained for flow cytometry.

Table 3.1: Patient Clinical and Demographic Characteristics.

	RRMS (n=33)	**Control (n=22)**
Age (years; mean±SD)	47.4±9.93	47.2±11.7
Sex (#(%))		
Female	26(78.8)	17(77.3)
Male	7(21.2)	8(22.7)
EDSS		
Range	0-4	
Median (IQR)	1.5(2)	
Disease Duration (years; mean±SD)	14.5±9.49	

3.2.2 Nanotracking Analysis

To obtain particle concentration within plasma samples, nanotracking analysis (NTA) was performed. Plasma samples were diluted 1:1000 in 0.1μm filtered PBS and analysed by NanoSight NS3000 (Malvern Panalytical, Malvern UK) equipped with a sCMOS camera and 488nm laser using NanoSight NTA software version 3.4. Hardware and analysis settings were as follows: Laser type: Blue488, Camera Level: 13, Camera gain: 3, Slider Shutter: 1232, Slider Gain: 219, Frame Rate: 25.0 FPS, Temperature: 25.0℃, Detect Threshold: 4, Blur size: auto, Max Jump Distance: auto.

3.2.3 Plasma Staining Protocol

Cryopreserved plasma samples were thawed to room temperature on the benchtop and centrifuged for 20 minutes at 2,000xg prior to experimentation. Based on particle concentration measurements obtained by NTA, the volume of sample stained represented ~$1x10^9$ particles in a final volume of 5μL with 0.1μM filtered PBS. Prior to staining, all antibodies were centrifuged at 12,500xg for 10 minutes to remove any antibody aggregates. Optimal antibody concentration was determined for each antibody based on previously published protocols and internal optimization studies (Supplementary Figure 3.1). Concentrations used ranged from 12.5-50ng antibody per ~10^9 particles (Supplementary Table 3.1). Samples were stained for 1 hour at room temperature. To establish optimal gating parameters, negative staining controls consisting of equal volumes of 0.1μm filtered PBS and plasma samples were also stained (Supplementary Figure 3.2), and staining specificity was confirmed by treating stained samples with 1x

Triton-X 100 to lyse EVs (Supplementary Figure 3.3, (Welsh et al., 2020). Unless otherwise specified, prior to data collection, all samples were diluted to a final concentration of $1x10^8$ particles/mL in 400µL diluted in 0.1µm filtered PBS.

3.2.4 EV Flow Cytometry

All experiments were designed following relevant criteria, based on instrument choice and study/analysis design according to the MIFlowCyt-EV framework (Supplementary Table 3.2, (Welsh et al., 2020).

EVs in stained plasma samples were quantified by flow cytometry using a CytoFLEX (Beckman Coulter) configured to use Violet (405nm) side scatter (SCC) as the trigger parameter, with the threshold set at 800, as described previously (Brittain et al., 2019). These settings were tested using a cocktail of size-calibrated beads (ApogeeMix for Flow Cytometer Performance Assessment (Apogee) and recorded for 30 seconds. The V-SSC detector gain was set at 100 and phycoerythin (PE) fluorescence was measured using a 585/42nm bandpass filter with a detector gain set at 340; settings were chosen based on general guidelines for threshold and gain settings and on balancing between minimizing noise and using 0.1µm filtered PBS as a background control (Brittain et al., 2019). PE was chosen as the fluorophore for all markers based on its size, brightness, and ability to separate cleanly from background (Tang et al., 2019). Samples were run for 10 minutes at the slow flow rate (10µL/min). PE positivity was determined by setting gates based on the unstained plasma sample, and the plasma samples stained with anti-CD9-PE

and anti-CD45-PE (Supplementary Figure 3.4). The EV gate was set independently for each plasma sample and remained constant for the analysis of all cell markers.

3.2.5 PBMC Staining and Flow Cytometry

PBMC flow cytometry was performed on a majority subset of the cohort based on sample availability. PBMCs (200,000 cells) were stained with 1µL LIVE/DEAD™ fixable violet stain (Invitrogen/Thermo) in 1mL PBS and incubated for 30 minutes at 4ºC in the dark. The cells were washed with 4mL PBS containing of 1% bovine serum albumin, 2mM EDTA, and 2mM sodium azide. Cells were decanted and resuspended in 100µL and added to a DURAclone IM Phenotyping BASIC tube (Beckman Coulter), mixed and incubated at 4ºC for 30 minutes. The cells were washed and fixed with 100µL 2% paraformaldehyde. Data was acquired using the CytoFLEX (Beckman Coulter).

3.2.6 Data Analysis

Flow cytometry data were analyzed using FlowJo™ v10.0 software (FlowJo, LLC, Ashland, OR). All statistical analyses were performed using Prism 9 (GraphPad Sofware Inc. San Diego, CA). No differences in overall $CD45^+$ counts were observed between RRMS and healthy controls when normalized using CD9, therefore counts for lymphocyte subpopulations in both PBMC and EV analyses were normalized to $CD45^+$ counts within each individual sample. For EV analyses, $CD45^+$ and $CD61^+$ counts were normalized to $CD9^+$ counts. Outliers were identified using the robust regression and outlier removal (ROUT) method (Motulsky & Brown, 2006). Participants were deemed outliers and removed from all analyses if they were identified as such in measurements

from two or more cellular markers; one individual was excluded from each group. Final cohorts consisted of 32 RRMS and 21 healthy controls. Data were analyzed by using a Mann-Whitney test. Correlations between circulating EVs, PBMC populations, and clinical data were assessed using the Spearman correlation test. For all analyses, data are presented as mean±SEM, unless otherwise noted, and α set to 0.05.

3.3 Results

3.3.1 Quantification of plasma-derived extracellular vesicles by flow cytometry.

NTA of healthy control human plasma revealed a size distribution with a single peak of 62.5nm (Figure 3.1a). While using flow cytometry calibrated for EV detection as previously described, individual populations of size calibrated beads ranging from 80-1,500nm are clearly visible (Figure 3.1b). Plasma samples were then stained with the EV markers CD9 and CD63 (Théry et al., 2018). Detection of $CD9^+$ and $CD63^+$ EVs are shown in Figure 3.1c, using both unstained plasma and antibody diluted in 0.1μm filtered PBS as background controls. Positive staining of CD9 on EVs in plasma was stronger and more consistent than CD63 positivity, therefore CD9 was chosen as the general EV marker for the duration of the study. To determine optimal particle concentration for the purpose of separating background from positive staining, a dilution series was conducted from $1.25x10^8$-$3.13x10^7$p/mL (Figure 3.1d). Based on the results of the dilution series, $1.0x10^8$ was used as the final dilution factor for data collection.

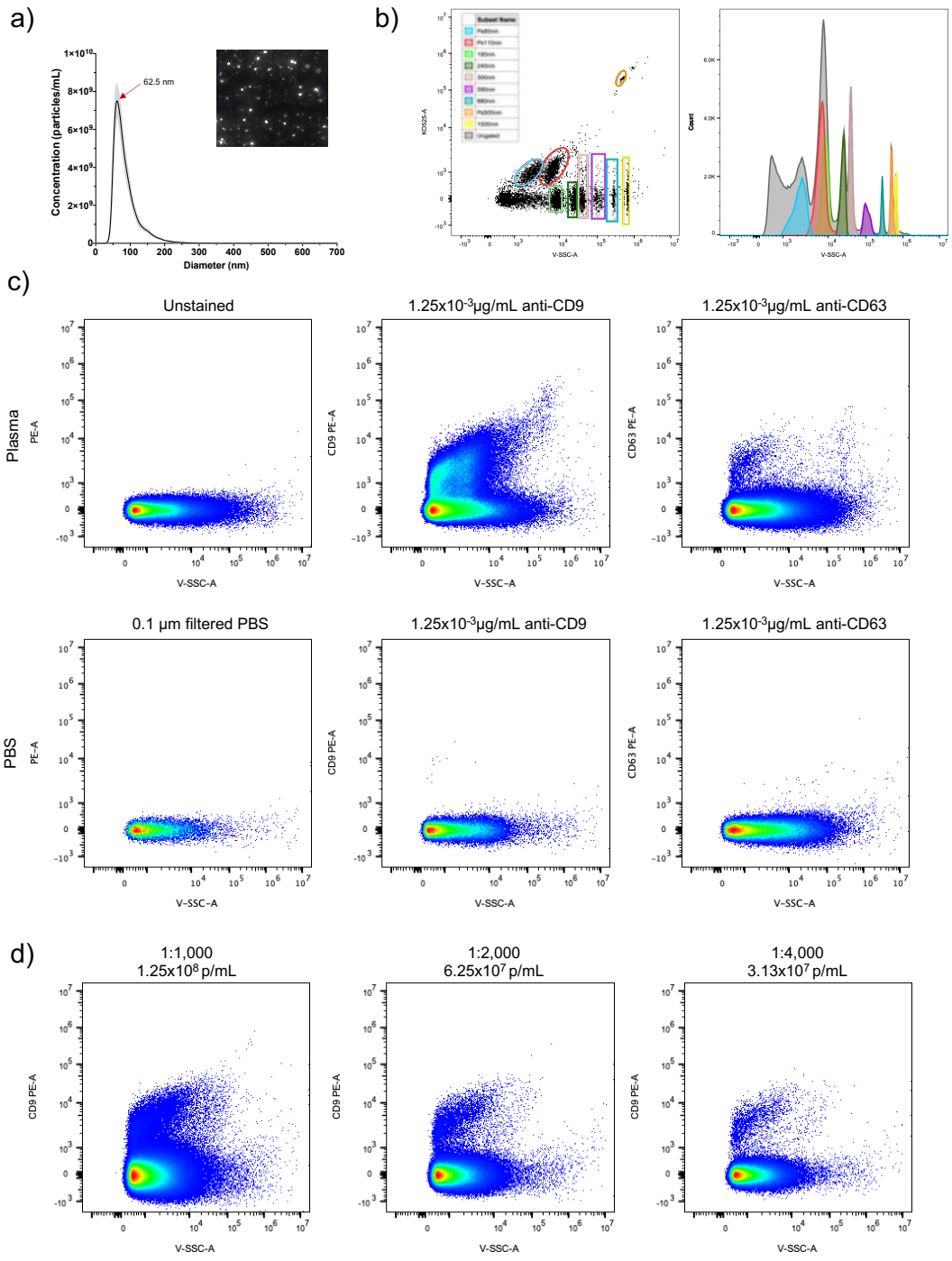
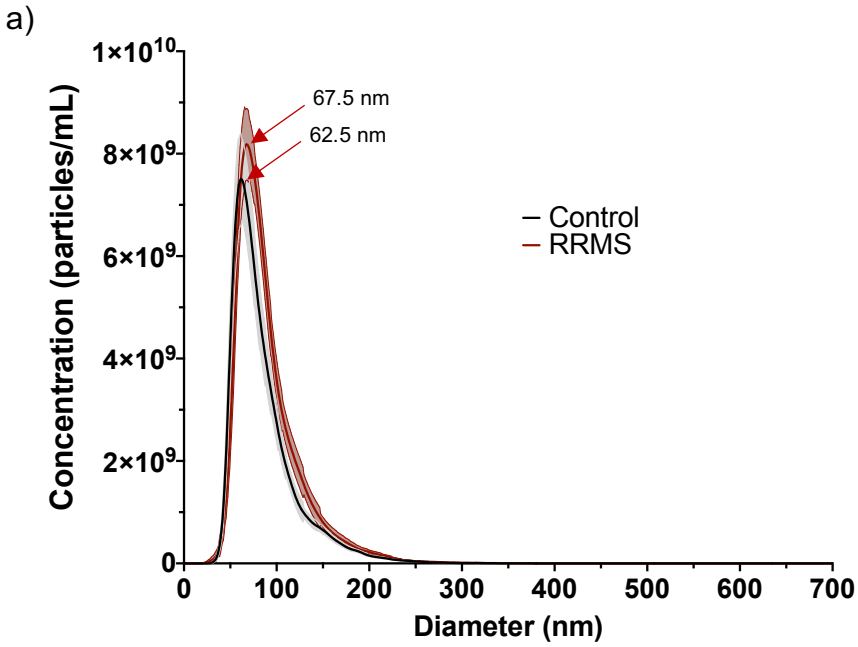

a)
b)
c)
d)
Concentration (particles/mL)
Diameter (nm)
62.5 nm
V-SSC-A
Count
Unstained
1.25x10-3µg/mL anti-CD9
1.25x10-3µg/mL anti-CD63
Plasma
PE-A
CD9 PE-A
CD63 PE-A
0.1 µm filtered PBS
PBS
1:1,000
1.25x10^8 p/mL
1:2,000
6.25x10^7 p/mL
1:4,000
3.13x10^7 p/mL

Figure 3.1: EVs in plasma are detectable by flow cytometry. a) NTA particle size distribution with representative video screenshot (inset) of control human plasma. b) Detection of size calibrated beads (Apogee) using violet side scatter (V-SSC-H) as the threshold parameter. Three of the bead populations were fluorescently labelled with the KO525 fluorophore and separated based on size (along the X axis) and fluorescence (along the Y axis). Histogram (right) reveals separation of all size beads based on V-SSC. c) Positive staining of EVs labelled with antibodies against the EV markers CD9 and CD63, using antibodies diluted in 0.1μm filtered PBS as negative controls. d) Plasma dilution series showing separation of PE positivity from background. Final antibody concentration was 0.019, $9.38x10^{-3}$ and $4.69x10^{-3}$ μg/mL for 1:1000, 1:2000 and 1:4000 dilutions.

3.3.2 Increased leukocyte derived EVs are observed in RRMS patient plasma compared to healthy controls

Plasma from healthy control and RRMS patients was analyzed by NTA and displayed similar size distribution curves (Figure 3.2a). Plasma particle concentration measured in healthy control samples was $3.98x10^{11}\pm3.66x10^{10}$ p/mL and did not differ from RRMS samples, which were measured at $4.56x10^{11}\pm3.65x10^{10}$ p/mL (Figure 3.2b; p=0.28). Plasma particle concentration was not associated with age (Pearson's r=0.226, p=0.326) or sex (p=0.107; data not shown)

When comparing sub-populations of immune cell-derived EVs between healthy control and RRMS samples, we observed differences in all lymphocyte-derived populations investigated. Figures 3.3a displays representative flow plots of all markers analyzed in healthy control and RRMS samples. Significant increases were observed in RRMS compared to healthy control in the following populations (RRMS vs Healthy Control, Figure 3.3): $CD3^+$ (0.139±0.013 vs 0.093±0.017; p=0.036), $CD4^+$ (0.042±0.005 vs 0.022±0.003; p=0.002), $CD8^+$ (0.120±0.009 vs 0.079±0.008; p=0.002), $CD14^+$ (0.177±0.019 vs 0.114±0.021; p=0.013), and $CD19^+$ (0.147±0.015 vs 0.085±0.011; p=0.002). No difference was observed between the CD4/CD8 ratio between RRMS and healthy control patients (Figure 3.3g; 0.309±0.022 vs 0.267±0.023, p=0.264). We also investigated ratios of platelet-derived EVs ($CD61^+$) and all leukocyte-derived EVs ($CD45^+$) normalized to $CD9^+$ counts; no significant differences between RRMS and healthy control samples were observed for either $CD61^+$ (Figure 3.3h, 1.099±0.085 vs 1.705±0.542, p=0.301) or $CD45^+$ (Figure 3.3i; 0.085±0.018 vs 0.086±0.019, p=0.230).



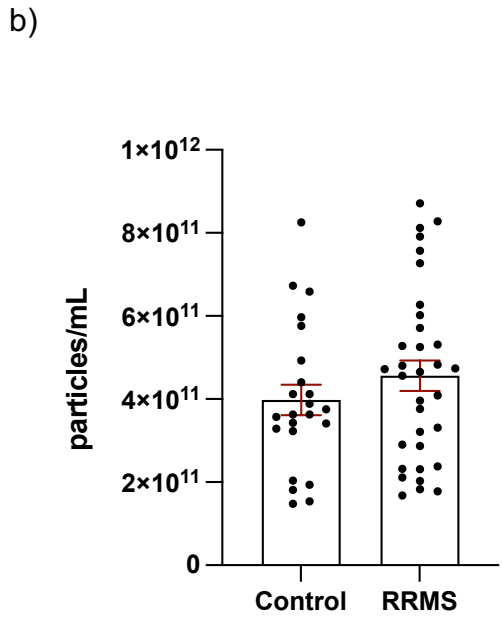


Figure 3.2: Comparison of EVs in control and RRMS plasma. a) NTA size distribution of control and RRMS plasma. b) Comparison of EV concentration measured by NTA between control and RRMS plasma samples.

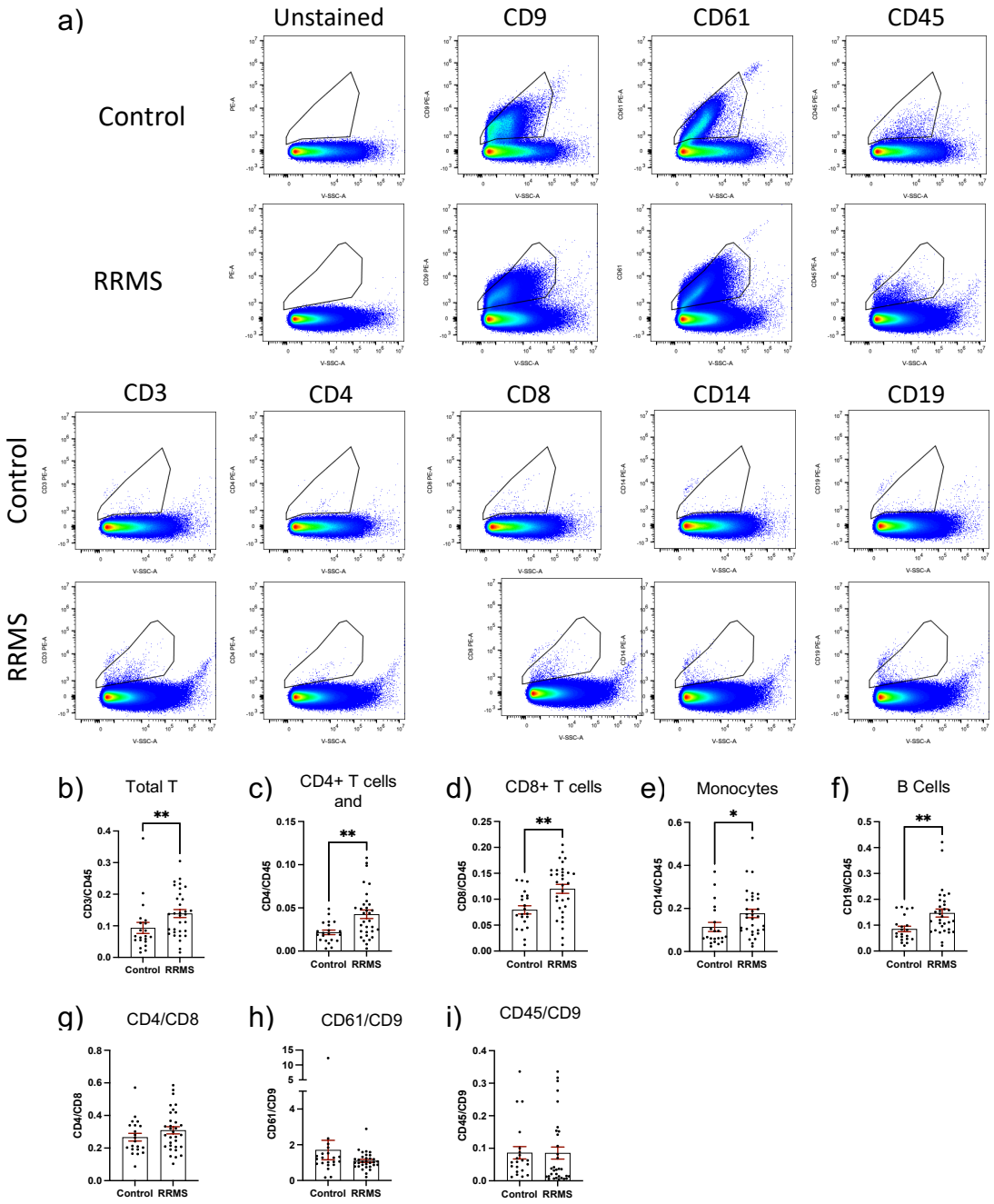


Figure 3.3: Comparison between RRMS and Control plasma reveals increased lymphocyte derived EVs. a) Representative flow cytometry plots displaying PE+ staining in plasma from control and RRMS cases. b-i) Analysis of lymphocyte subsets normalized to CD45+ counts reveals significantly higher amounts of CD3+, CD4+, CD8+, CD14+ and CD19+ populations in RRMS compared to healthy controls. *p<0.05 and **p<0.01.

