A Study of Potential Plasma Biomarkers of Disease Conversion and Progression in Multiple Sclerosis

by

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Abstract

Multiple sclerosis (MS) is a complex and heterogeneous disease of the central nervous system (CNS) characterized by leukocyte infiltration, myelin damage, local gliosis, and axonal injury. Relapsing-remitting MS (RRMS) is the most common disease course. Although the pathogenesis remains unclear, it is believed that MS is initiated by a breakdown of immune tolerance to CNS antigens due to genetic and/or environmental factors. Clinically Isolated Syndrome (CIS) is a demyelinating event isolated in time that is compatible with the possible future development of MS. In patients with MS, comorbid psychiatric disorders, such as depressive disorders and anxiety disorders, are common. There has been increasing evidence of the role of neuroinflammation in both MS and neuropsychiatric conditions, suggesting that inflammatory changes may contribute to disease occurrence, interaction and progression. Currently no established plasma markers for MS exist. Limited diagnostic cerebrospinal fluid (CSF) markers have been identified, however they do not correlate with disease severity or prognosis.

In this study, plasma levels of amino acids [glutamate, glutamine, aspartate, asparagine, glycine, arginine D-serine, L-serine, L-serine-O-phosphate (LSOP), alanine, and taurine] and neuroactive steroids (allopregnanolone, pregnanolone, pregnenolone, epiallopregnanolone, DHEA, and THDOC) were measured in controls (n=17), CIS (n=31) and RRMS (n=33) patient cohorts to identify trends with progression and severity of symptoms, and potential neuroinflammatory biomarkers of MS. Demographic and clinical variables including treatment with disease modifying therapies (DMTs) and corticosteroids, comorbidities, and changes in Expanded Disability Status Scale (EDSS), original Multiple Sclerosis Severity Scale (MSSS), updated MSSS, and Age-Related

MSS (ARMSS) scores were determined via retrospective chart review to assess for correlates of change in disability scores. A three-step hierarchical regression was then conducted with change in disability score as the dependent variable.

Plasma concentrations of the amino acids alanine, arginine and glutamine were significantly lower in the RRMS group compared to controls, while concentrations of LSOP and taurine were found to be significantly higher in the RRMS group compared to controls. Aspartate and taurine were also significantly higher in the RRMS group compared to the CIS group. Alanine and arginine concentrations were significantly lower in the CIS group compared to controls. Median plasma concentration of the neuroactive steroid allopregnanolone was significantly higher and the concentration of epiallopregnanolone was significantly lower in the RRMS group compared to the CIS group. Among clinical variables, the likelihood of depression was significantly greater in the RRMS group compared to controls. Initial EDSS scores were significantly higher in the RRMS group compared to the CIS group and both initial and final ARMSS scores were higher in the RRMS group compared to the CIS group. During the median study period of 1.85 years, 51.6% of CIS patients converted to RRMS.

In terms of correlates of change in disability scores, an increase in EDSS scores across both groups was significantly and inversely correlated with plasma pregnanolone concentrations ($r = -0.267$; $p = 0.041$) and positively correlated with disease duration at final EDSS assessment ($r = 0.310$; $p = 0.013$). A three-step hierarchical regression to assess predictors of EDSS change scores found that step 1 (age, sex and disease duration) accounted for 14.3% of the variance, with disease duration at final EDSS assessment significantly associated with an increase in change score. Adding pregnanolone and

THDOC to the model (step 2) further explained 11.0% of the variance. Plasma concentrations of THDOC were significantly positively associated with increases in EDSS, whereas pregnanolone concentrations were inversely associated but not significant.

Based on these findings, some plasma amino acids and neuroactive steroids are promising as biomarkers of MS disease progression in the context of neuroinflammation and immunomodulation, and warrant further investigation. The identification of neuroinflammatory biomarkers in MS is fundamental to the diagnosis, prognostication and treatment of this debilitating condition.

Preface

This thesis is the original work of Catherine Cheng. This research was conducted at the University of Alberta under the supervision and guidance of Drs. Christopher Power and Glen Baker. The analysis of clinical variables and use of human plasma was approved by the University of Alberta Human Research Ethics Board (Biomedical) for which consent documents were signed by all study participants. My role in this project included clinical chart review, data collection, contributions to data analysis, creation of research tables and figures, and manuscript and thesis preparation. D. Gomez of the Fujiwara lab assisted with statistical analysis and preparation of figures in this thesis. This research received funding support from an Alberta Innovates Health Solutions (AIHS) CRIO grant and the University of Alberta.

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List of Abbreviations

Abbreviation	
9HPT	9-Hole Peg Test
Aβ	Amyloid β-peptide
ACTH	Adrenocorticotropic hormone
AD	Alzheimer's disease
ADMA	Asymmetric dimethylarginine
ALLO	Allopregnanolone
ALS	Amyotrophic lateral sclerosis
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ARMSS	Age Related Multiple Sclerosis Severity Score
ASN	Asparagine
BAFF	B-cell activating factor
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
CCL4	Macrophage inflammatory protein-1β
CCL11	Eotaxin
CHI3L1	Chitinase-3-like protein 1
CIS	Clinically isolated syndrome
CLIP1, or CLIP-170	CAP-Gly domain containing linker protein 1
CNS	Central nervous system
CSF	Cerebrospinal fluid
CXCL13	C-X-C motif chemokine 13
DAAO	D-amino acid oxidase
DDO	D-aspartate oxidase
DHDOC	Dihydrodeoxycorticosterone
DHEA	Dehydroepiandrosterone, 3β-hydroxy-5-androstene-17-one
DMTs	Disease modifying therapies
EAAT	Excitatory amino acid transporter
EAE	Experimental autoimmune encephalitis
EBNA-1	Epstein-Barr nuclear antigen-1
EBV	Epstein Barr virus
EDSS	Expanded Disability Status Scale
FTLD	Frontal temporal lobar degeneration
$GABA_A$	γ-Aminobutyric acid A
Gd	Gadolinium
GDVII	George's disease 7
GDH	Glutamate dehydrogenase
GluR1	Glutamate receptor 1
GS	Glutamine synthetase
GSL	Glycosphingolipid
^{1}H-MRS	Proton magnetic resonance spectroscopy
HPA	Hypothalamic-pituitary-adrenal

IL-1B	Interleukin-1B
JCV	John Cunningham virus
lncRNAs	Long non-coding RNAs
LSOP	L-Serine-O-phosphate
MAGNIMS	Magnetic Imaging in Multiple Sclerosis
MAP2	Microtubule-associated protein 2
MDD	Major depressive disorder
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple sclerosis
MS-DSS	MS disease severity scale
MSFC	Multiple Sclerosis Functional Composite
MSSS	Multiple sclerosis severity score
NAA	N-Acetylaspartate
NASs	Neuroactive steroids
NAWM	Normal-appearing white matter
NMO	Neuromyelitis optica
NMR	Nuclear magnetic resonance
NMDA	N-Methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAID	Nonsteroidal antiinflammatory drug
OCBs	Oligoclonal bands
PAD	Peptidyl arginine deiminase
PASAT	Paced Auditory Serial Addition Test
Phgdh	Phosphoglycerate dehydrogenase
PML	Progressive multifocal leukoencephalopathy
PPMS	Primary progressive multiple sclerosis
PREG	Pregnanolone
PREGNEN	Pregnenolone
PS	Phosphatidylserine
ROS	Reactive oxygen species
RRMS	Relapsing-remitting multiple sclerosis
SHMT	Serine hydroxylmethyl transferase
SPMS	Secondary progressive multiple sclerosis
SR	Serine racemase
T25FT	Timed 25-foot walk
THDOC	Tetrahydrodeoxycorticosterone
THP	Tetrahydroprogesterone
TLRs	Toll like receptors
TMEV	Theiler’s murine encephalomyelitis virus
TO	Theiler’s original
TSPO	Translocator protein
TUG1	Taurine up-regulated 1