3.3.3 Levels of lymphocyte derived EVs in circulation do not correlate with clinical and demographic variables in MS.

Levels of circulating EVs displaying CD3, CD4, CD8, CD14 or CD19 were analyzed to identify correlations with age, EDSS, and disease duration in MS cases (Table 3.2). The only significant correlations observed were between $CD14^+$ EVs and age (Spearman r=-0.391; p=0.03), and $CD14^+$ EVs and disease duration (Spearman r=-0.362; p=0.045). However, age and disease duration were significantly correlated with one another (data not shown; Pearson's r= 0.694; p<0.001). No correlations were observed between EV populations and age in the healthy control group (data not shown).

Table 3.2: Spearman correlation coefficients of lymphocyte EV populations with clinical and demographic variables in MS patients. * Indicates significance at $\alpha \leq 0.05$

		Age	EDSS	Disease Duration
CD3/CD45	**r**	-0.182	-0.103	-0.105
	p-value	0.325	0.588	0.575
CD4/CD45	**r**	-0.107	-0.112	0.039
	p-value	0.568	0.548	0.834
CD8/CD45	**r**	-0.107	-0.134	-0.015
	p-value	0.566	0.48	0.938
CD14/CD45	**r**	-0.391	-0.186	-0.362
	p-value	0.03*	0.324	0.045*
CD19/CD45	**r**	-0.265	-0.165	-0.34
	p-value	0.15	0.383	0.061

3.3.4 Immunophenotype of circulating leukocytes are not correlated with circulating EV subpopulations

Circulating leukocytes from cryopreserved whole PBMC populations were quantified from a majority subset of the individuals included in the EV analysis (11 controls and 23 RRMS, selected based on sample availability). Populations of $CD45^+$, $CD3^+$, $CD4^+$, $CD8^+$, $CD14^+$, and $CD19^+$ cells were quantified by flow cytometry and displayed as $\%CD45^+$. Representative flow plots and gating strategies for PBMC quantification are displayed in Figure 3.4a. In whole PBMCs, no significant differences were observed between RRMS and healthy control cases for $CD3^+$, $CD4^+$, $CD8^+$, or $CD14^+$ cells (Figure 3.4b-f). A significant increase in $CD19^+$ cells (Figure 3.4f; $p=0.001$) was observed in RRMS patients compared to healthy controls.

Quantification of circulating lymphocytes were plotted against EVs displaying the same cellular markers, to determine whether increased levels of circulating EVs from specific lymphocyte populations were correlated with circulating levels of the parent cells. No significant correlations were observed between cells and EVs displaying any markers investigated in this study (Figure 3.5). We also investigated whether a correlation existed when only RRMS cases were considered; no significant correlations were observed (data not shown).

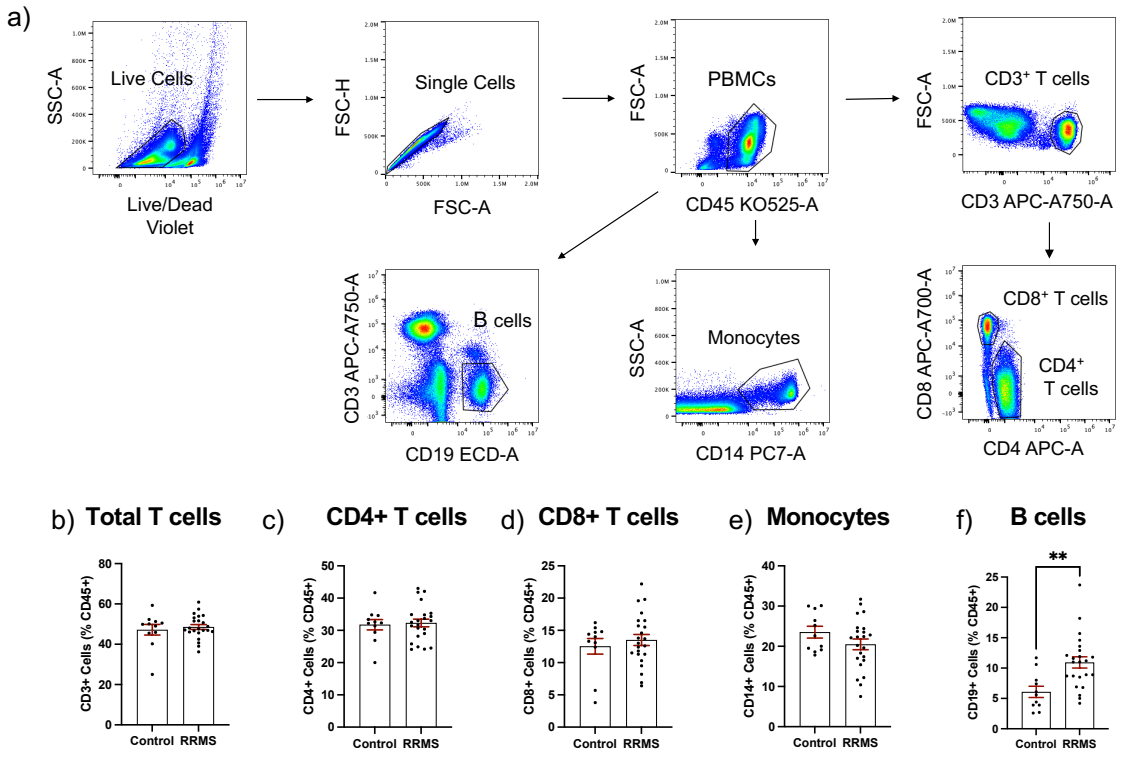


Figure 3.4. Circulating PBMC immunophenotypes in RRMS and controls a) Representative flow cytometry plots display the PBMC gating strategy used to quantify levels of circulating lymphocyte populations. No significant differences in circulating b) total T cells, c) CD4+ T cells and monocytes, d) CD8+ T cells or e) monocytes were observed between RRMS and control. f) a significantly increased number of $CD19^{+}$ cells were observed in RRMS patients compared to controls **$p<0.01$.

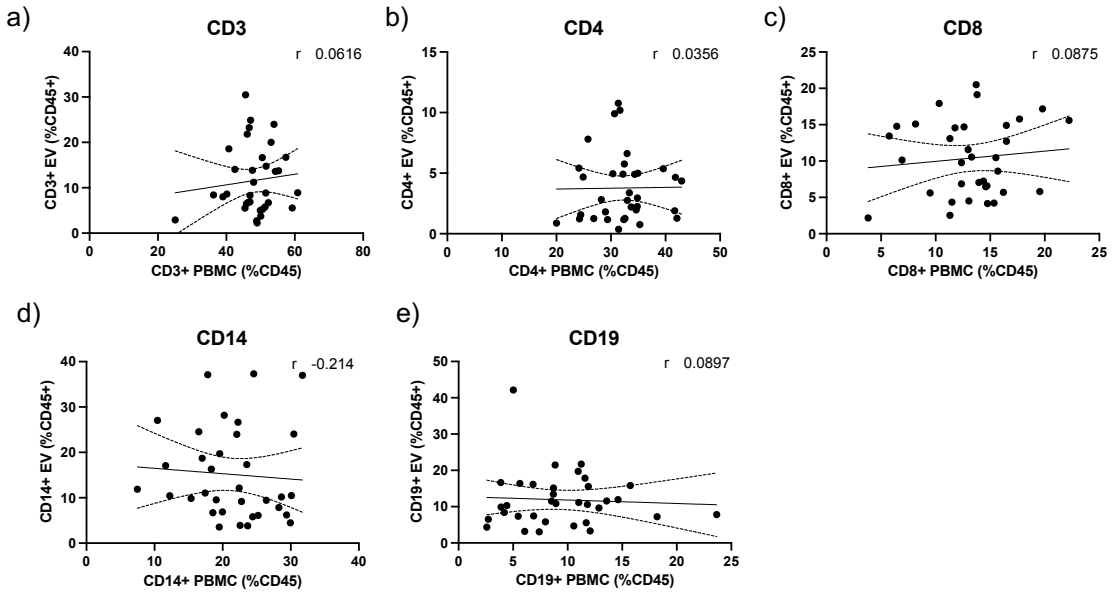


Figure 3.5. Circulating PBMC immunophenotype does not correlate with circulating lymphocyte-derived EVs Plasma levels of EVs derived from a) total T cells, b) CD4+ T cells and monocytes, c) CD8+ T cells, d) monocytes and e) B cells are not correlated with circulating levels of circulating parent lymphocyte populations

3.4 Discussion

The objective of this study was to evaluate circulating EVs secreted from MS-relevant lymphocyte populations, including T cells, B cells, and monocytes, and to determine whether these populations differed from healthy controls. Herein, we document that despite only limited changes to levels of circulating lymphocytes between RRMS and healthy control cases, significant increases in EV populations secreted from major lymphocyte populations were measured in treatment-free RRMS patients with low disability.

Flow cytometry has long been used as a method of quantifying and phenotyping cell populations. In addition to cells, larger EVs have also been quantified using this technique in many body fluids, including plasma and CSF (Verderio et al., 2012; Sáenz-Cuesta et al., 2014; Zinger et al., 2016; Groen et al., 2020; Gelibter et al., 2021). However, the full size range of EVs (30-1,000nm) extends within the level of background noise of conventional cytometers, creating a challenge of resolving a true range of positive signals (Welsh et al., 2017; Marostica et al., 2020). The CytoFLEX instrument was specifically designed to mitigate this challenge by incorporating innovative optics, which results in low background and high signal (Brittain et al., 2019). In addition, the 405nm laser for light scatter detection results in higher scatter from smaller particles further increasing signal (Brittain et al., 2019). Using this instrument, we were able to resolve polystyrene beads down to 80nm in diameter (Figure 3.1b). We also successfully resolved EVs labelled with the well-characterized EV markers CD9 and CD63 (Figure 3.1c), and cell-specific markers of several major leukocyte populations (Figure 3.3a).

When analyzing EV populations in patient and healthy control cases, we observed no overall differences between total particle size and concentration as measured by NTA (Figure 3.2). Despite this, we did observe a significant increase in EVs displaying immune cell-specific markers of T cells (both cytotoxic and helper), monocytes, and B cells (Figure 3.3c-g). No differences in the $CD4^+/CD8^+$ ratios between RRMS and healthy control cases (Figure 3.3h), nor in the amount of leukocyte-derived ($CD45^+$) or platelet-derived ($CD61^+$) EVs when normalized to the general EV marker CD9 (Figure 3.3i-j). Three previous studies have measured levels of EVs derived from various leukocyte populations within the plasma of RRMS patients, each using a different instrument. An early investigation demonstrated that levels of plasma microparticles (MP) displaying CD45 or CD14 were not different between untreated RRMS and healthy controls (Sáenz-Cuesta et al., 2014). Similar results were shown in two additional studies, which also observed no change in $CD14^+$ MPs (Zinger et al., 2016; Groen et al., 2020). One of these studies reported no change in T-cell derived particles ($CD3^+$, (Zinger et al., 2016), which is supported by a more recent study that investigated subpopulations of T cells whereby no differences in $CD4^+$ or $CD8^+$ particles were measured between RRMS and control cases (Groen et al., 2020).

In contrast to these studies, we observed significant increases in EVs from all leukocyte populations investigated, namely T cells, B cells, and monocytes. Similarly to our patient cohort, the aforementioned studies consisted of modest group sizes with low disability. Our RRMS cohort was larger by 9-17 individuals, depending on the study considered, which may have played a role in the discrepancies observed. Failure to detect these changes in the previous studies may be due, in part, due to the instrumentation. For

example, the most recent study, performed by Groen and colleagues, specify their instrument was capable of differentiating particles >200nm in diameter (Groen et al., 2020), which is likely to omit data from smaller EVs. In the current study, the instrument used to collect data uses novel technologies to optimize small particle detection (Brittain et al., 2019). Our NTA and flow cytometry data, which is consistent with others using the CytoFLEX, demonstrates that smaller diameter EVs (<100nm) are present in bodily fluids and can be readily phenotyped. The CytoFLEX can resolve beads of known diameters <100nm suggesting that we are also able to detect EVs smaller than 100nm (Figure 3.1b, (Brittain et al., 2019). Based on theoretical calculations, Brittain and colleagues (2019) surmise the CytoFLEX can detect EVs down to 12nm in diameter, and that EVs in the 30nm diameter range cluster around the 60nm polystyrene bead. Since the earlier studies were unable to resolve the smaller vesicle populations, it could be this population that is driving the differences we observe in particles derived from leukocyte populations.

An important finding of the current study is that despite observing no changes in levels of circulating T cells or monocytes between healthy control and RRMS cases (Figure 3.4), we did see a significant increase in circulating EVs bearing cell-specific markers for all these cell populations (Figure 3.3b-f). Our cellular phenotyping data is consistent with a previous report from a study with similar cohort size, which documented no major changes in the levels of circulating leukocyte populations in MS compared to healthy controls (Schafflick et al., 2020). The only exception being that we found a significant increase in circulating $CD19^+$ B cells in RRMS (Figure 3.4f). Recent evidence suggests that circulating levels of $CD19^+$ B cells in MS cases is heterogenous (Abbadessa et al., 2021). Therefore, the increase in B cells observed in the current study may be due

to unintentional random sampling of a population of MS cases skewed towards higher levels of B cells. Additional investigations into B cell populations in MS with larger cohorts are indeed needed before concluding whether differences in circulating B cells truly exist between RRMS and healthy control cases. In this study, we also noted a significant increase in $CD19^+$ B cell-derived EVs, however, despite also observing increased $CD19^+$ B cells, these levels were not correlated (Figure 3.5e), suggesting that the increase in $CD19^+$ EVs is not likely a product of increased levels of $CD19^+$ cells within circulation, but perhaps rather due to activation status.

Our results suggest that despite not observing differences in the levels of circulating immune cell subsets, EVs derived from these cells are elevated in RRMS compared to healthy controls. Previous studies have provided evidence suggesting that cells release more EVs under pro-inflammatory vs. control conditions and can transfer inflammatory signals via EVs to neighboring cells, and even cells distant in the body (Verderio et al., 2012; Dickens et al., 2017). While our results do not directly show that this propagation occurs, they allude to a possible mechanism whereby a cells' pro-inflammatory activities can propagate in RRMS cases via secretion of EVs. Further investigations into the functional relevance of these EV populations within the circulation in RRMS will provide interesting insights into whether specific EVs can drive pathological activity in these otherwise non-inflamed cases with low disability.

Diagnosing MS is complex and involves multiple invasive and expensive procedures often including magnetic resonance imaging (MRI), and a lumbar puncture (Paul et al., 2019). Additionally, with the diagnostic criteria requiring dissemination in both space and time, patients are often left undiagnosed for extended periods of time

(Thompson et al., 2018). The drive to identify more specific biomarkers for MS is active but remains unmet. The identification of blood-based biomarkers is of particular interest, as sample collection is simple, cost effective and non-invasive. Complex patterns of circulating EVs and their contents could prove valuable for identifying markers of complex conditions like MS (Marostica et al., 2020). Secreted EVs can cross the blood-brain-barrier (BBB) and can transfer inflammatory signals to recipient cells in the absence of additional inflammatory triggers or BBB breakdown (Verderio et al., 2012; Dickens et al., 2017). Therefore, immune-derived EVs from blood and/or CSF can also possibly inform on potential ongoing pathological processes in the CNS or the periphery in the absence of obvious changes to a patient disability or concrete demyelinating event. In fact, recent evidence suggests that levels of myeloid cell-derived EVs in the CSF may serve as predictive biomarkers for disease course and disability accrual in MS (Gelibter et al., 2021). Whether this extends to myeloid cell-derived EVs measured in the periphery has yet to be investigated and may represent an important metric in blood to consider.

Further investigation into this hypothesis using larger group sizes and cases with increased level of disability is, of course, required before the utility of leukocyte derived EVs as a biomarker for MS is fully understood. It will be important to determine whether these changes persist or change with relapse activity or with progression, and whether they are sensitive to specific DMT use. Our data suggest that levels of plasma EVs do not correlate with disability as measured by EDSS (Table 3.2), but the limited disability of our sample may have prevented identification of any true associations that may exist. This study was limited to using EDSS as a measure of disability, as we did not have access to sufficient MRI data. It will be critically important for future studies to determine whether

EV populations are associated with other more objective markers of disability and disease activity, including MRI and neurofilament light chain measures.

This study provides evidence that plasma levels of EV populations secreted from T cells, B cells, and monocytes are elevated in untreated RRMS cases with low disability, despite no change to inflammatory activity in these patients as measured by PBMC flow cytometry. These results suggest a possible future clinical utility of measuring circulating EV populations as a biomarker in MS. While the current study provides an important initial step in this direction, future studies investigating circulating EVs during the time of MS diagnoses, and longitudinally throughout the disease process are needed.

3.5 Supplementary Material

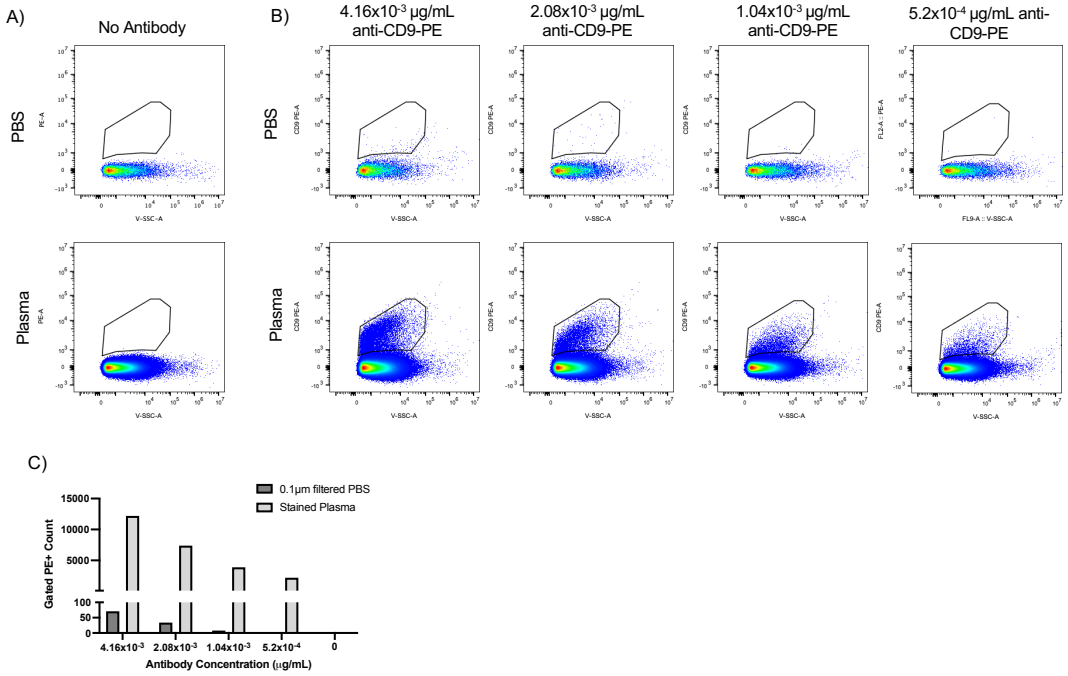
Supplementary Table 3.1: Antibodies used in phenotypic analysis of EVs. All antibodies were purchased from BD Biosciences, except for CD63-PE which was purchased from Beckman Coulter.

Antigen	Clone	Secreting Cell Type	Amount Used	Final Concentration (ug/mL)
Anti-CD9	M-L13	EVs	12.5ng	1.25×10^{-3}
Anti-CD63	CLBGran/12	EVs	12.5ng	1.25×10^{-3}
Anti-CD45	H130	Leukocytes	25ng	2.5×10^{-3}
Anti-CD61	VI-PL2	Platelets	25ng	2.5×10^{-3}
Anti-CD3	HIT3a	T cells	25ng	2.5×10^{-3}
Anti-CD4	RPA-T4	CD4+ T cells and macrophages	25ng	2.5×10^{-3}
Anti-CD8	HIT8a	CD8+ T cells	25ng	2.5×10^{-3}
Anti-CD14	M5E2	Monocytes	25ng	2.5×10^{-3}
Anti-CD19	HIB19	B cells	50ng	5.0×10^{-3}

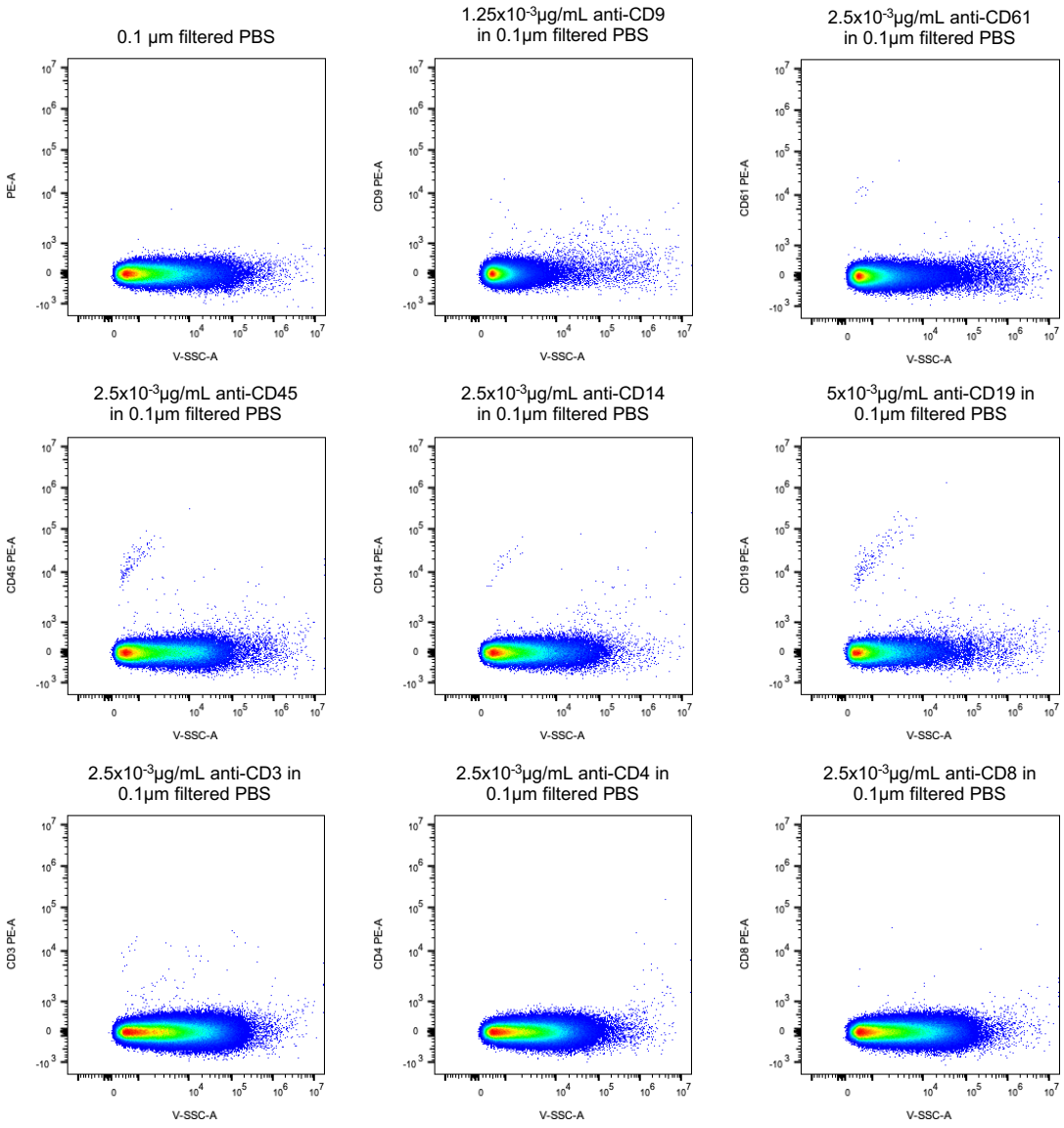
Supplementary Table 3.2: MIFlowCyt-EV framework checklist.

Framework Criteria	**Please complete each criterion**
1.1 Preanalytical variables conforming to MISEV guidelines.	Relevant preanalytical variables are detailed in the methods section of the manuscript.
1.2 Experimental design according to MIFlowCyt guidelines.	MIFlowCyt checklist items 1.1, 1.2, and 1.3 are available in the manuscript
2.1 Sample staining details	Sample staining procedure is recorded in the methods.
2.2 Sample washing details	Samples were not washed. Instead, unbound antibodies were diluted to a final concentration where they contributed minimally to positive fluorescence. See supplementary figure 1 for this process
2.3 Sample dilution details	Final dilutions and diluents used are recorded in the methods
3.1 Buffer alone controls.	Acquisition settings remained consistent throughout the duration of data collection. Buffer-only controls were ran and analysed once for each day of collection. All samples were recorded for 10 minutes.
3.2 Buffer with reagent controls.	Buffer with reagent controls were not ran and analysed with every sample, however, they were ran sporadically throughout the data collection phase, and always recorded and analysed using the same settings and concentration as test samples. Buffer with reagent controls resulted in negligible positive staining (supplementary figure 2 and figure 1).
3.3 Unstained controls.	unstained controls of each sample were recorded and analyzed at the same settings during the same experiment as stained samples.
3.4 Isotype controls.	Isotype controls were not used or analyzed.
3.5 Single-stained controls.	All experiments in this manuscript contained only single stains.
3.6 Procedural controls.	Buffer only and buffer with reagent controls were treated procedurally in an identical manner as test samples.
3.7 Serial dilutions.	Serial dilutions were conducted early on while determining optimal final particle concentration for analysis. Once 1x10^8 particles/mL was chosen as a final concentration serial dilutions were not performed as the concentration was standardized across individuals.
3.8. Detergent treated EV-samples	Triton-X was used as a detergent control; See supplementary figure 3.
4.1 Trigger Channel(s) and Threshold(s).	Relevant details are described in Materials and methods.

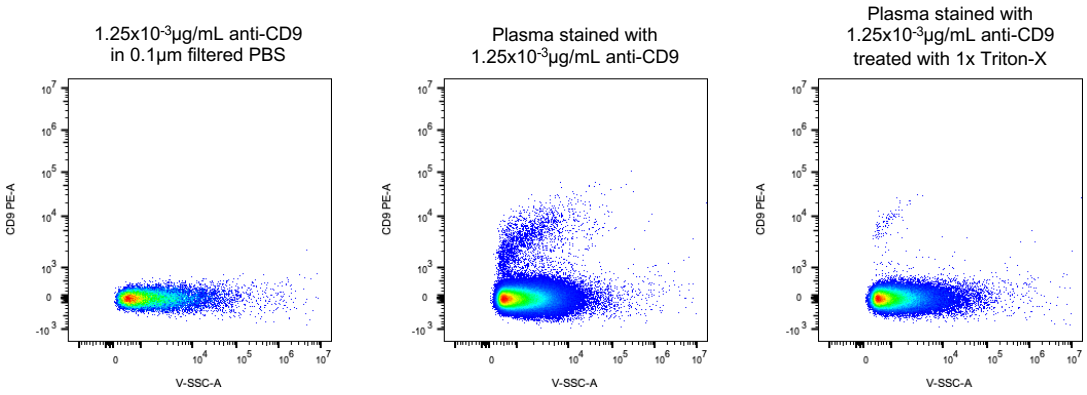
4.2 Flow Rate / Volumetric quantification.	Flow rate was set to 10μL/min (slow flow rate) using the CytEXPERT software. Calibration was not performed
4.3 Fluorescence Calibration.	Fluorescence calibration as not performed.
4.4 Light Scatter Calibration.	Light scatter calibration was not performed
5.1 EV diameter/surface area/volume approximation.	EV diameter, surface area or volume was not estimated using flow cytometry.
5.2 EV refractive index approximation.	EV refractive index was not approximated
5.3 EV epitope number approximation.	EV epitope was not approximated
6.1 Completion of MIFlowCyt checklist.	Details are available in the manuscript.
6.2 Calibrated channel detection range	Fluorescence or light scatter calibration was not performed
6.3 EV number/concentration.	EV number/concentration was not reported as a raw number, but instead normalized to the CD45+ count obtained from the same patient sample. Total particle concentration in each sample was obtained by nanotracking analysis.
6.4 EV brightness.	EV brightness was not evaluated
7.1. Sharing of data to a public repository.	The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.



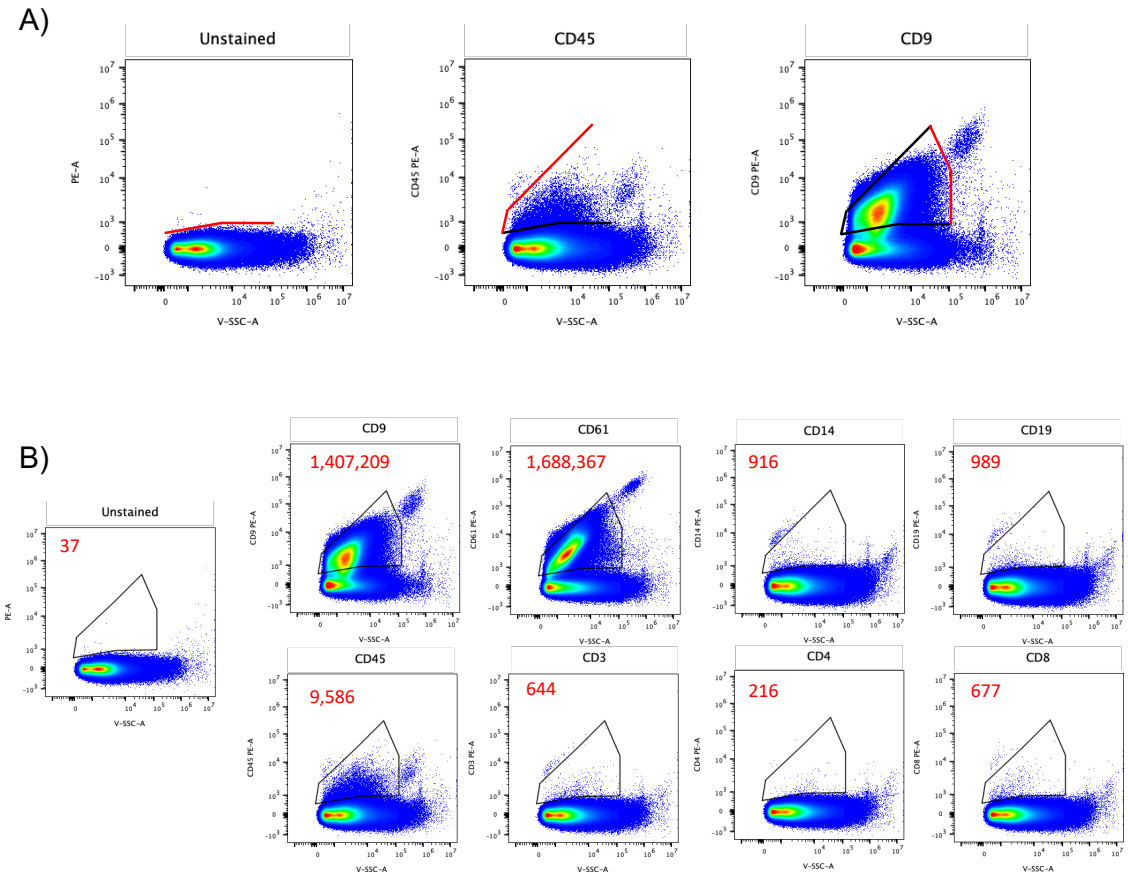
Supplementary Figure 3.1: Representative antibody titration series to determine optimal concentration. A) Background instrument noise was assessed with 0.1um filtered PBS and unstained plasma at a final concentration of $1x10^{8}$ particles/mL in 0.1um filtered PBS B) Representative antibody titration series for anti-CD9 PE. Each antibody was tested at a final concentration range of 4.16x10-3 to 5.2x10-4 ug/mL to assess background when diluted in PBS alone, and signal when staining plasma samples ($1x10^{8}$ particles/mL). C) Gated PE+ counts show a linear relationship between PE+ events in both stained and unstained samples. For this antibody, a final concentration of $1.25x10^{-3}$ ug/mL was chosen based on minimum noise generation and sufficient positive staining. This process was repeated for all antibodies used in this study. Plots displayed here represent 2-minute captures at a flow rate of 10uL/min.



Supplementary Figure 3.2: Antibody in 0.1um filtered PBS Negative Controls. Based on results of antibody optimization final concentrations of each antibody were diluted in 0.1um filtered PBS to confirm minimal contribution of unbound antibodies and antibody aggregates to background PE fluorescence. Plots displayed in this figure represent 10-minute captures at a flow rate of 10uL/min.



Supplementary Figure 3.3: Triton-X Negative Control. Representative series showing CD9-PE+ events (middle) are eliminated with treatment of plasma with 1x Triton-X (right).



Supplementary Figure 3.4: Gating Strategy. A) Stepwise addition of the gate beginning by setting the lower limit based on the unstained sample, followed by the left angled limit based on CD45+ antibody aggregates and the top/left limits based on CD9 stained sample. B) Application of the gate to a representative sample illustrating the total number of positive events counted. Plots here represent 10-minute captures at a flow rate of 10uL/min

Chapter 4 – Immunophenotyping human cerebrospinal fluid: CXCL10 and neurofilament light chain are associated with CSF immune cells with additional pathophysiological relevance in multiple sclerosis.

4.1 Introduction

Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease characterized by inflammatory insult to the central nervous system (CNS) that results in demyelination and subsequent neurodegeneration. In Canada, over 90,000 individuals are living with MS (MSIF, 2020). In the majority of clinical settings, a diagnosis of MS is made according to the McDonald Criteria, which necessitates the dissemination of CNS lesions in both space and time, and is often based on clinical assessment, magnetic resonance imaging, and immunoglobulin G (IgG) synthesis within the cerebrospinal fluid (CSF) (Thompson et al., 2018).

While CSF is important for diagnostic purposes in MS, it also represents an important sampling site for many potential pathologic molecules that may shed light on the disease process (Link & Tibbling, 1977). CSF is produced in the four ventricles by the choroid plexus (CP), a highly specialized tissue rich in vasculature (Ghersi-Egea et al., 2018). Once produced, CSF flows through the ventricular system and enters the brain parenchyma via paraarterial spaces where it mixes with interstitial fluid (ISF; (Iliff et al., 2012; Iliff et al., 2013; Ringstad et al., 2018). ISF returns to the CSF pool surrounding the brain by paravenous spaces, and CSF is reabsorbed into the bloodstream by arachnoid granulations in the subarachnoid space (Upton & Weller, 1985; Go et al., 1986; Iliff et al., 2012; Iliff et al., 2013; Raper et al., 2016). Critically, the flow of CSF, together with the continuous exchange of ISF, allows the exchange of macromolecules and proteins (e.g. antigens and cytokines) between the CSF and the brain parenchyma.

The CSF compartment represents an important immunological niche within the CNS (Croese et al., 2021). Peripherally born immune cells circulate in the CSF and

contribute to the homeostatic immunological surveillance of the CNS. Immune cells circulating within the CSF are primarily composed of T cells, specifically CD4+ T cells with an effector memory phenotype (de Graaf et al., 2011; Han et al., 2014). In addition, lesser numbers of B cells, natural killer (NK) cells, dendritic cells (DCs), and monocytes are also present (Han et al., 2014; Esaulova et al., 2020; Schafflick et al., 2020). In MS, compared to non-inflammatory neurological disease (NIND) controls, studies have documented a shift in the diversity of cell types present in the CSF, particularly in B cell populations, suggesting that the CSF may provide a rich source of potential biomarkers to be exploited to better understand and diagnose MS (Esaulova et al., 2020; Ramesh et al., 2020; Schafflick et al., 2020).

CSF has been previously investigated to identify the repertoire of cytokines and chemokines present in the CSF compartment in MS. Cytokines and chemokines are critical for the induction, maintenance, and resolution of inflammatory processes throughout the body, and it stands to reason that inflammatory conditions, like MS, would be reflected in increased pro- and decreased anti-inflammatory molecules. Profiling studies measuring levels of cytokines and chemokines in the CSF of MS patients have revealed significant increases in several cytokines, including CCL5, CCL7, CXCL10, and GM-CSF, compared to NIND controls (Khaibullin et al., 2017). Importantly, many of the cytokines upregulated in MS were related to Th1-mediated immune responses (Khaibullin et al., 2017). This concept is supported by earlier studies, which also documented a similar upregulation of Th1-related cytokines including IFNg, TNF, and IL-6 (Maimone et al., 1991; Rodríguez-Sáinz Mdel et al., 2002). However, additional studies contradict this notion leaving a lack of consensus in the literature (Hauser et al., 1990; Burman et al.,

2014; Kothur et al., 2016).

It is likely that the cytokine and chemokine profiles in CSF can be a rich source of potential biomarkers. However, exactly which marker (or combination of markers) is the most valuable remains of debate and depends on type of biomarker being considered (i.e., Diagnostic, prognostic, *etc.*). Significant efforts have recently focused on neurofilament light chain (NfL) as a marker for monitoring axonal injury during the disease process of MS (Benkert et al., 2022). However, elevations in CSF NfL are not unique to MS (significant increases are observed in almost every neurodegenerative condition evaluated) and is further compounded by NfL being significantly correlated with age in healthy populations (Yilmaz et al., 2017; Bridel et al., 2019). Nevertheless, NfL has remained an important measure in MS and has already been adopted as a secondary outcome measure in several clinical trials amongst the newer DMTs.

CXCL10 (formerly IP-10 (IFNg inducible protein 10)) is a chemokine induced in response to pro-inflammatory stimulation, particularly IFN-γ, TNF and LPS, and is involved in the chemoattraction of macrophages, monocytes and activated T and NK cells (Vazirinejad et al., 2014). In addition, CXCL10 is involved in the modulation of T cell development and function, promoting T cell adhesion to endothelial cells, and the induction of NK cell migration and cytolysis (Vazirinejad et al., 2014). CXCL10 binds to its receptor CXCR3 that is most commonly expressed on T cells and other immune cells (Vazirinejad et al., 2014). CXCR3 is a G-protein coupled receptor with three known human isoforms and an incompletely understood intracellular signaling pathway, that results in chemotaxis and other cellular processes (Vazirinejad et al., 2014). In MS, elevations in CXCL10 have been measured compared to controls (Balashov et al., 1999;

Simpson et al., 2000; Sorensen et al., 2001; Mahad et al., 2002; Scarpini et al., 2002; Roberts et al., 2015; Khaibullin et al., 2017). Indeed, it has been noted that increases in CSF CXCL10 were associated with numbers of cells circulating within the CSF as measured by hemocytometer (Sorensen et al., 2001). In addition, increased CSF CXCL10 appeared to be associated with higher disability (Khaibullin et al., 2017).