1. Introduction

1.1. Multiple sclerosis overview and subtypes

Multiple sclerosis (MS) is a neuroinflammatory and autoimmune disease of the central nervous system (CNS) characterized by leukocyte infiltration, demyelination, gliosis, and ensuing axonal injury (Noorbakhsh, Baker, & Power, 2014). MS is a complex and highly heterogeneous disease, with varying clinical presentations, disease trajectory and therapeutic response. (Comabella & Montalban, 2014). Often with first onset in early adulthood and a higher overall prevalence in women, MS affects over 2.5 million people worldwide (Raphael, Webb, Stuve, Haskins, & Forsthuber, 2015). Individuals with an affected first-degree relative have a 2-4% risk of MS, compared to the general population risk of approximately 0.1%, and concordance in monozygotic twins is 30 to 50% (Reich, Lucchinetti, & Calabresi, 2018). Common clinical presentations of MS include mononuclear vision loss, double vision, limb weakness or sensory loss and ataxia (Reich et al., 2018). With disease progression, impaired mobility and cognition may present. Although the pathogenesis remains unclear, it is believed that MS is initiated by a breakdown of immune tolerance to CNS antigens due to a combination of genetic and environmental factors.

Several subtypes of MS have been identified. Clinically Isolated Syndrome (CIS) is a demyelinating event isolated in time that is compatible with the possible future development of MS. Depending on the localization of the original lesion, studies suggest between 10 and 85% of CIS converts to MS pathology (Miller, Chard, & Ciccarelli, 2012). Time to conversion is highly variable, and CIS can remain isolated without conversion to MS in approximately one-third of patients, especially for initial

presentations of idiopathic unilateral optic neuritis or myelitis (Fisniku et al., 2008). An abnormal magnetic resonance imaging (MRI) scan at the time of presentation is a strong clinical predictor of conversion to MS, with a long-term risk of 60-82% compared to 8-25% in those with a normal initial MRI scan (Brownlee & Miller, 2014). The presence of unmatched oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) is also an independent risk factor (Brownlee & Miller, 2014). HLA-DRB1*1501 is a strong genetic risk factor for MS and CIS conversion, with an odds ratio of approximately three (Hauser et al., 2000). Additional risk factors for diagnosis of or conversion to MS include younger age at presentation, CIS with cognitive impairment, low levels of serum vitamin D, infectious exposures including Epstein Barr virus (EBV) and smoking (Brownlee & Miller, 2014).

Relapsing-remitting multiple sclerosis (RRMS) is the most common disease phenotype, typically affecting young adults (mean age of onset 30 years), and is estimated to affect 80-85% of patients (Brownlee, Hardy, Fazekas, & Miller, 2017; Milo & Miller, 2014). RRMS is characterized by periods of relapse followed by periods of remission, with periods of full or partial recovery and a lack of disease progression between episodes. Primary progressive multiple sclerosis (PPMS) affects 10-15% of individuals with MS, typically presents at an older age (mean age of onset of 40 years), and has a slow progressive course with an increase in disease burden and neurological disability over time without relapse, and with potential periods of plateaus (Milo & Miller, 2014). RRMS can further convert to secondary progressive multiple sclerosis (SPMS), where an initial RRMS phenotype evolves into a progressive course with increasing disability, with or without relapses and remissions. Among patients with

RRMS, approximately 50% will convert to SPMS after 10 years and 90% after 25 years (Milo & Miller, 2014).

A diagnosis of MS requires objective evidence of CNS lesions that are disseminated in time and space, with alternative diagnoses excluded. The McDonald Criteria, initially produced in 2001 and revised in 2005 and 2010, are used for diagnosis and integrate the Swanton/MAGNIMS (Magnetic Imaging in Multiple Sclerosis) criteria with clinical and laboratory findings (Milo & Miller, 2014). In December 2017, the fourth and latest version of the McDonald Criteria was published (Carroll, 2018). Important changes include the endorsement of CSF OCBs, which may now substitute for a second clinical event or MRI finding to fulfill the dissemination in time criteria. In addition, symptomatic and asymptomatic MRI lesions, as well as cortical and juxtacortical lesions are now considered in fulfilling the dissemination in space and time criteria. The 2017 McDonald criteria do not impact previously diagnosed cases of MS using prior criteria, and do not affect the role of currently approved therapies.