In the CNS, astrocytes are likely the major source of CXCL10 (Ransohoff et al., 1993). CXCR3 is expressed on CNS cells, including neurons, oligodendrocytes, and microglia (Xia et al., 2000; Biber et al., 2002; Nelson & Gruol, 2004; Rappert et al., 2004; Omari et al., 2005). In the CNS, CXCL10 is involved in the recruitment of microglia to sites of inflammation and impair oligodendrocyte progenitor cell differentiation (Rappert et al., 2004; Moore et al., 2015). Astrocytes also express CXCR3 and are critically responsible for maintaining glutamate homeostasis in the brain (Ransohoff et al., 1993; Rothstein et al., 1996). Little is known about the autocrine effects of astrocyte CXCL10 signaling, however, signalling through CXCR3 can induce changes in the activation of NF-κB, a transcription factor known to regulate glutamate transporter expression in astrocytes (Ghosh et al., 2011; Chung & Liao, 2016). Glutamate is the major excitatory neurotransmitter in the brain. Glutamate released from presynaptic cells interacts with receptors on postsynaptic cells propagating the neural signal. Excess glutamate in the synaptic cleft is transported into surrounding astrocytes by glutamate transporters to be recycled for future use (Danbolt, 2001). If these astrocytic glutamate transporters become faulty, glutamate can build up in and around the synapse and continue to interact with the receptors. This leads to overactivation of the postsynaptic cell, which, if left unrectified, can lead to neurodegeneration in a process known as

excitotoxicity (Rothstein et al., 1996; Wang & Qin, 2010).

While CXCL10 and NfL have long been recognized as being dysregulated in CNS inflammation and MS, a comprehensive evaluation of their associative levels and their effects on cellular infiltration into the CNS compartment have yet to be investigated. Herein, we demonstrate an increase in CSF CXCL10 in MS compared to NIND controls. We also demonstrate that CXCL10 is associated with increased T cells within the CSF compartment, but not with central or peripheral expression of its receptor CXCR3. While NfL was not observed to be different between RRMS and controls, CSF NfL was significantly associated with CD8+ T cells, and NK cells in the CSF. In addition, we show that CSF from MS cases contains factors that cause phenotypic changes in astrocytes that can be recapitulated using recombinant CXCL10 alone.

4.2 Materials and Methods

4.2.1 Patient Cohorts and Demographic Information

All studies using human samples followed Canadian Institutes of Health Research guidelines, and institutional review board approval at Memorial University of Newfoundland (Health Research Ethics Authority). Cerebrospinal fluid (CSF) samples were obtained by lumbar puncture (LP) from patients recruited from the neurology clinic at the (St. John's, NL) or Horizon Health (Saint John, NB). For CSF analyses, all patients were DMT-naïve at time of collection; 39 patients were treated with steroids at time of clinical relapse (1-166 months prior to LP). For plasma analyses, RRMS patients were recruited through the Health Research Innovation Team in Multiple Sclerosis (HITMS), an MS patient registry and biorepository at Memorial University of Newfoundland, St. John's NL, Canada, or Neurology clinic at Memorial University of Newfoundland, St. John's NL. All clinical parameters were determined based on neurological assessment by a MS neurologist. For all cohorts, to demonstrate real-world relevance, all RRMS patients were eligible for inclusion in the study. Patients were only excluded based on inadequate sample availability, or missing demographic or clinical variables. Relevant basic clinical and demographic information are presented in Table 4.1.

Table 4.1. Human body fluid patient cohorts and demographic information.

1. CSF Cohort for Bioplex (n=57)	
Age (years; mean±SD)	43.0±15.6
Sex (%)	
Males	24.6%
Females	75.4%
Diagnosis at CSF Draw (%)	
RRMS	66.6%
NIND	33.3%
Last Relapse (MS only, months; mean±SD)	13.7±19.9
2. CSF Cohort for ELISA (n=84)	
Age (years; mean±SD)	44.2±13.7
Sex (%)	
Males	24.2%
Females	75.8%
Diagnosis at CSF Draw (%)	
RRMS	73.8%
NIND	26.20%
Last Relapse (MS only, months; mean±SD)	19.2 ± 30.8
3. Plasma Cohort (n=116)	
Age (years; mean±SD)	44.1 ± 10.2
Sex (%)	
Males	25.8%
Females	74.1%
Diagnosis at Blood Draw (%)	
RRMS	100%
Last Relapse (months; mean±SD)	60.0±58.9
Disease Duration (years; mean±SD)	11.6±7.36
DMT use (%)	
None	16.4%
GA	17.2%
Natalizumab	3.45%
Fingolimod	11.2%
DMF	29.3%
Teriflunomide	12.9%
IFNβ-1a	9.48%

4.2.2 Preparation of Human CSF samples

CSF was drawn by lumbar puncture, and 2.5mL was immediately separated from the tube and centrifuged to pellet cells to be stained for flow cytometry. The remaining sample was also centrifuged (300xg) to pellet cells. CSF was aliquoted, frozen at -80℃, and transferred to liquid nitrogen. Pelleted cells not used for flow cytometry were lysed in QIAzol® (Qiagen) and stored at -80 for subsequent RNA isolation and qPCR analysis.

4.2.3 Preparation of Human Blood Samples.

Human peripheral blood mononuclear cells (PBMCs) were isolated using a protocol as described previously (Moore et al., 2013). All studies using human samples followed Canadian Institutes of Health Research guidelines and institutional review board approval at Memorial University of Newfoundland (Health Research Ethics Authority). Briefly, peripheral blood was collected from consenting individuals into EDTA-coated tubes (BD Vacutainer®). Peripheral blood mononuclear cells were isolated from whole blood using Ficoll density gradient centrifugation (Thermo Fisher) and SepMate™ Tubes (StemCell Technologies). Once isolated, PBMCs were cryopreserved and stored in liquid nitrogen. Prior to experimentation, cells were thawed quickly and immediately fixed and stained for flow cytometry.

4.2.4 Cytokine and Neurofilament Light Measurements

All assays were performed according to manufacturer's instructions. CSF levels 27 cytokines, including CXCL10, were measured by Bio-Plex Pro™ Human Cytokine

27-plex Assay and analyzed on a Bio-Plex® 200 system. Cytokine measurements in CSF and plasma and in cell culture supernatants were obtained for human IL-6 and CXCL10 (R&D Systems) by ELISAs. CSF levels of NfL were measured by ELISA (Uman Diagnostics), according to manufacturer's instructions. Measurements below the detection limit of the assay were excluded from analysis unless otherwise specified.

4.2.5 CSF Cell Flow Cytometry

CSF cells were quantified by flow cytometry in a subset of patients characterised in Table 4.2. Cells from 2.5mL CSF from each sample were pelleted by centrifugation (300xg). CSF supernatants were aliquoted and frozen at -80℃ and transferred to liquid nitrogen for long term storage. Cells were resuspended in 100uL PBS containing of 1% bovine serum albumin, 2mM EDTA, and 2mM sodium azide and applied to a DURAclone IM Phenotyping BASIC tube (Beckman Coulter), mixed and incubated at 4ºC for 30 minutes. The cells were washed in buffer outlined above and fixed with 100µL 2% paraformaldehyde. Data were acquired using the CytoFLEX (Beckman Coulter) and all cells in the sample were quantified and phenotyped.

Table 4.2 Patient Characteristics for CSF cell flow cytometry

RRMS (n=27)	
Age (years; mean±SD)	44.0±10.8
Sex (%)	
Males	37.5%
Females	62.5%
Last Relapse (months; mean±SD)	7.66±11.1

4.2.6 Peripheral PBMC Flow Cytometry

Flow cytometry was used to quantify levels of circulating immune cells expressing CXCR3 in 30 individuals: 10 healthy controls and 20 RRMS cases (Table 4.3). Of the RRMS cases, 10 had high CSF CXCL10 and 10 had low CSF CXCL10 measured by ELISA, defined as above or below the mean value of the dataset, this cut-off value was 569 pg/mL. PBMCs used for flow cytometry were thawed quickly from cryopreservation and were stained with 1µL LIVE/DEAD™ aqua (Invitrogen/Thermo) in 1mL PBS and incubated for 30 minutes at 4ºC in the dark. The cells were washed with 4mL PBS containing of 1% bovine serum albumin, 2mM EDTA, and 2mM sodium azide. Cells were pelleted and resuspended in 100µL and added to an antibody cocktail containing anti-CD3-PerCP (BD Biosciences), anti-CD4-FITC (BioLegend), anti-CD8-APC/Cy7 (BioLegend), anti-CD14-APC (BD Biosciences), anti-CD19-PacBlue (BioLegend), and anti-CXCR3-PE (BD Biosciences) mixed and incubated at 4ºC for 30 minutes in the dark. Isotype control samples were incubated with the same antibody cocktail but replacing the anti-CXCR3-PE antibody with anti-IgG1k-PE (BD Biosciences). The cells were washed and fixed with 100µL 2% paraformaldehyde. Data were acquired using the CytoFLEX (Beckman Coulter). 100,000 cells were captured per sample.

Table 4.3 Patient Characteristics for PBMC flow cytometry

1. RRMS, Low CSF CXCL10 (n=10)	
Age (years; mean±SD)	40.0±8.7
Sex (%)	
Males	30%
Females	70%
Last Relapse (months; mean±SD)	34.4±30.9
2. RRMS, High CSF CXCL10 (n=10)	
Age (years; mean±SD)	49.7±21.2
Sex (%)	
Males	40%
Females	60%
Last Relapse (months; mean±SD)	9.8±9.8
3. Healthy Control (n=10)	
Age (years; mean±SD)	48.4±11.0
Sex (%)	
Males	30%
Females	70%

4.2.7 Isolation of Human Fetal Astrocytes and Treatments.

Human fetal CNS tissue was obtained from consenting donors (Health Sciences Centre – General Hospital, St. John's, NL). The obtained tissue was digested in a solution containing 2mL PBS with 1mL 2.5% trypsin (Thermo/Life Technologies) and 200uL DNase (1300ug/mL stock; Sigma), incubated at 37°C for 15 minutes on a nutator, triturating once halfway. Digestion was stopped by addition of 1mL HI-FBS, and tissue was passed through a 70um cell strainer, washed with 5-10mL PBS, and collected by centrifugation at 300xg for 10 minutes at 4°C. Cells were washed once more with pre-warmed media consisting of DMEM with 5% heat-inactivated fetal bovine serum, 1x P/S and 1x GlutaMAX®, resuspended in warmed media and seeded in a T12.5 tissue culture treated flask. Cells were left to adhere and expand for one week, and then grown to over-confluence before each passage. Half media changes were performed when appropriate. Human fetal astrocytes (HFAs) were used for experiments between pass 5-7 and plated at a density of $1x10^5$ cells/mL in experimental vessels. Experiments were conducted on cultures grown to 70-80% confluence.

HFA cultures were treated with 100% patient-derived CSF samples for 24h. Demographic and clinical information of the patients are displayed in Table 4.4. In addition, HFA cultures were treated with 10ng/mL IL-1β as a positive control, and with recombinant human CXCL10 at a concentration of 1µg/mL for 24h. Following treatment, supernatants were collected and stored at -80°C, and cells were lysed in QIAzol® for subsequent RNA isolation and qPCR analysis.

Table 4.4. Patient Characteristics of CSF samples used for HFA treatments

1. RRMS CSF (n=8)	
Age (years; mean±SD)	44.0±10.8
Sex (%)	
Males	37.5%
Females	62.5%
Last Relapse (months; mean±SD)	7.66±11.1
2. NIND CSF (n=7)	
Age (years; mean±SD)	36.7±10.4
Sex (%)	
Males	28.6%
Females	71.4%
Diagnosis at CSF Draw (%)	
Idiopathic Intracranial Hypertension	71.4%
Chronic Headache Syndrome	14.3%
Migraine	14.3%

4.2.8 RNA Isolation and qPCR Analyses

Cells used for RNA analysis were lysed in QIAzol® and stored at -80°C. Total RNA was isolated using the RNeasy® Micro Kit with a DNAse treatment step (Qiagen, Hilden, DE) and quantified by NanoDrop℠ 1000 spectrophotometer (Fischer Scientific Waltham MA). 200ng total RNA was reverse transcribed using M-MLV reverse transcriptase kit (Invitrogen, Burlington CA). Gene expression assays were performed using TaqMan Fast Universal PCR Master Mix (Applied Biosystems, Waltham MA) and TaqMan probes and primers for SLC1A2 (GLT-1), SLC1A3 (GLAST), CXCL10 and CXCR3 (Applied Biosystems, Waltham MA). RT-qPCR was performed on the Applied Biosystems Viia7 Real-Time qPCR system. Data were analyzed by the ΔΔCT method normalised to the endogenous control genes 18S or GAPDH.

4.2.9 Statistical Analysis

Statistical analyses were conducted using Prism 9 (GraphPad Software Inc. San Diego, CA). Flow cytometry data were analyzed using FlowJo™ v10.0 software (FlowJo, LLC, Ashland, OR). Data are presented as mean±SEM unless otherwise indicated and $p \leq 0.05$ was considered significant. In all analyses the data were assessed for normality by the Shapiro-Wilk test, and nonparametric tests were performed if the data were determined not to be normally distributed.

Bio-Plex and ELISA data were analyzed by unpaired t-test comparing RRMS and NIND cases. For Bio-Plex, values measured using extrapolation below the lowest standard were included in the analyses. The percentage of extrapolated values for each

cytokine is documented in Table 3.3. Note that no values of CXCL10 were extrapolated. CSF CXCL10 measured by ELISA and Bio-Plex™ in matching samples were analyzed by simple linear regression and Pearson correlation test. CXCL10 in CSF and/or plasma and CSF NfL were compared to demographic variables using T tests (sex), simple linear regression and Pearson's correlation test (disease duration, age, time since last relapse) or Spearman's correlation (EDSS), and one way ANOVA (DMT use).

Flow cytometry data were analyzed and gated independently for each sample. For CSF flow cytometry, raw cell counts were correlated with CXF CXCL10 concentration using Spearman's correlation test. Peripheral CXCR3 expression in individual PBMC populations was analysed between HC and two RRMS groups (high or low CSF CXCL10) using one-way ANOVA and were correlated to CSF CXCL10 using Spearman's correlation test. qPCR for CXCR3 in cells isolated from CSF was expressed as 1/CT and correlated to CXCL10 levels by Spearman's correlation test.

Cytokine measurement from HFAs in culture were analyzed by one-way ANOVA, and qPCR data were analyzed by ΔΔCT method normalised to the endogenous control genes 18S or GAPDH and analysed by one-sample T test compared to a theoretical mean of 1, the value of matched no treatment conditions.

4.3 Results

4.3.1 BioPlex analysis of RRMS CSF compared to NIND controls

To obtain an immunological profile of cytokines in CSF from RRMS patients and controls, levels of 27 cytokines were measured by Bio-Plex Pro™ Human Cytokine 27-plex Assay and analyzed on a Bio-Plex® 200 system. Demographic information of patients in this analysis are presented in part one of Table 4.1. Results of the Bio-Plex® analysis are displayed in Table 4.5. When comparing levels of cytokines between NIND and RRMS cases, we observed a significant increase in CSF levels of CXCL10 in RRMS cases (1628±251pg/mL) compared to NIND controls (771±80.2pg/mL, p=0.021, Figure 4.1a). In addition, we observed a significant decrease in MCP-1 in RRMS cases compared to controls (p=0.031). No significant difference was observed between groups for any other of the 25 cytokines measured in the assay (Table 4.5).

Table 4.5: Comparison of CSF cytokines measured by BioPlex between RRMS and NIND cases.

Cytokine	% Detected		% Extrapolated		t	df	P-value
IL-1β	RRMS	100%	RRMS	55.3%	1.894	55	0.0635
	NIND	100%	NIND	21.1%			
IL-1RA	RRMS	100%	RRMS	0%	1.591	55	0.117
	NIND	100%	NIND	0%			
IL-2	RRMS	100%	RRMS	78.9%	1.033	55	0.306
	NIND	100%	NIND	25.6%			
IL-4	RRMS	100%	RRMS	0%	1.304	55	0.198
	NIND	100%	NIND	5.26%			
IL-5	RRMS	84.2%	RRMS	59.4%	0.654	46	0.516
	NIND	84.2%	NIND	50.0%			
IL-6	RRMS	100%	RRMS	13.2%	1.361	55	0.197
	NIND	100%	NIND	0%			
IL-7	RRMS	100%	RRMS	2.63%	1.192	55	0.238
	NIND	100%	NIND	5.26%			
IL-8	RRMS	100%	RRMS	0%	0.934	55	0.354
	NIND	100%	NIND	0%			
IL-9	RRMS	100%	RRMS	5.26%	0.984	55	0.330
	NIND	100%	NIND	5.26%			
IL-10	RRMS	92.1%	RRMS	20.0%	1.228	51	0.225
	NIND	94.7%	NIND	16.7%			
IL-12p70	RRMS	97.4%	RRMS	32.4%	0.856	54	0.407
	NIND	100%	NIND	36.8%			
IL-13	RRMS	100%	RRMS	0%	0.074	55	0.941
	NIND	100%	NIND	0%			
IL-15	RRMS	47.4%	RRMS	0%	0.350	24	0.729
	NIND	42.1%	NIND	25.0%			
IL-17	RRMS	100%	RRMS	63.2%	1.353	55	0.181
	NIND	100%	NIND	47.4%			
CXCL10	RRMS	100%	RRMS	0%	2.375	55	0.021*
	NIND	100%	NIND	0%			
Eotaxin	RRMS	100%	RRMS	0%	0.387	55	0.699
	NIND	100%	NIND	0%			
FGF Basic	RRMS	97.4%	RRMS	100%	1.177	53	0.245
	NIND	94.7%	NIND	88.9%			

Table 4.5 continued

Cytokine	% Detected		% Extrapolated		t	df	Pvalue
G-CSF	RRMS	100%	RRMS	0%	1.672	55	0.100
	NIND	100%	NIND	0%			
GM-CSF	RRMS	89.4%	RRMS	20.6%	0.369	49	0.713
	NIND	89.4%	NIND	11.8%			
IFN-γ	RRMS	73.7%	RRMS	10.7%	0.401	41	0.690
	NIND	78.9%	NIND	20.0%			
MCP-1	RRMS	100%	RRMS	0%	2.219	55	0.031*
	NIND	100%	NIND	0%			
MIP-1a	RRMS	100%	RRMS	0%	1.821	54	0.074
	NIND	94.7%	NIND	0%			
PDGF-bb	RRMS	42.1%	RRMS	50.0%	1.104	23	0.281
	NIND	47.4%	NIND	22.2%			
MIP-1b	RRMS	100%	RRMS	0%	1.550	55	0.127
	NIND	100%	NIND	0%			
RANTES	RRMS	100%	RRMS	26.3%	0.018	55	0.956
	NIND	100%	NIND	26.3%			
TNFα	RRMS	100%	RRMS	34.2%	0.736	55	0.465
	NIND	100%	NIND	15.8%			
VEGF	RRMS	76.3%	RRMS	3.44%	0.287	43	0.776
	NIND	84.2%	NIND	6.25%			

4.3.2 CSF CXCL10 is elevated in RRMS cases

Following the results of the BioPlex assay, we validated the CXC10 results in a larger cohort (Part 2, Table 3.1). In general, levels of CXCL10 were approximately 40% of the levels measured by Bio-Plex (ELISA mean 539.6±40.9pg/mL vs BioPlex mean 1342.5±177.0pg/mL) but were highly correlated with one another when selectively comparing measurements obtained from the same patient sample ($r=0.888$; $p<0.0001$, Figure 4.1b). CSF CXCL10 measured by ELISA in this larger cohort displayed the same significant increase in CXCL10 in RRMS cases (569±49.9pg/mL) compared to NIND controls (339±53.0pg/mL, $p=0.012$, Figure 4.1c). CSF CXCL10 was not correlated with patient age ($r=0.188$; $p=0.134$; Figure 4.1d), was not associated with patient sex (males 779±115pg/mL vs females 571±61.4pg/mL, $p=0.104$, Figure 4.1e) and was not significantly different between samples collected ≤7 months (below median time since previous relapse) from most recent relapse (597±65.7pg/mL) than those collected >7months (456±66.2pg/mL, $p=0.139$, Figure 4.2f). There was also no association between CSF CXCL10 and whether the attack prior to LP was treated with steroids or not (treated 552±64.6pg/mL vs untreated 539±74.0pg/mL, $p=0.901$, data not shown).

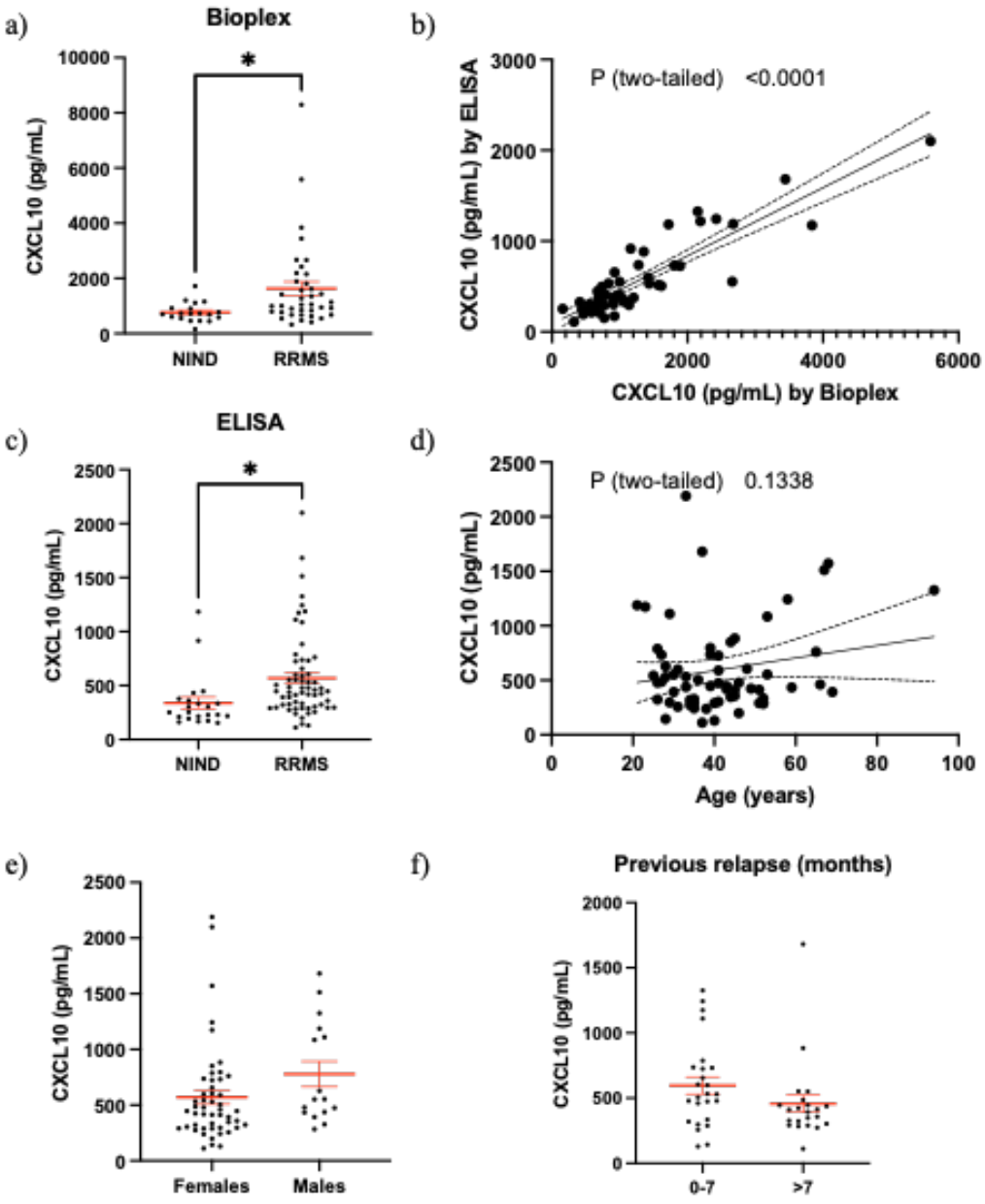


Figure 4.1 CXCL10 is elevated in RRMS compared to NIND. a) CSF CXCL10 is significantly elevated in RRMS cases compared to NIND controls when measured by BioPlex™. b) CXCL10 concentrations measured by BioPlex™ and ELISA are significantly correlated with one another. c) ELISA measurements confirmed that CXCL10 is elevated in RRMS cases compared to controls in a larger cohort. d) CXCL10 was not correlated with age. e) levels of CXCL10 were no different between males and females. f) levels of CXCL10 were comparable between those <7 months past their most recent previous relapse and those <7 months.

4.3.3 Plasma CXCL10 is associated with disease duration, but not with patient age, relapse activity, EDSS, sex or DMT use.

To determine whether CXCL10 in MS increases at a systemic level, or whether it is unique to the CSF, plasma CXCL10 and CSF CXCL10 were correlated with one another in matched samples from the ELISA cohort. This analysis revealed that CSF and plasma CXCL10 were not correlated (Part 2, Table 4.1; r=0.0847; p=0.516; Figure 4.2a), and there was no difference observed in plasma CXCL10 between RRMS (65.4±10.1pg/mL) and NIND cases (79.8±24.0pg/mL, p=0.515, Figure 4.2b). We therefore turned to the HITMS biorepository to generate a larger plasma cohort consisting of only RRMS cases to identify whether plasma CXCL10 was significantly associated with any clinical or demographic variables (Section 3, Table 4.1).

Using this dataset, we determined that plasma CXCL10 was significantly correlated with disease duration (r=0.232, p=0.012, Figure 4.2c), but not with patient age (r=0.009, p=0.922, Figure 4.2d), number of months since previous relapse (r=0.066, p=0.478, Figure 4.2e), or EDSS (r=0.0006, p=0.995, Figure 4.2f). There was also no association between plasma CXCL10 and patient sex (males 377±39.1 vs females 374±21.2, p=0.951, Figure 4.2g) or DMT use (F=1.081, p=0.380, Figure 4.2h).

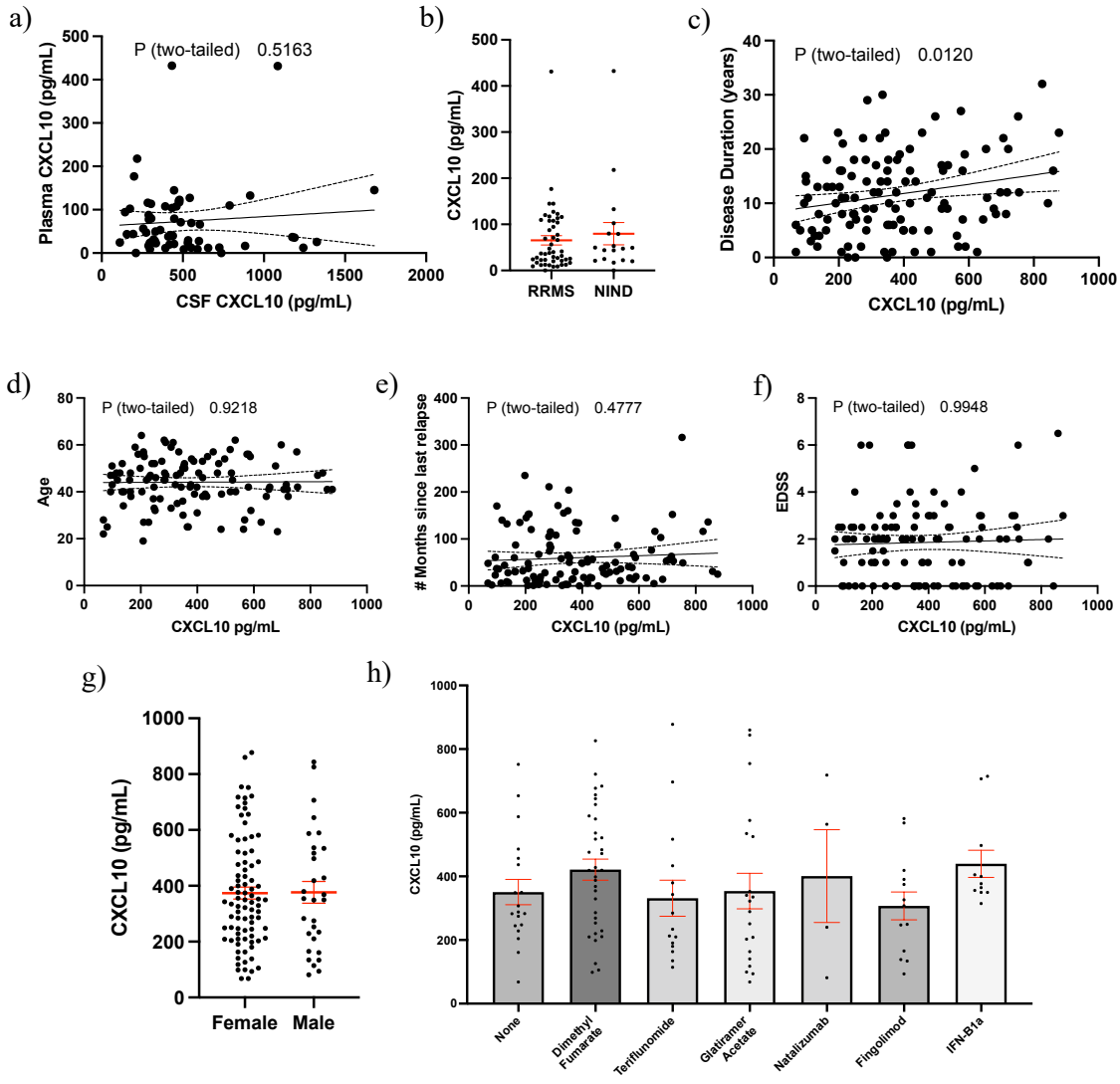


Figure 4.2 Analysis of CXCL10 in plasma. a) levels of CXCL10 in the plasma and CSF are not correlated with one another. b) Levels of CXCL10 in plasma are no different between RRMS and NIND cases. c) Plasma CXCL10 is significantly correlated with disease duration reported in years, but not with patient d) age e) number of months since most recent clinical relapse or f) EDSS score. g) Plasma CXCL10 levels were no different between males and females and h) were not associated with the use of any DMT.

4.3.4 CSF CXCL10 is associated with increased T cell presence in the CNS

Cells from 2.5mL CSF from 27 RRMS cases (Table 4.2) were pelleted by centrifugation and stained for flow cytometry to immunophenotype all the cells present in the sample. The gating strategy adopted to quantify each cell population is displayed in Figure 4.3a. Leukocytes were identified from the single cell population using anti-CD45, and this CD45+ gate was used to identify subsequent populations of CD3+ T cells, CD56+ NK cells, CD14+ monocytes and CD19+ B cells. CD3+ T cells were subsequently subdivided into CD4+ and CD8+ populations. Absolute numbers of each cell population were then correlated to CSF CXCL10 concentration measured by ELISA. This analysis revealed that CSF CXCL10 was significantly correlated with the total number of cells present in the CSF sample ($r=0.383$, $p=0.048$, Figure 4.3b), total T cells ($r=0.471$, $p=0.013$, Figure 4.3c), CD4+ T cells ($r=0.457$, $p=0.017$, Figure 4.3d), and CD8+ T cells ($r=0.427$, $p=0.026$, Figure 4.3e), but not with monocytes ($r=0.191$, $p=0.341$, Figure 4.3f), B cells ($r=0.306$, $p=0.121$, Figure 4.3g), or NK cells ($r=0.351$, $p=0.071$, Figure 4.3h).

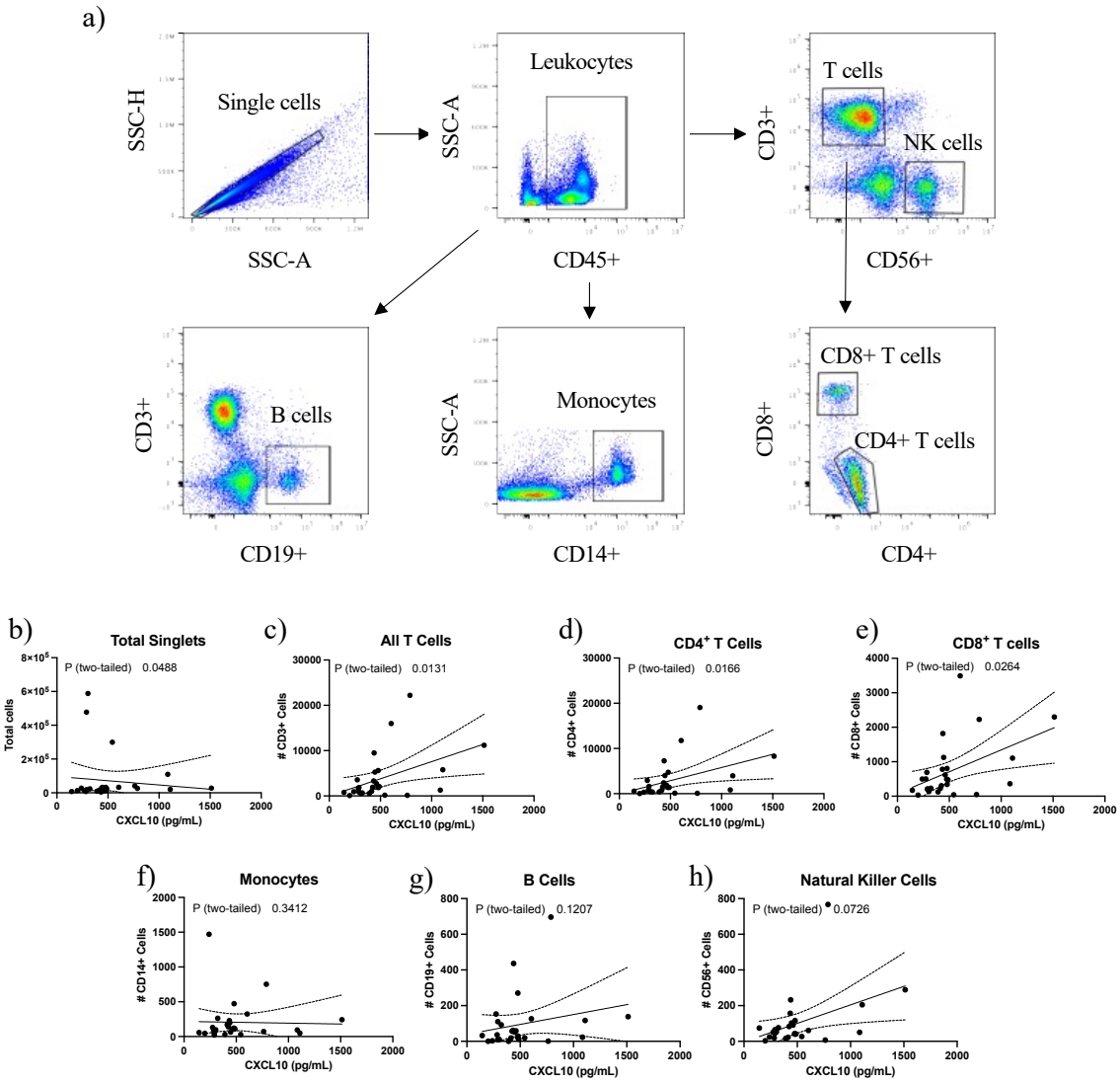


Figure 4.3. CXCL10 is correlated with T cells in the CSF. a) Gating strategy to identify populations of immune cell subsets in the CSF. Quantification of numbers of different immune cells in the CSF were correlated with CSF CXCL10 concentration and reveals significant correlations with b) total singlets, c) total CD3+ T cells, d) CD4+ T cells, and e) CD8+ T cells, but not with f) CD14+ monocytes, g) CD19+ B cells, or h) CD56+ natural killer cells.

4.3.5 CSF NfL is associated with increased presence of CD8+ T cells and NK Cells in the CSF

NfL was quantified in CSF samples from both the CSF cohorts analyzed in Figures 1 and 3. In the cases immunophenotyped using the BioPlex™ assay (Part 1, Table 4.1), CSF NfL was not significantly different in RRMS (1121±286pg/mL) compared to NIND (1010±523pg/mL) cases (p=0.386; Figure 4.4a) and was not significantly correlated with CSF CXCL10 (Pearson's r=0.158, p=0.215, Figure 4.4b). In RRMS cases analysed in the larger cohort quantified by ELISA (part 2, Table 4.1), CSF NfL was not correlated with patient age (Pearson's r=-0.149, p=0.245, Figure 4.4c), and was not significantly different in females (1009±223pg/mL) compared to males (650±123; p=0.969, Figure 4.4d). However, CSF NfL was significantly increased in individuals who had their LP within 7 months of a previous relapse (1310±387pg/mL) compared to those whose LP occurred >7 months after relapse (540±121pg/mL, p=0.009, Figure 4.4e).

CSF NfL was also correlated to the numbers of infiltrating leukocytes in the CSF from patients described in Table 3.2 (Figure 4.4f-l). Significant correlation was observed between CSF NfL concentration and CD8+ T cells in the CSF (Spearman r=0.452, p=0.018, Figure 4.4i) and CD56+ NK cells (Spearman r=0.420, p=0.029, Figure 4.4l), but not with total cells quantified in the CSF (r=0.135, p=0.519, Figure 4.4f), CD3+ T cells (r=0.336, p=0.0862, Figure 4.4g), CD4+ T cells (r=0.321, p=0.102, Figure 4.4h), CD14+ monocytes (r=0.100, p=0.618, Figure 4.4j) or CD19+ B cells (r=0.362, p=0.0637, Figure 4.4k).

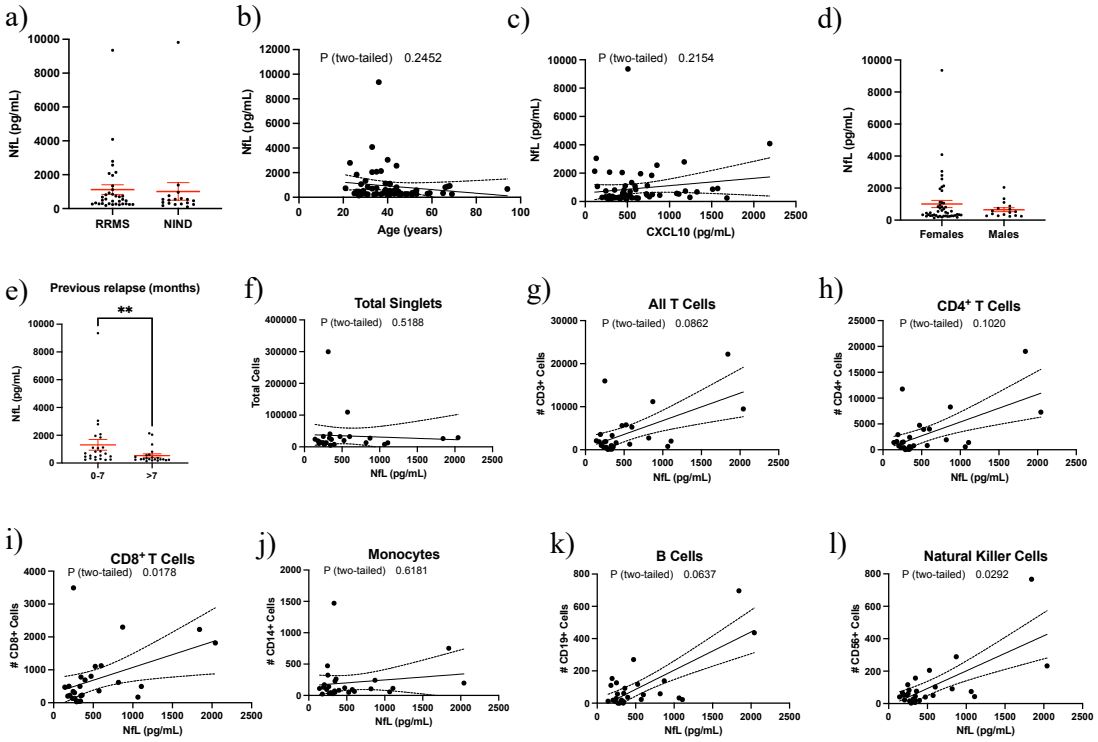


Figure 4.4 CSF NfL levels are correlated with CD8+ T cells and NK cells in the CSF. a) CSF levels of NfL were no different between RRMS and NIND cases in this cohort. b) CSF NfL and CXCL10 are not correlated. c) NfL in the CSF was also not correlated with age or associated with d) sex. e) CSF NfL was significantly elevated in individuals 0-7 months following their last clinical relapse compared to those >7 months. CSF NfL was not correlated with f) total cells in the CSF, g) all CD3+ T cells, or h) CD4+ T cells but were correlated with i) CD8+ T cells. There was also no correlation observed between j) CD14+ monocytes or k) CD19+ B cells but were correlated with k) CD56+ natural killer cells.