1.2. Current Understanding – the pathogenesis of multiple sclerosis

Although significant advances have been made, the pathogenesis of MS is not fully understood. Significant debate also remains as to whether the initial cause of MS originates intrinsic or extrinsic to the CNS (Stys, Zamponi, van Minnen, & Guerts, 2012). In addition, one growing hypothesis suggests that regardless of initial immunologic patterns of demyelination, a single patient-dependent immune effector mechanism dominates in each individual (Reich et al., 2018). Traditionally, the peripheral activation and subsequent migration of an autoimmune cascade, primarily led by autoreactive CD4+

T-helper cells (Th1/TH17 cell subsets) targeting myelin self antigens has been generally accepted to cause or propagate the inflammatory process in MS (Karussis, 2014). Growing evidence suggests that the disease process is much more complex, with the immune system playing a significant role in the pathogenesis of MS via multiple innate and adaptive processes and cell types involved (Yadav, Mindur, Ito, & Dhib-Jalbut, 2015). Oxidative stress has been implicated as a key mediator of both inflammatory and neurodegenerative processes in both MS and animal models of MS (Yong, Chartier, & Quandt, 2018). In the early phases of MS, CNS antigen-specific immune activation likely occurs first in the periphery via activation of autoreactive T cells as a result of direct cross reactivity, molecular mimicry or bystander activation; and subsequently crosses the blood-brain barrier (BBB) into the CNS. However, in the progressive phase, immune activity within the CNS predominates (Hemmer, Kerschensteiner, & Korn, 2015).

Amplification of initial myelin-reactive T cell response via an inflammatory cascade involves myeloid cells, including microglia and infiltrating macrophages (Skaper, Facci, Zusso, & Giusti, 2018). Pathogenic mechanisms of disease resulting in inflammation, plaque formation and neurodegeneration are highly heterogeneous and, in addition, largely believed to be patient- and site-dependent and further influenced by environmental factors. Further support for immune and inflammatory contributions to MS disease pathology is the association of low levels of vitamin D, a biologically active hormone and potent immunomodulator, with an increase in MS disease severity, including conversion from CIS to MS (Shaheen, Sayed, Daker, AbdelAziz, & Taha, 2018). Experimental studies in mice models have also shown that the administration of

1,25-dihydroxyvitamin D prevents experimental autoimmune encephalitis (EAE) onset and progression (Becklund, Hansen, & DeLuca, 2009).

From a histopathological perspective, demyelinating plaques accompanied by gliosis are a hallmark of MS. Active lesions in the early stages of MS are characterized by breakdown of the BBB with inflammatory perivascular infiltrates of macrophages, glial cells, $CD8^+$ T cells and B cells, regions of on-going demyelination, reactive gliosis, axonal loss and increased oligodendrocytes involved in remyelination (Meltzer, Costello, Frohman, & Frohman, 2018; Moore, 2010). Subsequently significant heterogeneity exists – lesions may become "shadow plaques" with early remyelination and remain protective against further axonal loss, become inactive with a demyelinated core, or remain chronically active, displaying continual progressive and expanding demyelination at the lesion border with a core of macrophages and fibrillary gliosis (Frohman, Racke, & Raine, 2006; Moore, 2010). Tissue injury and clusters of activated microglia and macrophages have been observed in normal-appearing white matter (NAWM) and may represent the earliest stage of MS lesion development (Yadav et al., 2015).

Following demyelination, axonal damage and subsequent transection and loss may occur. Proposed mechanisms include direct immune cell attack, indirect injury as a result of harmful substances, such as free radicals, cytokines, chemokines and oxidative products in an inflammatory microenvironment, elevated nitric oxide (NO), $CD8^+$ T-cell-mediated axonal transection and glutamate-mediated excitotoxicity (Meltzer et al., 2018). Chronic axonal loss is perpetuated by a chronic energy imbalance in supply and demand. Demyelinated axons have a higher metabolic need as a result of Na^+/K^+-ATPase pump leakage in the absence of myelin and increases in and redistribution of sodium channels

as an adaptation to restore axonal conduction. Increased metabolism is supported by findings of higher mitochondrial content in demyelinated axons (Zambonin et al., 2011). Furthermore, glutamate excitotoxicity and reversal of the sodium-calcium exchanger may result in calcium influx, triggering intracellular cascades of calcium-mediated injury and resulting neurodegeneration (Frohman et al., 2006).