4.3.6 Peripheral expression of CXCR3 does not differ between HC and RRMS, and is not associated with CSF CXCL10 levels

Flow cytometry was conducted on previously cryopreserved PBMCs from healthy controls and RRMS cases with high or low CSF CXCL10 levels (Table 4.3). Individual leukocyte populations were identified based on expression of cell-specific tetraspanins, and co-expression of CXCR3 was assessed based on the gating strategy outlined in Figure 4.5. Cut-off values for positive CXCR3 expression were determined individually for each sample.

Quantification of double positive staining revealed no difference in CXCR3 expression between HC and RRMS groups, in any of the markers tested. Of the whole PBMC population, 22.2±2.22% were double positive for CD3 and CXCR3 in HC, compared to 19.7±3.43% and 23.0±6.16% in the RRMS groups (high CSF CXCL10 and low CSF CXCL10 respectively, Figure 4.6a, p=0.805). Additionally, 0.402±0.112% of PBMCs were double positive for CD14 and CXCR3 in the HC group compared to 0.582±0.133% (high CSF CXCR3) and 0.591±0.109% (low CSF CXCR3) in the RRMS groups (Figure 4.6b, p=0.128). Finally, 0.863±0.138% were double positive for CD19 and CXCR3, compared to 1.18±0.257% and 0.971±0.249% in the RRMS groups (high CSF CXCL10 and low CSF CXCL10 respectively, Figure 4.6c, p=0.818).

Of the CD3+ population, 17.4±1.68% were double positive for CD4 and CXCR3 in the HC group, compared to 19.2±2.25% and 18.9±4.07% in the RRMS groups (high CSF CXCL10 and low CSF CXCL10 respectively, Figure 4.6d, p=0.629). In addition, 13.9±1.61% were double positive for CD8 and CXCR3 in the HC group, whereas 15.4±2.14% (high CSF CXCR3) and 17.0±2.40% (low CSF CXCR3) were observed

double positive for CD8 and CXCR3 in the RRMS groups (Figure 4.6e, p=0.668). CSF CXCL10 levels were also correlated to the proportion of cells expressing CXCR3 in PBMCs and no significant correlations were observed (Table 4.6). Flow cytometry data were also analysed for mean fluorescent intensity, and as percentage of the parent population and results were no different than those obtained and presented in Figure 6 and Table 4.6 (data not shown). Importantly, qPCR for CXCR3 was performed on cells collected from MS CSF, and we observed that the expression of *cxcr3* mRNA in CSF cells was not correlated with CSF CXCL10 concentrations (Spearman's r=-0.009, p=0.969; Supplementary Figure 4.1).

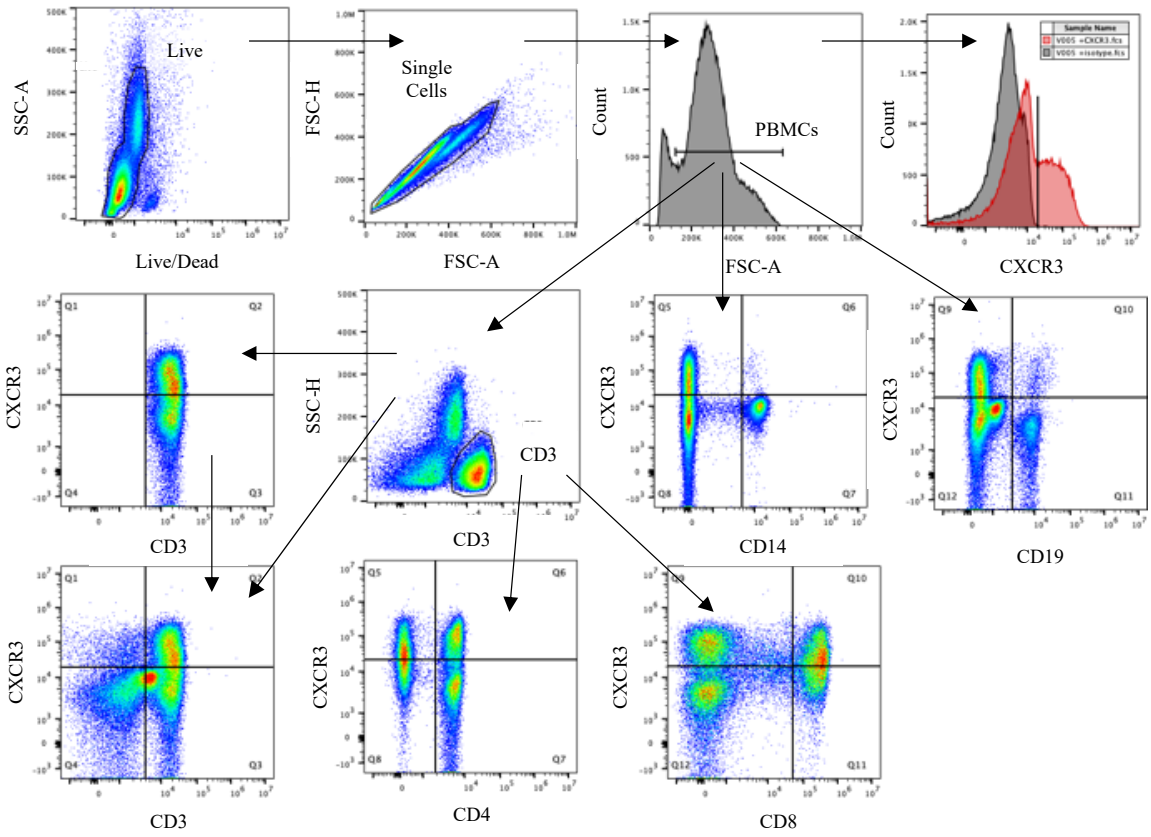


Figure 4.5. Peripheral PBMC gating strategy. Live cells were selected based staining with a Live/Dead stain, then single cells selected based on FSC grouping. PBMCs were gated from the background using FSC-A. From the PBMC population, CXCR3 positive staining was identified using a cut-off based on comparing antibody staining (red) to its isotype (grey). CD3+ cells were selected from the PBMC population by gating using CD3 and SSC-H, and then split into CD4+ and CD8+ subpopulations. CD14+ monocytes and CD19+ B cells were gated from the whole PBMC populations using positive staining.

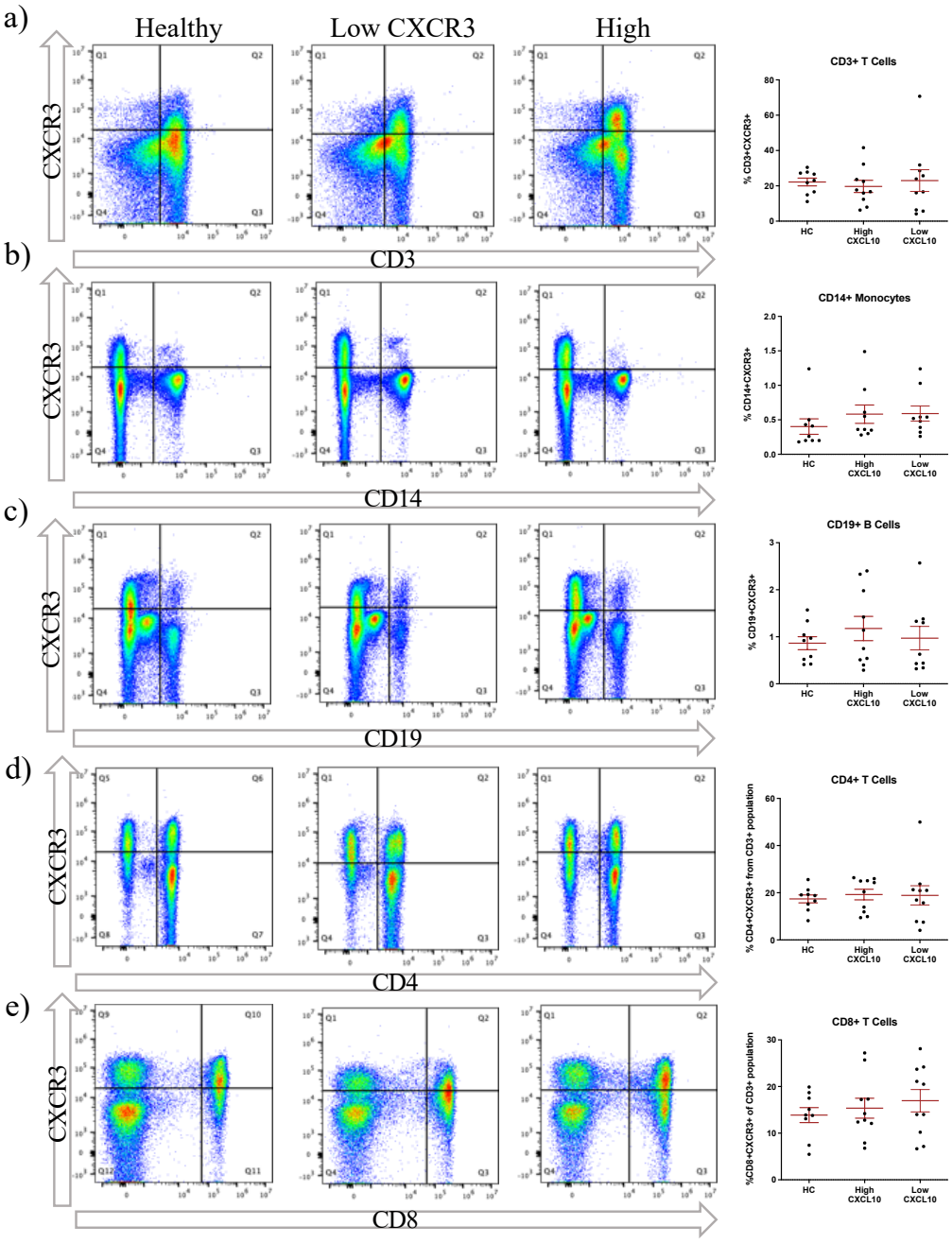


Figure 4.6 CSF CXCL10 is not associated with peripheral expression of CXCR3. Representative flow plots and quantifications reveal no difference between CXCR3 expression in a) CD3+ T cells, b) CD14+ monocytes, c) CD19+ B cells, d) CD4+ T cells or CD8+ T cells between healthy controls and RRMS cases with high or low CSF CXCL10 concentrations.

4.3.7 Astrocytes in vitro treated with human CSF downregulate glutamate transporter expression

Astrocytes are critical support cells in the brain and can contribute to neurodegeneration via excitotoxicity when stimulated with pro-inflammatory cytokines. They are the main source of CXCL10 in the CNS, and express CXCR3 suggesting an autocrine feedback loop may exist. Therefore, to further elucidate a pathological relevance of the pro-inflammatory phenotypes of MS CSF, we cultured HFAs with 100% human CSF from NIND and RRMS cases for 24h. Clinical and demographic information for these groups is presented in table 4.4. ELISAs for IL-6 and CXCL10 were performed pre- and post-treatment, and cells were lysed for RNA and protein extraction (Figure 4.7a). Prior to treatment, IL-6 was undetected in all CSF samples, but after 24h, astrocytes secreted increased IL-6 in response to both RRMS (329±91.1pg/mL) and NIND (250±70pg/mL) CSF compared to no-treatment controls (NT, 14.9±4.42pg/mL, Figure 4.7b). In contrast, while levels of CXCL10 were higher in CSF samples (RRMS: 547±56.4pg/mL; NIND: 301±32.7) post-treatment compared to NT (18.3±7.23pg/mL, Figure 4.7c), compared to their pre-treatment concentrations, both RRMS and NIND CSF had reduced CXCL10 after the treatment period (RRMS: 42.3±3.27% reduction, NIND: 28.5±7.94% reduction, Figure 4.7d). This change was not significantly different between RRMS and NIND ($p=0.069$).

HFAs treated with the highly potent pro-inflammatory cytokine IL-1β significantly reduced expression the glutamate transporter *glt-1* (mean fold change 0.59, $p=0.0024$) but not *glast* (mean fold change 0.613, $p=0.095$, Figure 4.7e). Treatment with both MS and NIND CSF also resulted in downregulation of *glt-1* mRNA ($p<0.0001$ and

p=0.0241 respectively, Figure 4.7f) and *glast* mRNA (p=0.001 and 0.0203 respectively, Figure 4.7g). Finally, western blot analysis of protein levels of GLT-1 revealed certain CSF samples are capable of drastically reducing protein levels of GLT-1 in HFAs following 24h exposure (Figure 4.7h).

4.3.8 CXCL10 alone causes downregulation of glutamate transporters in astrocytes in vitro

While many factors in CSF could account for the change in HFA phenotype that occurs following treatment, we investigated whether treating HFAs with recombinant CXCL10 could recapitulate the downregulation in glutamate transporters observed. Preliminary results (n=3) suggest that treatment with 1µg/mL recombinant CXCL10 for 24h resulted in the downregulation of *glt-1* (mean fold change 0.651, p=0.0114) mRNA but not *glast* (mean fold change 0.836, p=0.361, Figure 4.7i).

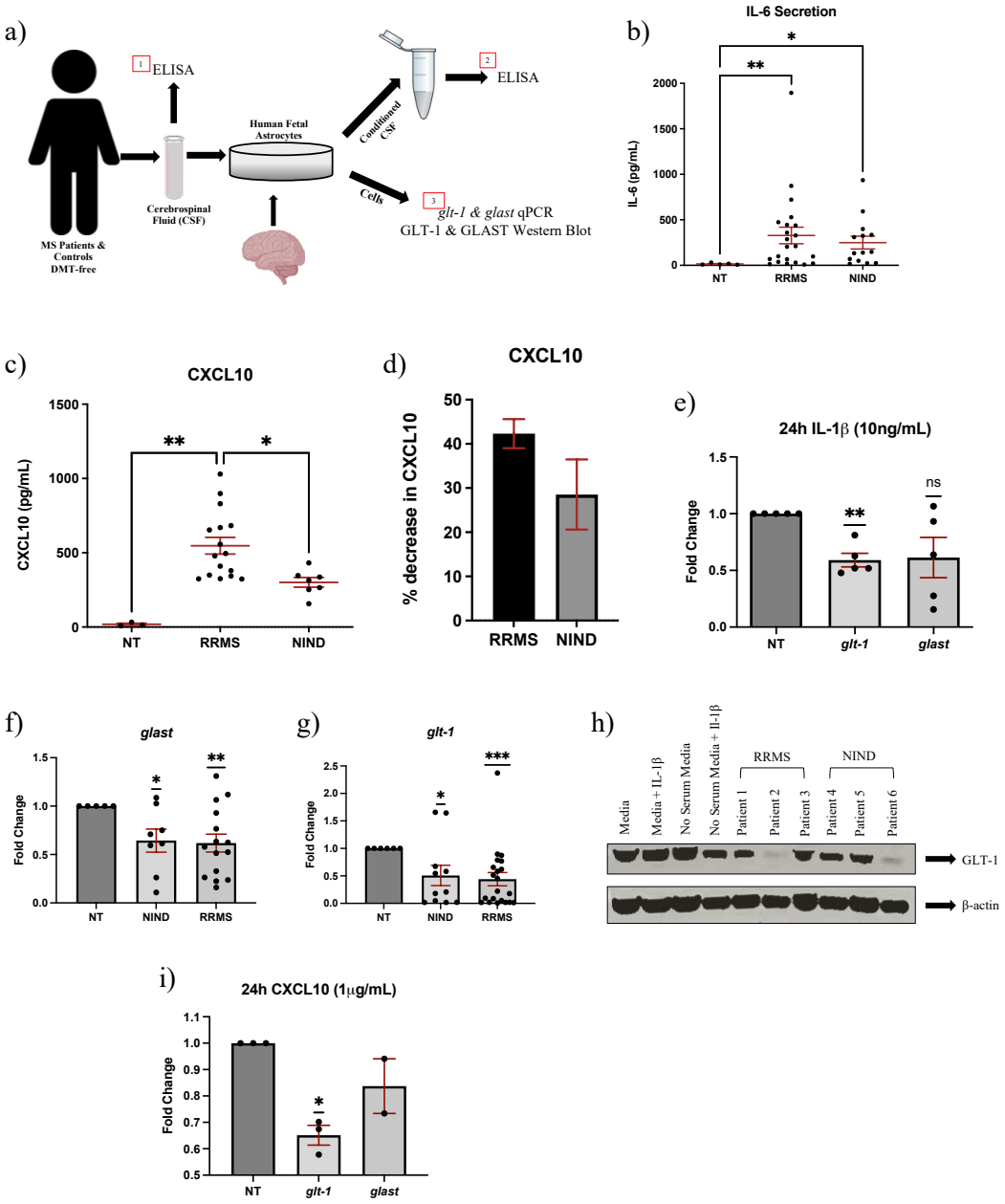


Figure 4.7. HFAs are sensitive to human CSF and CXCL10. a) Experimental overview. IL-6 and CXCL10 was measured in CSF samples by ELISA (1) before and (2) after treatment with 100% CSF for 24h. (3) Cells were collected for RT-qPCR and western blots. b) following treatment HFAs secreted IL-6, which was undetected prior to treatment. c) CXCL10 measured in the CSF was significantly higher than NT conditions but were decreased from pre-treatment measurements. d) CXCL10 levels post-treatment displayed as % decrease compared to pre-treatment measurements. qPCR for *glt-1* and *glast* in HFAs following e) IL-1β treatment, and CSF treatments (f and g). h) western bot for GLT-1 in HFAs following treatments. i) qPCR for *glt-1* and *glast* following 24h treatment with recombinant CXCL10 (1μg/mL).

4.4 Discussion

The first goal of the current study was to profile and compare cytokine and chemokine profiles in human RRMS and NIND CSF samples and investigate the pathological relevance to MS. Using both an investigational cohort (via BioPlex) and validation cohort (via ELISA), we demonstrated that CXCL10 levels in the CSF were significantly elevated in RRMS compared to controls; levels were significantly correlated with numbers of T cells present in the CSF but were unrelated to the expression of the CXCL10 receptor, CXCR3, on immune cells in either the CSF or periphery. We also demonstrated that levels of CSF NfL were significantly correlated with numbers of CD8+ T cells and NK cells in the CSF. Finally, to elucidate direct pathological consequences of increased CXCL10 within the CSF, we demonstrated that exposing human astrocytes to CSF samples resulted in a pro-inflammatory phenotypic change, a result that was recapitulated by treatment with CXCL10 alone.

Phenotyping CSF via a BioPlex™ immunoassay to simultaneously measure 27 cytokines/chemokines in each sample revealed little difference between RRMS and NIND CSF. Herein, we demonstrated that only CXCL10 and MCP-1 were differentially regulated in RRMS compared to controls (Table 4.5). We observed a significant increase in CXCL10, and a significant decrease in MCP-1. These results are in line with previous studies that have documented similar results (Sorensen et al., 2001; Mahad et al., 2002; Scarpini et al., 2002; Iwanowski et al., 2017; Khaibullin et al., 2017). A consensus of the literature suggests this finding may not be unique to MS, but rather reflective of an inflammatory CNS environment that occurs in MS and several other inflammatory neurological conditions/diseases (Franciotta et al., 2001; Mahad et al., 2002; Scarpini et

al., 2002).

In our analysis, differential levels of several cytokines and chemokines were examined and no significant differences in levels of pro- (e.g.. IL-1β, TNF, IL-17 and IFNγ) or anti-inflammatory (e.g. IL-4, IL-10, and IL-13) cytokines were noted between MS and NIND cases. This result contrasts with a similar study comparing 20 MS cases and 20 spinal hernia cases, in which significant increases were observed in 10 cytokines analysed: IL-2RA, CCL5, CCL11, CXCL1, CXCL10, IFNγ, MIF, SCF and TRAIL (Khaibullin et al., 2017). Several of these proteins were not measured in our assay, including IL-2RA, CXCL1, MIF, SCF and TRAIL, so it is possible that our cohort also exhibits increases in these cytokines. A more recent study investigated the levels of the same 27 cytokines investigated in the present study in a group of progressive MS cases, including SPMS and PPMS cases, and while many were not different between the groups, a significant difference in several cytokines in patients displaying active disease compared to non-active cases were observed (Scarpini et al., 2002; Donninelli et al., 2021). These results suggest the likelihood that inflammatory cytokines in the CSF are more related to disease activity rather than progression, a concept that is supported by an early study that found significantly increased CSF and serum CXCL10 in active compared to stable cases (Franciotta et al., 2001). If this is indeed the case, the discrepancies between our study and those previously published could be due to random sampling of cases with low disease activity at the time of LP.

Following results of the initial investigation cohort, a validation cohort was used to confirm our findings. Using this cohort, we also demonstrated that CXCL10 levels in

the CSF CXCL10 were not associated with patient age (Figure 4.1d), sex (Figure 4.1e), nor previous relapse activity (Figure 4.1f). We also demonstrated that within individual patients, CXCL10 levels in the plasma vs. CSF were not correlated (Figure 4.2a), thereby suggesting that plasma CXCL10 levels cannot be indicative or predictive of levels within the CSF (Figure 4.2b). Similar results have been previously reported in which significant increases in CXCL10 were observed in CSF samples of MS patients vs. NIND cases, but not in blood (Iwanowski et al., 2017; Khaibullin et al., 2017). Taken together, these results suggest that the blood compartment may not necessarily be reflective of the inflamed CNS compartment and further emphasizes the need to study CSF to help discover possible novel biomarkers in diseases like MS.

In RRMS, we sought to determine whether plasma levels of CXCL10 were significantly associated with clinical and/or demographic variables. To address this, we used a large cohort consisting of 116 RRMS patients that were previously enrolled in a longitudinal patient registry and biorepository (Health Research Innovation Team in Multiple Sclerosis, HITMS). Results of this analysis demonstrated that plasma CXCL10 was significantly correlated with disease duration, but not with age, months since last relapse, or EDSS (Figure 4.2c-f). We also observed no differences in plasma CXCL10 levels between males and females (Figure 4.2g), nor did we observe any significant association with use of various DMTs (Figure 4.2h). While several previous studies have compared CSF to blood levels of CXCL10, few have investigated any associations between serum/plasma levels and clinical variables apart from DMT use. One study compared clinical and demographic variables in serum CXCL10 in MS cases; the only significant correlation observed was between serum CXCL10 and age, but only in a

progressive subgroup of MS cases (Iwanowski et al., 2017).

Since CXCL10 is a chemokine known to induce migration of leukocytes, we used flow cytometry to immunophenotype cells found within the CSF of MS cases. When correlated to levels of CSF CXCL10, we found that CXCL10 was significantly correlated with total singlets (i.e. total cells) in CSF (Figure 4.3b), in addition to T cells (both CD4+ and CD8+; Figure 4.3c-e). No other significant correlations were observed between CSF CXCL10 and monocytes, B cells or NK cells in the CSF (Figure 4.3f-h). Previous studies investigating the association between CSF CXCL10 and immune cell infiltration (quantified by hemocytometer) has yielded conflicting results (Sorensen et al., 2001; Scarpini et al., 2002). Consistent with our results, Sorensen et al., observed a significant correlation between the number of cells in the CSF with the concentration of CXCL10 in the MS group that was absent in the NIND group (Sorensen et al., 2001). Conversely, others have noted no association between CSF CXCL10 and CSF cell counts. In this study, the authors combined active/nonactive RRMS and PMS cases and did demonstrate significant differences between these groups (Scarpini et al., 2002). In addition, the failure of both studies to sub-categorize the cells into different populations may also underlie the discrepancies seen between these two studies and with the results presented here. To our knowledge, this is the first study to offer a comprehensive analysis of the relationships between CXCL10 and specific immunophenotypes of cells in the CSF.

In parallel with these works, several other studies have used flow cytometry to quantify expression of the CXCL10 receptor, CXCR3, on cells within the CSF and periphery; these studies have also presented conflicting results (Sørensen et al., 2002; Teleshova et al., 2002; Matsui et al., 2005). One study documented an increase in

CXCR3-expressing T cells in the blood and CSF in MS cases compared to noninflammatory controls, and that this difference was largely driven by untreated patients since patients treated with IFN-β were no different from controls (Teleshova et al., 2002). Interestingly, this study also found a higher proportion of CXCR3+ T cells in the CSF compared to the blood in matched samples, suggesting that the CXCR3/CXCL10 axis is an important driver of T cell infiltration into the CNS. In this study however, no differences in the CSF CXCL10 levels were observed between MS and NIND cases (Teleshova et al., 2002). A separate study published the same year reported similar results that the proportion of T cells expressing CXCR3 (CD4+ and CD8+ subsets) were higher in the CSF compared to blood. However, no difference between the MS and the control groups (NIND and healthy) were noted (Sørensen et al., 2002). This study also reported that the percentage of CXCR3+ T cells in blood and CSF did not correlate with time from symptom onset to LP, presence/absence of OCB, CSF total leukocyte count, MRI abnormalities, or with CSF CXCL10 concentration (Sørensen et al., 2002). A third study documented significantly higher percentage of CD4+CXCR3+ cells in the blood of active RRMS patients compared to stable, which may have implications for the above-mentioned studies, as neither separated their cohorts into active vs non-active cases (Matsui et al., 2005). Nevertheless, this study also reported no correlation between CXCR3+ T cell populations in the blood or the CSF and CSF CXCL10 (Matsui et al., 2005).

In the previous studies mentioned above, an investigation of the overall presence of T cells (both expressing and not expressing CXCR3) and a comparison to CXCL10 levels was not performed. These results could explain why our results demonstrate a

significant correlation between CSF CXCL10 and the numbers of T cells in the CSF, while others did not. As the CSF flow cytometry performed in this study was a routine protocol performed as part of the general immunophenotyping of patients and samples enrolled in the biorepository, flow cytometry for CXCR3 on CSF cells was not included, which is a limitation of our current study. We did perform qPCR for CXCR3 on pooled cells in the CSF samples and found that CXCR3 expression was not correlated with CSF CXCL10, suggesting that CSF CXCL10 is not related to the expression of CXCR3 on leukocytes in the CSF. It is possible, however, that once in the CNS, cells migrating based on a CXCL10/CXCR3 axis lose expression of CXCR3 once they have reached the high CXCL10 environment in the CNS. However, our data suggests that peripheral CXCR3 expression is not different between MS cases and controls and has no association with CSF CXCL10 (Figure 4.6). These data lead to the question of whether the CXCR3/CXCL10 axis represents the major function in the CNS in MS cases. Several other routes of entry for leukocytes to enter the brain exist (Takeshita & Ransohoff, 2012). Therefore, the CXCL10/CXCR3 axis is not solely responsible for the infiltration of leukocytes and may represent the route of entry of only a minority of cells.

We also sought to determine whether levels of NfL were related to numbers of cells and immune cell subsets in the CSF. In our cohort, we report that CSF NfL was not significantly different in RRMS compared to NIND (Figure 4.4a), nor was it associated with age (Figure 4.4b), or sex (Figure 4.4c). While NfL has previously been shown to be significantly correlated with age, this was not observed in this study (Gisslén et al., 2016; Yilmaz et al., 2017; Benkert et al., 2022). Samples included in this study were collected initially for inclusion in a biorepository of patients presenting to a neurology clinic in St.

John's, Newfoundland and Labrador, Canada often prior to formal diagnosis. All patients included in the study did receive formal diagnosis of clinically definite MS, or another neurological condition. However, as the LP was performed during a period of uncertainty, and possibly during periods of clinical exacerbation regardless of final diagnosis, NfL levels are likely more related to disease activity especially in the younger patients. This is supported by our results showing a significant increase in CSF NfL in RRMS patients who underwent the LP within 7 months of a clinical exacerbation compared to those >7 months (Figure 4.4d). This is supported also by the literature; NfL is known to be elevated during periods of MS relapse and is associated with new MRI lesions and remains elevated for 3 months following the development of the lesion (Disanto et al., 2017; Rosso et al., 2020). In addition, while a significant correlation with age is seen in the healthy population, evidence suggests that this association is lost in MS (Manouchehrinia et al., 2020b).

CD4+ T cells are thought to underlie lesion development in MS lesions, (Trapp et al., 1998; Lucchinetti et al., 2000; Benkert et al., 2022). However, CD8+ T cells outnumber CD4+ cells, and have been suggested to contribute to lesion development and persistence (Machado-Santos et al., 2018; Nicol et al., 2018). It is interesting that we did observe a significant correlation between CSF NfL concentration and CD8+ T cells in the CSF of MS cases (Figure 4.4h), supporting a wealth of existing data describing the toxicity of CD8+ T cells in MS (Kaskow 2018). We also observed a significant correlation between CSF NfL and NK cells in the CSF (Figure 4.4k), but not with total CSF cells (Figure 4.4e), total T cells (Figure 4.4f), CD4+ T cells (Figure 4.4g), monocytes (Figure 4.4i) or B cells (Figure 4.4j).

The impact of NK cells in MS pathology is unclear, as murine NK cells have different expression of CD56 and different receptor profiles which makes it difficult to elucidate their role using animal models (Mimpen et al., 2020). However, recent studies suggest an important role of NK cells in MS. NK cells are known to express many of the genes linked to susceptibility in MS, and $CD56^{bright}$ cells have been shown to be immunomodulatory in MS by killing autoreactive CD4+ T cells (Bielekova et al., 2006; Jiang et al., 2011; IMSGC, 2019). Recent evidence suggests an important role of the peripheral NK/CD4+ T cell ratio in measuring disease activity in MS, and results suggest that this ratio is significantly negatively correlated with plasma NfL levels (Mimpen et al., 2021). The effect of NK cells on MS pathology is a subject that is currently debated (Laroni, 2019; Liu & Shi, 2019; Segal, 2019). Our results suggest a significant positive correlation between CSF CD56+ NK cells and CSF NfL which may represent central modulation of autoreactive T cells in the CNS; this represents an interesting future direction for which further investigation is warranted.

To elucidate direct mechanistic and/or pathophysiological significance of elevated CXCL10 levels within the CSF of MS, we extended our study to investigate the phenotypic effects of treating primary human astrocytes with CSF from patients with MS and other non-inflammatory controls (Figure 4.7a). This treatment resulted in the secretion of IL-6 by astrocytes treated with either RRMS or NIND CSF (Figure 4.7b), but a decrease in CXCL10 compared to pre-treatment measures (Figure 4.7d). We also demonstrated that treatment with the highly pro-inflammatory cytokine IL-1β (Figure 4.7e) and human CSF (Figure 4.7f-h) downregulated the expression of two important glutamate transporters GLT-1 and GLAST. This was not unique to MS, as the NIND CSF

was also capable of downregulating GLT-1 and GLAST to a similar degree.

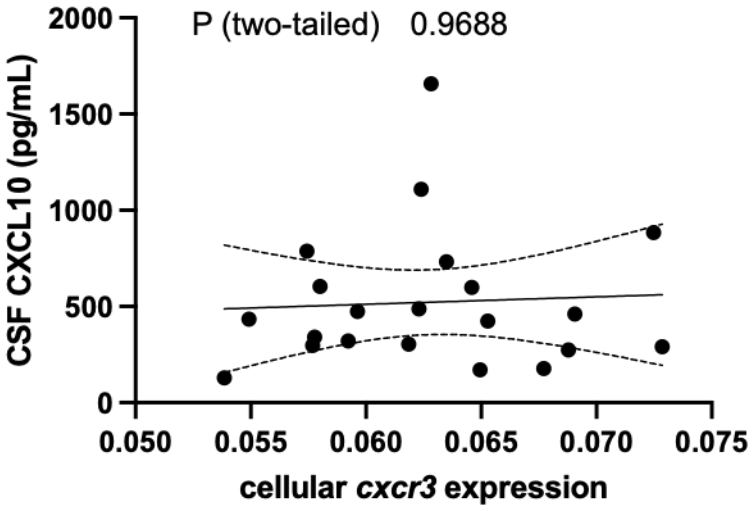
Many MS-relevant inflammatory mediators including TNF and IL-1 have previously been shown to be able to influence glutamate transporter expression and cause dysregulated glutamate uptake in both human and mouse *in vitro* models (Hu et al., 2000; Mandolesi et al., 2013). Additionally, decreased GLT-1 levels are observed in lesions in *post-mortem* brain tissue from MS patients suggesting a role in glutamate transporter dysregulation in MS (Newcombe et al., 2008; Vercellino et al., 2009). We expand these findings by providing evidence that CSF from neurological disorders, including MS, are capable of downregulating glutamate transporters on astrocytes providing evidence that the CSF harbors factors capable of inducing pathological changes in astrocytes that could result in excitotoxicity-mediated neurodegeneration. Furthermore, we show that treatment with recombinant CXCL10 alone is capable of downregulating *glt-1* on HFAs.

Astrocytes themselves are major producers of CXCL10 and are known to potently upregulate the secretion of CXCL10 in response to many pro-inflammatory cytokines, including IL-1β. This suggests an autocrine signaling pathway by CXCL10 in astrocytes. This result is supported by a recent study that investigated CXCR3 knockout animals and the effect on glia *in vitro* and in an *in vivo* model of MS (Chung & Liao, 2016). However, this study suggests an immunoregulatory role of CXCR3 signaling on astrocytes that contrasts the glutamate transporter downregulation we observed here. This study demonstrated that CXCR3 knockout animals exhibit an exacerbated EAE course characterized by significantly increased Th17 cell infiltrates into the spinal cord, increased expression of cytokines required for Th17 expansion including IL-6, IL-17, IL-23 and CCL20, and increased glial activation in spinal cord sections (Chung & Liao,

2016). Primary astrocyte cultures derived from CXCR3 knockouts also exhibited increased production of IL-23 and CCL20, suggesting CXCR3 signaling in astrocytes is an important regulator of Th17 polarization in the CNS during EAE (Chung & Liao, 2016). The study performed by Chung and colleagues also demonstrated that CXCR3 signaling in a glioblastoma cell line negatively regulates the transcription factor NF-κB (Chung & Liao, 2016). This is likely the link that could result in the simultaneous reduction in Th17-inducing cytokine production and glutamate transporter expression, as NF-κB is thought to positively regulate transcription of GLT-1 in astrocytes (Ghosh et al., 2011). Future research investigating the intracellular signaling of CXCR3 signaling on astrocytes is warranted to elucidate the mechanism of glutamate transporter downregulation and the effects on astrocyte phenotype.

In summary, the present study provides evidence that CSF CXCL10 is elevated in RRMS cases compared to NIND, and that it is not significantly associated with clinical or demographic variables. We also show that plasma CXCL10 is significantly correlated with disease duration, but with no other clinical or demographic variables investigated. We provide evidence that CSF CXCL10 is significantly correlated with the numbers of T cells in the CSF, but this is unrelated to expression of CXCR3 in the CSF or in the periphery. Finally, we have demonstrated that human astrocytes exposed to CSF derived from MS and NIND cases results in IL-6 secretion and downregulation of glutamate transporters; CXCL10 alone may be sufficient to cause the downregulation of glutamate transporters *in vitro*. Taken together, these results support the importance of elevated CXCL10 in MS and other neurological disorders and supports an alternative pathological relevance of CXCL10 outside of immune cell trafficking into the CNS.

4.5 Supplementary Figures



Supplementary Figure 4.1. CSF CXCL10 is not correlated with *cxcr3* expression in pooled cells in CSF.

Chapter 5 – Summary and Future Directions

5.1 Summary

Immune activation throughout the course of MS occurs systemically, including the CNS, but can be measured in the periphery. The aim of this thesis was to identify novel candidate biomarkers in blood plasma and CSF of MS cases, while also elucidating their relevance to pathological disease mechanisms in RRMS. I hypothesized that both plasma and CSF contain inflammatory mediators that can help diagnosis and disease monitoring in RRMS and contribute to ongoing pathological mechanisms in the CNS. Here, I identified three candidate biomarkers with pathophysiological relevance to RRMS.

Initial studies focused on plasma-based markers of inflammatory activity. I identified the presence of an anti-inflammatory cytokine IL-1RA in plasma samples of RRMS cases and determined that levels of IL-1RA were significantly correlated with disability independent of patient age, sex, disease duration of use of DMTs. *In vitro* and *in situ* investigations suggested that IL-1RA is produced and secreted within the inflamed CNS, which suggests it may be an endogenous mechanism to mediate local inflammation. I also determined that EVs secreted from individual immune cell subsets were elevated in RRMS plasma despite only minimal changes in circulating parent cell populations, raising the possibility of whether inflammatory signals/mediators can be transferred throughout the body by EVs in MS. The final study presented in this thesis immunophenotyped RRMS CSF samples and identified CXCL10 as being differentially regulated in MS compared to controls and was associated with T cells presence in the CNS. Taken together, these results contribute to the body of literature identifying

immune-relevant markers in body fluids being differently regulated in RRMS and provides pathophysiological insights to ongoing disease mechanisms.

5.1.1 Interleukin-1 Receptor Antagonist is Associated with Disability in MS

IL-1RA is an endogenous antagonist to the IL-1R1 receptor. Signaling through the IL-1 receptor by its cognate agonist IL-1α and IL-1β results in the activation of NF-κB and subsequent triggering of potent pro-inflammatory signaling; the binding of IL-1RA in place of an agonist prevents this pro-inflammatory signaling pathway and dampens the response (Dinarello, 2018). The study presented in Chapter 2 used ELISA to measure plasma IL-1RA and used multiple regression to determine that plasma IL-1RA levels are significantly correlated with EDSS, independent of patient age, sex, disease duration or DMT use.

The increased levels of plasma IL-1RA observed in RRMS cases with higher disability presented in Chapter 2 seems counterintuitive, as logic suggests that increases in the anti-inflammatory cytokine would be associated with decreased disability. However, the results presented in this thesis using primary human macrophages and microglia indicate that IL-1RA is secreted in conjunction with IL-1β in response to activation of the NLRP3 inflammasome. We also showed that in mixed active/inactive lesions, myeloid cells at the hypercellular edge of the lesion express IL-1RA. This tandem secretion may represent an endogenous mechanism meant to maintain some level of homeostasis in the midst of increased IL-1β (Nicoletti et al., 1996; Corr et al., 2011; Dosh et al., 2019; Nitta et al., 2019). These data suggest that while this situation may be the

case locally, circulating levels of IL-1RA, IL-1β and IL-18 are not comparable in the plasma, nor are they significantly correlated with one another. As such, IL-1β concentration was not significantly associated with disability. Plasma IL-1β concentrations were significantly lower than IL-1RA, possibly due to a more stringent degradation process once secreted as a safeguard to prevent systemic inflammation.