Restoration of myelin sheaths to demyelinated axons in MS lesions is also a variable process, with remyelination ranging from extensive to absent. Remyelination occurs simultaneously to opposing demyelination and pro-inflammatory processes. Early experimental evidence in the cuprizone mouse model suggests that in addition to a long-term protective role, remyelination may aid in immediate axonal restoration following demyelinating insult (Schultz et al., 2017).

In keeping with an immune-mediated mechanism, there is evidence that conversion from CIS to MS may result from an inability of the immune system to suppress effector B cell production. In a study of the peripheral blood cells of 17 CIS patients at first attack, where follow-up was conducted at three years, patients who converted to MS were compared to those who had not. Patients who converted to MS had significantly increased levels of total B cells and suppressed unswitched memory B cell and plasma cell frequencies (Aktura et al., 2018). This finding supports the role of inflammation in the disease conversion and pathogenesis from CIS to MS.

1.3. Animal models of MS

Multiple pathological mechanisms have been implicated in the manifestation of MS disease and various animal models have been used to study specific aspects of the

disease process. Animal models that are currently used widely to study MS include experimental autoimmune encephalomyelitis (EAE) models, Theiler's murine encephalomyelitis virus (TMEV) infection in mice and the myelinotoxic cuprizone model.

EAE models are widely used and useful for studying the autoimmune component of MS. EAE is a group of disorders characterized by neuroinflammation, myelin damage and neurodegeneration as a result of T cell-mediated infiltration-related mechanisms, which are hallmarks of relapsing and remitting disease (Zendedel, Beyer, & Kipp, 2013). In EAE models, animals are immunized with a CNS-related antigen, inducing an autoimmune response and MS-like CNS disease, and the combination of peptide and mouse strain determines the disease phenotype exhibited. For example, induction of EAE using myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide results in paralysis of the tail and hind limbs after 10-14 days and the development of an acute monophasic EAE with no clinically related progression (Kipp, Nyamoya, Hochstrasser, & Amor, 2017). Most EAE models result in development of monophasic or relapsing-remitting disease, although a few progressive EAE models have been established (Sato, Omura, Martinez, & Tsunoda, 2018).

TMEV is a viral model of MS, with two subgroups – George's disease 7 (GDVII) and Theiler's original (TO). The GDVII subgroup is highly neurovirulent and results in death within 1-2 weeks of intracerebral infection, whereas the TO subgroup exhibits an acute polioencephalomyelitis 1 week after infection, followed by chronic inflammatory demyelinating disease 1 month after infection (Sato et al., 2018). In the TMEV model, CD4+ helper cells play an important role in the pathogenesis of demyelination. Unlike

EAE models, TMEV induces an inflammatory demyelinating illness in mice only and infected mice develop a progressive disease course (Procaccini, De Rosa, Pucino, Formisano, & Matarese, 2015).

The cuprizone model is a toxin-induced demyelination model where T cells are not relevant for cuprizone-induced demyelination and breakdown of the BBB is not a characteristic feature (Kipp et al., 2017). Animals are fed a cuprizone diet, and though the exact mechanism is not understood, administration of the copper chelating reagent cuprizone inhibits mitochondrial enzyme functions that require copper as a co-factor, resulting in oxidative stress and subsequent oligodendrocyte apoptosis and demyelination, microgliosis and astrocytosis (Kipp et al., 2017; Procaccini et al., 2015). Primary oligodendrocyte damage leads to innate immune activation within the CNS, with complete demyelination after 4-5 weeks. Endogenous remyelination and repair occur when cuprizone intoxication ceases, making the cuprizone model particularly useful in studying mechanisms of remyelination (Praet, Guglielmetti, Berneman, Van der Linden, & Ponsaerts, 2014).