This study also analysed IL-1RA in the CSF and found significant correlation between IL-1RA and NfL. In the CSF, IL-1RA levels were not different between RRMS and NIND cases but were significantly increased in OIND cases. In addition, CSF samples positive for IL-1β had significantly higher measured IL-1RA. These results further suggest that the release of IL-1RA can occur as a compensatory mechanism in response to increased IL-1β. These results also provide additional evidence that inflammatory states in the CNS and the periphery are not always tightly matched and represent separate compartments, even in the context of a systemic condition.

5.1.2 Circulating Extracellular Vesicles

Extracellular vesicles are released from cells during homeostatic and pathological conditions; they bear markers specific for their cell of origin and can be measured in all major body fluids. In the study presented in Chapter 3, EVs were quantified in plasma samples from RRMS and controls using an EV flow cytometry protocol developed and optimised in house. Nanotracking analysis was used to determine that size profiles and overall particle concentration were no different between control and RRMS samples. Flow cytometry was then used to immunophenotype EVs in circulation and identify the

levels of immune cell derived EVs and compare them to numbers of parent cells in circulation. The study provides evidence that levels of circulating EVs derived from all immune cell subtypes investigated are elevated in untreated RRMS compared to healthy controls. Results presented showed only minimal changes to levels of circulating parent-cell subsets, suggesting that the increase in EVs in circulation is not simply reflective of increased cells in circulation.

Levels of circulating EVs may instead be reflective of the activation status of the cells. In fact, several studies have shown increased EV release under pro-inflammatory circulation, particularly from myeloid cells, astrocytes, and endothelial cells (Minagar et al., 2001; Jimenez et al., 2005; Verderio et al., 2012; Dickens et al., 2017). Importantly, data also suggests that EVs are capable of harboring cargo reflective of a pro-inflammatory parent cell and transfer that inflammatory signal to recipient cells affecting their phenotype (Jy et al., 2004; Verderio et al., 2012; Dickens et al., 2017). Whether this is the case in MS, and the identification of factors secreted inside EVs that contribute to pathology remains to be seen and is a topic of future research, discussed below.

5.1.3 Immunophenotyping CSF

CSF is an important body fluid that harbors a wealth of information about the CNS. CSF is continuously exchanged with the ISF and contains molecules from the parenchyma that could provide critical information regarding ongoing inflammatory activity. The study presented in Chapter 4 measured 27 cytokines and NfL in the CSF of MS and NIND cases, and found striking similarities between the two, with very few

cytokines being identified as differentially regulated. Only CXCL10 and MCP-1 were significantly different between RRMS and NIND – CXCL10 was significantly elevated, whereas MCP-1 was significantly decreased.

Experiments were designed to follow up on the significant increase in CXCL10 observed in RRMS cases. It was determined that CSF CXCL10 was not correlated with any clinical or demographic variable investigated, and serum CXCL10 was only associated with disease duration. We then investigated whether the chemotactic effects of CXCL10 were represented in RRMS with increased immune cells in the CSF and found that CXCL10 levels were significantly correlated with T cells (both CD4+ and CD8+) in the CSF suggesting the chemotactic properties of CXCL10 may result in this T cell infiltration. In contrast, CSF NfL was not different between RRMS cases and NIND controls and was not significantly associated with patient age or sex but was elevated in RRMS patients ≤7 months past their most recent clinical relapse compared to those >7 months. CSF NfL was also significantly correlated with CSF CD8+ T cells and CD56+ NK cells. These data suggest that CD8+ T cells and NK cells may participate in pathological activities that result in increased NfL in circulation.

Further experiments were conducted to investigate the CXCL10-mediated chemotaxis of immune cells subsets by quantifying CXCR3 expression on immune cell subsets in the periphery. This experiment provided evidence that peripheral immune cell expression of CXCR3 was no different between MS cases and healthy controls, and that CSF CXCL10 levels did not affect CXCR3 levels on T cells, B cells, monocytes, or NK cells. We also performed qPCR on pooled CSF cells and found no correlation between expression of CXCR3 mRNA and CSF CXCL10. Taken together, these data suggest that

elevated CSF CXCL10 may serve an alternate purpose than T cell chemotaxis. To investigate this, an experiment was designed to determine the effects of treating primary human astrocytes with CSF. This treatment resulted in the secretion of IL-6, a pro-inflammatory cytokine that is indicative of astrocyte activation, and the downregulation of mRNA and protein for two glutamate transporters GLT-1 and GLAST. However, this was not unique to RRMS CSF samples, and was also observed when HFAs were treated with CSF from NIND cases. Evidence presented suggests that the downregulation of GLT-1 may be regulated, at least in part, by CXCL10, as treatment with recombinant CXCL10 was sufficient to significantly downregulate *glt*-1 in HFAs.

5.2 Future Directions

The research presented in this thesis represents three cross sectional studies identifying novel exploratory biomarkers in MS with pathological relevance to ongoing disease processes. From these works, the opportunity for countless future studies arises. While results herein present important preliminary data in the utility of these biomarkers, future works should assess biomarker specificity for MS, provide longitudinal analyses investigating how the biomarkers change over the course of the disease within individual patients, assess the applicability across the MS disability and disease spectrum, and assess the functional relevance of the altered immune-relevant molecules identified here on MS pathology.

5.2.1 Assessing Biomarker Specificity

One of the limitations to currently used and strongly researched biomarkers are the lack of specificity for MS. MRI, OCB, NfL and many circulating cytokines are also significantly increased in systemic inflammatory diseases and/or other neurodegenerative conditions (Comabella & Montalban, 2014). While healthy control, and other neurological disease control groups were employed whenever relevant, direct comparison between other neurological conditions, both inflammatory and non-inflammatory, are critical for assessing whether the markers identified in this thesis are specific to MS or indicative of changes ubiquitous to neurodegeneration and neuroinflammation.

The ideal biomarker in MS is a marker that is unique to MS and is absent in other conditions, including MS mimics (Comabella & Montalban, 2014). With so many

conditions involving an inflammatory component, it is unlikely that these markers of general inflammation will be specific to MS. However, results suggest that the identified markers in this thesis are not directly related to discrete inflammatory events. For example, plasma IL-1RA and CSF CXCL10 were not associated with time since most recent clinical relapse, and plasma immune cell derived EVs were not associated with levels of the parent cell populations. Thus, assessing specificity to MS will still provide valuable information and further determine the possible utility of using these biomarkers in future clinical practice.

5.2.2 Investigating Biomarkers Across the Disability and Disease Spectrum

EDSS scores are well known to incompletely reflect the complete picture of MS disability, most notably in that the assessment places stronger weight on walking ability than on other physical and cognitive symptoms. Scores are often subjective and reflect the neurologist's experience assessing MS cases. In addition, changes in EDSS score represent measurable clinical symptoms that can occur due to relapse or progression and distinguishing between the two requires the documentation of changes serially over a period of years.

The results presented in Chapter 2 suggest that IL-1RA is elevated in RRMS, regardless of relapse activity as measured by months since last documented relapse. In addition, as IL-1RA is elevated with increased disability in the absent of concomitant increase in the pro-inflammatory markers IL-1β and IL-18. Taken together, these results suggest that IL-1RA has the potential to act as an objective indicator of disability

worsening that is not dependent on heightened inflammation associated with active relapse. The data presented in Chapter 3 also suggest that levels of circulating immune cell derived EVs are not related to the numbers of circulating parent cells. These results also suggest that measuring more subtle markers of systemic inflammation may prove valuable in identifying changes that occur between major inflammatory events. Critical future experiments would identify patients in active relapse and assess levels of IL-1RA and immune cell derived EVs and investigated across the spectrum of EDSS scores including those with elevated levels of disability. Treatment effect of various DMTs and their mechanisms of action should be investigated to determine whether the biomarkers are useful in monitoring treatment responses.

Finally, in progressive subtypes of MS, inflammatory mechanisms take a back seat to neurodegenerative processes. This is clear in the fact that immunomodulatory DMTs used to manage RRMS fail to prevent conversion to SPMS and lose their efficacy once progressive disease takes over (Cree et al., 2016). One area in which biomarker discovery in MS has failed is identifying a biomarker differentiating RRMS from a progressive course. This is true both diagnostically and prognostically; no objective biomarker is available for differentiating RRMS from PPMS upon initial presentation, nor for identifying cases that have transitioned from RRMS to SPMS, or those at significantly higher risk of transitioning at any given time during the disease course. Future studies investigating IL-1RA, plasma EVs, or CXCL10 should investigate whether any of these molecules can mark progression.

A major limitation to the studies presented in this thesis is the lack of available MRI data to use in the evaluation of these potential novel biomarkers. As new and

worsening lesions are currently the gold standard to objectively monitor disease worsening, investigating now these potential biomarkers are or are not associated with MRI activity is critical to determine their utility moving forward.

5.2.3 Longitudinal Investigation of Candidate Biomarkers

Each of the studies presented in this thesis represented a cross sectional design. Critical future studies must investigate the identified biomarkers with a longitudinal design. While the cross-sectional studies presented here provide important preliminary investigations identifying IL-1RA, immune cell-derived EVs and CXCL10 as possible biomarkers with pathological relevance in RRMS, investigating changes in these makers over time will provide critical evident about their clinical utility moving forward. MS is a complex, heterogenous and long-term condition, and it will be critical to understand how these markers change as the disease evolves over its natural course, and with treatment. The HITMS biorepository is now in its 7th year in operation, and many patients have provided 4-5 samples over the course of the years allowing us to begin planning longitudinal analyses using patient plasma samples.

Experiments are currently being designed to investigate how IL-1RA and plasma EVs change in an individual with increasing disability (as measured by EDSS), and with increasing axonal damage (as measured by NfL) over time. In addition, patients identified as CIS or RIS when presenting to the neurology clinic for diagnostic purpose will be followed and recruited once a clinically definite MS diagnosis is obtained in order to investigate the prospective use of plasma EV patterns in the early stages of disease to aid

in obtaining earlier diagnoses. Finally, the effects of DMTs on plasma IL-1RA, plasma EVs, and CSF CXCL10 will be investigated by comparing pre- and post-DMT initiation samples within individuals starting new therapies.

5.2.4 Identifying Functional Relevance of Altered Immune-Relevant Molecules on MS Pathology.

One aspect that each the putative biomarkers identified in this thesis have in common is that not only do they have the potential to mark ongoing disease activity, but they themselves may be capable of enacting changes in the brain that actively contribute to pathology. Identifying how these molecules can contribute to pathology will add valuable information to our understanding of MS and may lead to the identification of future therapeutic targets.

First, the mechanism of IL-1RA has already been determined as having anti-inflammatory actions through blocking the IL-1R1 receptor and preventing its activation by IL-1β (Dinarello, 2018). At present, there is an ongoing phase 2 clinical trial assessing the safety, tolerability, and efficacy of anakinra, a synthetic recombinant IL-1RA currently approved to treat rheumatoid arthritis (clinicaltrials.gov NCT04025554). A primary outcome in this clinical trial is the disappearance of paramagnetic rims surrounding chronic active lesions, but secondary studies using serum and CSF collected throughout the trial will be used for biomarker identification and studying the mechanism of action of anakinra (clinicaltrials.gov NCT04025554). While this study will no doubt provide valuable information into the function of IL-1RA in during ongoing pathology,

more direct study is likely still required. Future studies will focus on the general homeostatic role of IL-1RA during the tandem secretion with IL-1β during activation of the NLRP3 inflammasome, and whether it is sufficient to prevent IL-1β-mediated damage in healthy individuals. The relationship between increased IL-1RA and increased disability will also be explored further, as this increase in an anti-inflammatory cytokine in the absence of any detectable increase of its pro-inflammatory counterpart (IL-1β) was not expected.

Second, as evidence indicates that proinflammatory mediators can be transferred from one cell to another by way of EVs, this will be further explored following the discovery presented in Chapter 3 that plasma from RRMS cases contain significantly higher numbers of immune cell derived EVs compared to controls. *In vitro* experiments are being planned to measure changes in EV secretion in whole blood samples as well as in immune cell subsets (particularly monocytes and monocyte-derived macrophages) using pro-inflammatory activation protocols. The cargo in EVs secreted under these paradigms will also be profiled to identify any pro-inflammatory proteins or microRNAs that may be differentially secreted. In addition, EVs can cross the BBB and have previously been shown to enact changes in parts of the body far away from where they were secreted (Dickens et al., 2017). Thus, these EVs can also be presented to cultured CNS cells like astrocytes, microglia, and oligodendrocytes to assess any phenotypic changes that EVs can enact on CNS resident cells. Not only will this generate a model that can be used to test the effects of DMTs on the secretion of EVs and the regulation of their cargo, but it will also contribute to the overall body of knowledge on the mechanism

of action of EVs, and how they are able to affect physiology in the brain under homeostatic and pathological states.

Finally, the data presented in Chapter 4 suggest an alternate relevance of increased CSF CXCL10 in RRMS from immune cell trafficking from the periphery. Astrocytes are likely the cellular source of CXCL10 in the CSF, and data presented here suggest an autocrine mechanism whereby CXCL10 acting on astrocytes that results in the downregulation of glutamate transporters. The mechanism by which this occurs is as of yet unclear and warrants further investigation. The role of each CXCR3 isotype, and their localization on astrocytes and in the CNS in general is currently lacking in the literature. The different CXCR3 isotypes appear to have opposing signaling pathways, and determining their individual contribution is critical. In addition, the most likely mediator of downstream signaling and gene regulation in CXCR3 signaling is the NF-κB transcriptional regulation. Therefore, critical future experiments will measure its activity and translocation into the nucleus to establish its role in regulating the expression of glutamate transporters and other gene targets in response to CXCL10. CSF samples were also shown to mediate pro-inflammatory phenotypic changes in astrocytes including IL-6 secretion and glutamate transporter downregulation; while CXCL10 likely mediates some of these effects, the role of other soluble factors should be investigated.

5.3 Conclusions

Under current criteria, MS diagnosis requires clinical assessment, MRI, and laboratory tests. Satisfying dissemination in both space and time criteria often takes time and leaves patients waiting for critical community and healthcare services, including DMTs. Evidence indicates that earlier and more aggressive DMT initiation produces more favorable long-term outcomes outlining the critical need for biomarkers that allow for faster time to clinically definite diagnosis (Cree et al., 2016; Brown et al., 2019). Despite decades of research, easily detectable body-fluid based biomarkers unique to MS have been elusive and have yet to be fully adopted by the field. This thesis identifies three novel candidate biomarkers with pathophysiological relevance to RRMS. While it remains to be seen whether these identified markers are sufficiently sensitive and specific to MS, and accurately reflect ongoing disease processes in MS over the disease spectrum, the results presented here provide the framework for which future studies can explore these questions.

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Appendices

Ethics Clearances

From: info@hrea.ca
Subject: HREB - Approval of Ethics Renewal 543673
Date: August 17, 2021 at 10:35 AM
To: Moore Craig(Principal Investigator) craig.moore@mun.ca
Cc: info@hrea.ca

Researcher Portal File #: 20200742

Dear Dr. Craig Moore:

This e-mail serves as notification that your ethics renewal for study HREB # 2019.190 – Identifying Mechanisms of Inflammatory-mediated Demyelinating Injury in Multiple Sclerosis – has been **approved**. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from October 8, 2021 to October 8, 2022.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an "Event".

The ethics renewal [will be reported] to the Health Research Ethics Board at their meeting dated [August 26, 2021].

Thank you,

Research Ethics Office

(e) info@hrea.ca
(t) 709-777-6974
(f) 709-777-8776
(w) www.hrea.ca
Office Hours: 8:30 a.m. – 4:30 p.m. (NL TIME) Monday-Friday

From: administrator@hrea.ca
Subject: HREB - Approval of Ethics Renewal 564424
Date: March 3, 2022 at 11:04 AM
To: Stefanelli Mark(Principal Investigator) cstefanelli@nl.rogers.com
Cc: Ploughman Michelle(Co-Principal Investigator) mploughm@mun.ca, Moore Craig(Co-Principal Investigator) craig.moore@mun.ca, Anthony Sarah(Key Contact) sarah.anthony@easternhealth.ca, administrator@hrea.ca

Researcher Portal File #: 20161208

Dear Dr. Mark Stefanelli:

This e-mail serves as notification that your ethics renewal for study HREB # 2015.103 – Health Research Innovation Team in Multiple Sclerosis (HIT MS) Provincial Portfolio – has been **approved**. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from April 30, 2022 to April 30, 2023.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an “Event”.

The ethics renewal [will be reported] to the Health Research Ethics Board at their meeting dated March 10, 2022.

Thank you,

Research Ethics Office

(e) info@hrea.ca
(t) 709-777-6974
(f) 709-777-8776
(w) www.hrea.ca
Office Hours: 8:30 a.m. – 4:30 p.m. (NL TIME) Monday-Friday

From: info@hrea.ca
Subject: HREB - Approval of Ethics Renewal
Date: July 27, 2021 at 3:48 PM
To: Moore Craig(Principal Investigator) craig.moore@mun.ca
Cc: info@hrea.ca

Researcher Portal File #: 20160977

Dear Dr. Craig Moore:

This e-mail serves as notification that your ethics renewal for study HREB # 2014.181 – Innate and Adaptive Immune Cell Mechanisms in Multiple Sclerosis – has been **approved**. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from September 8, 20212 to September 8, 2022.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an "Event".

The ethics renewal [will be reported] to the Health Research Ethics Board at their meeting dated [July 29, 2021].

Thank you,

Research Ethics Office

(e) info@hrea.ca
(t) 709-777-6974
(f) 709-777-8776
(w) www.hrea.ca
Office Hours: 8:30 a.m. – 4:30 p.m. (NL TIME) Monday-Friday

From: info@hrea.ca
Subject: HREB - Approval of Ethics Renewal
Date: August 12, 2021 at 10:04 AM
To: Moore Craig(Principal Investigator) craig.moore@mun.ca
Cc: info@hrea.ca

Researcher Portal File #: 20160976

Dear Dr. Craig Moore:

This e-mail serves as notification that your ethics renewal for study HREB # 2014.182 – Measuring Human Immune Cell Response in Neuroinflammatory Injury – has been **approved**. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from September 14 2021 to September 14, 2022.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an "Event".

The ethics renewal [will be reported] to the Health Research Ethics Board at their meeting dated August 12, 2021.

Thank you,

Research Ethics Office

(e) info@hrea.ca
(t) 709-777-6974
(f) 709-777-8776
(w) www.hrea.ca
Office Hours: 8:30 a.m. – 4:30 p.m. (NL TIME) Monday-Friday

From: info@hrea.ca
Subject: HREB - Approval of Ethics Renewal
Date: August 17, 2021 at 10:30 AM
To: Moore Craig(Principal Investigator) craig.moore@mun.ca
Cc: info@hrea.ca

Researcher Portal File #: 20161001

Dear Dr. Craig Moore:

This e-mail serves as notification that your ethics renewal for study HREB # 2014.216 – Investigating human neuro-immune mechanisms during inflammatory injury – has been **approved**. Please log in to the Researcher Portal to view the approved event.

Ethics approval for this project has been granted for a period of twelve months effective from October 10, 2021 to October 10, 2022.

Please note, it is the responsibility of the Principal Investigator (PI) to ensure that the Ethics Renewal form is submitted prior to the renewal date each year. Though the Research Ethics Office makes every effort to remind the PI of this responsibility, the PI may not receive a reminder. The Ethics Renewal form can be found on the Researcher Portal as an “Event”.

The ethics renewal [will be reported] to the Health Research Ethics Board at their meeting dated [August 26, 2021].

Thank you,

Research Ethics Office

(e) info@hrea.ca
(t) 709-777-6974
(f) 709-777-8776
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Instructor name	Dr. Craig Moore		

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