1.4. Current clinical measures of disease severity and progression

MS is a chronic demyelinating illness with highly variable disease severity and progression trajectory. Regular interval monitoring and the use of a reliable battery of clinical measures to track disease severity and progression is necessary and highly beneficial. Progressive residual disability with decline in physical functioning over time has been associated with decades of MS disease burden. In a study looking at the changes in broad spectrum disability in relation to disease severity over a ten-year period in

people with MS, Conradsson, Ytterberg, von Koch, & Johansson, (2018) found that both mild MS [defined in the study as an Expanded Disability Status Scale (EDSS) score between 0 and 3.5] and moderate/severe MS (EDSS score between 4 and 9.5) groups experienced a decline in walking, manual dexterity and cognition, with a larger decline in the moderate/severe group; however, only the moderate/severe group showed a long term significant physical impact including increased wheel-chair dependency and reduced participation in social/lifestyle activities. These findings suggest a need to identify individuals with greater disease severity early in the course of illness to reduce morbidity. Currently, no reliable and agreed upon clinical method or instrument to measure disease severity or predict disease progression in individuals with MS exists.

The most widely used and well-validated measure of MS disability and progression is the EDSS, which was first published by Kurtzke in 1983 (Kurtzke, 2015). The EDSS system is a clinician-administered assessment tool based on scores in eight functional systems of the central nervous system, and ranges from 0 (No disability) to 10 (Death due to MS). The EDSS system increases by ordinal 0.5 unit increments representing higher levels of disability and is used to quantify disability in MS and monitor changes in disability over time (Meyer-Moock, Feng, Maeurer, Dippel, & Kohlmann, 2014). An advantage to the EDSS is that its broad use enables cross-study comparisons, and it has been noted to be a robust measure over long periods of time. However, several limitations of the EDSS system have been raised including potential subjectivity of neurological examinations resulting in poor inter- and intra-rater reliability, and the unequal interval distances between units of severity with no clear

recommendation on the interpretation of change in EDSS (Meyer-Moock et al., 2014; van Munster & Uitdehaag, 2017).

The Multiple Sclerosis Functional Composite (MSFC), which was developed by the MS Society's Clinical Assessment Task Force, is an another clinical instrument used in clinical trials and created to measure MS disability progression based on a multidimensional metric with a three-part performance scale involving a timed 25-foot walk (T25FT) to assess leg function, the 9-Hole Peg Test (9HPT) to assess arm function, and the Paced Auditory Serial Addition Test (PASAT) to assess attention and cognitive function (Cutter, 1999). The integrated score is calculated based on z-scores, and although its use in clinical studies has increased, critics question the impact of the reference population on resulting standardized scores, making cross-trial comparisons problematic, the impact of practice and learning effects where participant scores improve over time due to learning on the 9HPT and PASAT, and the lack of willingness of many patients to undergo the PASAT. Numerous additional instruments have been created to assess MS disease severity and progression, although few have been noted to meet methodological standards required to be used in clinical trials (Meyer-Moock et al., 2014).

In attempts to measure disability progression using a single clinical assessment, in 2005 Roxburgh et al. created the Multiple Sclerosis Severity Score (MSSS), an algorithm that uses cross-sectional disability assessments to represent disease severity as a whole, based on relating EDSS score to the distribution of disability in patients with comparable disease durations between 1 and <30 years. This algorithm was applied to 9,892 patients from eleven countries and normalized to create the Global MSSS which has been found

to be a powerful and representative method to compare disease progression in groups of patients based on cross-sectional EDSS measurements made after the first year of disease duration (Roxburgh et al., 2005). A limitation of the MSSS however, is the use of disease onset which is often missing in clinical data and often imprecise due to retrospective assignment (Manouchehrinia et al., 2017).

As many clinical variables of MS have been associated with age in natural history studies, Manouchehrinia et al. subsequently adapted the MSSS algorithm by replacing disease duration with chronological age to develop the Age Related Multiple Sclerosis Severity Score (ARMSS), which ranks EDSS scores based on the patient's age at the time of assessment. An updated MSSS matrix was created based on applying the original MSSS algorithm proposed by Roxburgh et al. to their study population. The ARMSS is based on pooled disability data involving 26,058 patients and showed comparable power to detect disability differences between groups when compared to the updated and original MSSS (Manouchehrinia et al., 2017). Although less studied, the ARMSS score holds the advantage of using patient age, which is typically readily available, in place of disease duration, which is required by the MSSS.

In attempts to create a model with greater predictive value, Weidman et al. proposed a MS disease severity scale (MS-DSS), which was derived by making conceptual advancements to the MSSS including the addition of multiple clinical instruments to calculate a score called the CombiWISE score, the inclusion of prospectively acquired MRI scans if available, adjustment for disease modifying therapies (DMTs) use and the application of statistical learning in the form of gradient boosting machines to a training cohort (n =133). The final model achieved a correlation

of r = 0.5448 and was found to outperform the MSSS and ARMSS in the study's validation cohort (n=68) (Weideman et al., 2017). The MS-DSS authors recognize the complexity of applying their methodology and calculating the MS-DSS, but believe that predictive precision cannot be achieved without the collection of a robust range of features to capture the complexity of the MS disease process. In its current form, the MS-DSS model aims to aid clinical decision making relating to personalized treatment and holds the potential to identify further relationships and allow for greater predictive power. Due to its complexity and recent creation, the MS-DSS is not yet widely in use nor has it been tested on larger patient cohorts.

1.5. MS and neuropsychiatric interplay (Focus on immune/inflammatory role)

In patients with MS, comorbid psychiatric disorders such as depressive, anxiety and bipolar disorders are common. There has been increasing evidence for the common role of neuroinflammation in the pathogenesis of both MS and neuropsychiatric disorders, suggesting that inflammatory changes may contribute to disease occurrence, interaction and progression. In a study of population-based administrative health data where 44,452 persons with MS were compared to 220,849 controls matched for age, sex, and geographic area, Marrie et al. found that the incidence and prevalence of major depressive disorder (MDD), bipolar disorder, anxiety and schizophrenia were all higher in the MS population compared to the general population (Marrie et al., 2015). The presence of neuropsychiatric comorbidities have also been found to increase the severity of subsequent neurologic disability in patients with MS (McKay et al., 2018). Early diagnosis and appropriate management of psychiatric comorbidities may be a means to

reduce disability progression in MS. Mood and anxiety disorders may also be present as a result of subclinical intrathecal inflammation in patients with RRMS and may be a predictor of inflammatory reactivations of MS (Rossi et al., 2017).

Depressive disorders are the most common psychiatric comorbidity in MS and occur 2-3-times higher than in the general population and affect up to 50% of people with MS (Patten, Marrie, & Carta, 2017). Depression is a potentially treatable cause of morbidity and mortality in MS, and the presence of depression has been associated with decreased quality of life, increased physical disability, increase in non-adherence to disease-modifying therapies (DMTs) and increased suicidal ideation and completed suicide (Feinstein et al., 2014; Turner et al., 2016). Increasing evidence suggests that MDD and MS share several biological irregularities that may contribute to the pathogenesis of both conditions, including peripheral inflammation, neuroinflammation, chronic oxidative and nitrosative stress, mitochondrial dysfunction, gut dysbiosis and increased neuroendocrine activity and activated microglial pathology (Morris et al., 2018). In a subset of depressed patients with MS, increased levels of peripheral markers of inflammation, including elevated pro-inflammatory cytokines IL-1β, IL-2, IL-6, IL-10, IFN-γ, IFN-β and TNF-α have been found, indicating a significant association between immune dysfunction, inflammation and depression (Vattakatuchery, Rickards, & Cavanna, 2011). Serotonergic transmission is also altered in MS patients and serotonin may also play a protective role in the pathogenesis of MS by modulating the *in vitro* T-cell proliferation and cytokine production by effector T cells in RRMS patients (Sacramento et al., 2018).

In MDD, excessive activation of glutamate receptors by excitatory amino acids results in excitotoxicity and neuronal damage via the dysregulation of calcium homeostasis, triggering the production of free radicals and oxidative stress, mitochondrial dysfunction and subsequent cell death (Olloquequi et al., 2018) Thus there is growing interest and promise in the role of glutamate, and in particular antagonists of N-methyl-D-aspartate (NMDA) glutamate receptors such as ketamine, in the treatment of both neurological and psychiatric disorders. The amino acids glycine and D-serine are co-agonists at the NMDA receptor and have been shown in rat models to produce antidepressant effects through similar mechanisms to ketamine (Wei et al., 2017).

Research on the role of neuroactive steroids (NASs) in the pathogenesis of neuropsychiatric disorders has been promising and NASs have been found to effectively modulate multiple inhibitory and excitatory signaling pathways in the CNS, including those involving γ-aminobutyric acid A ($GABA_A$) and NMDA glutamate receptors, and play a modulatory role in processes such as neurotransmission, neurotoxicity, synaptic plasticity and cell migration (Blanco et al., 2018; MacKenzie, Odontiadis, Le Mellédo, Prior, & Baker, 2007). Growing evidence implicates the role of dysregulation of NAS production in numerous neuropsychiatric disorders including depression and schizophrenia, with growing evidence in anxiety, Parkinson's disease, cognitive impairment in Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS) (Durrant & Heresco-Levy, 2014). In depression, abnormal circulating levels of pregnane steroids, including decreased CSF and plasma concentrations of allopregnanolone (ALLO) and its isomer pregnanolone [3α,5β-tetrahyrdroprogesterone (3α,5β-THP), PREG)], both which both act as positive modulators of the $GABA_A$ receptor; and

elevated levels of epiallopregnanolone (3β,5α-THP), tetrahydrodeoxycorticosterone (THDOC) and its precursor dihydrodeoxycorticosterone (DHDOC), and dehydroepiandrosterone (DHEA) and DHEA-sulfate, which act as negative modulators of the $GABA_A$ receptor, have been identified as contributors to the down regulation of GABAergic activity seen in MDD (MacKenzie et al., 2007).

Preliminary imaging and brain pathology studies suggest findings of atrophy and lesion volume in the frontal and temporal regions in MS patients with depression (Feinstein et al., 2014). In a study of 59 patients with MS, Feinstein et al. found increased lesion volume in the medial inferior prefrontal cortex and anterior temporal atrophy in patients with MS and major depression, accounting for 42% of the variance in depression (Feinstein et al., 2004). In addition, subtle changes in normal appearing white and grey matter, including lower fractional anisotropy and higher mean diffusivity in the dominant temporal and inferior frontal lobes were independent predictors of depression in a sample of 62 MS patients (Feinstein et al., 2010). Imaging studies in patients with MS have also investigated the association between depression and hypothalamic-pituitary-adrenal (HPA) axis dysfunction in patients with MS, and although study findings are mixed, hyperactivity of the HPA axis with elevated cortisol, adrenocorticotropic hormone (ACTH), DHEA sulfate and failure of cortisol suppression with dexamethasone have been identified (Feinstein et al., 2014).

Taken together, growing evidence exists for the role of inflammation and immune response dysregulation in the pathogenesis of numerous neuropsychiatric disorders. The increased prevalence of neuropsychiatric disorders in individuals with MS, and

similarities in pathophysiology, particular with MDD, suggest the likelihood of an overlap and potential shared pathogenesis between MS and psychiatric disorders.

1.6. Role of biomarkers in MS – Current clinical biomarkers

For decades, significant efforts have been made to identify biomarkers of MS for diagnosis and disease activity. A biomarker is defined as an objectively measureable indicator of normal biological or pathogenic processes, or pharmacological response to therapeutic intervention (Biomarkers Definitions Working Group, 2001). Numerous biomarkers of MS have been proposed; however few have been replicated, validated or translated into clinical practice. As MS is a disease of the CNS, with growing evidence of peripheral inflammation and permeability at the BBB, potential biomarkers for MS have been proposed in CSF, peripheral blood, brain neuroimaging, tissue sampling via biopsy and urine samples (El Ayoubi & Khoury, 2017). Reproducibility, disease specificity and clinical relevance remain a challenge. Currently no established blood markers for MS exist. Several CSF markers have been identified, but they do not correlate with disease onset, severity or prognosis. The identification of biomarkers in MS is fundamental to the diagnosis, prognostication and treatment of this debilitating condition.

Biomarkers currently used in clinical practice of MS diagnosis and treatment include magnetic resonance imaging (MRI), IgG oligoclonal bands (OCBs) and levels, serum anti-John Cunningham virus (JCV) antibodies and serum anti-aquaporin 4 antibodies (Housley, Pitt, & Hafler, 2015). MRI imaging is most frequently used to monitor disease activity and to assist with treatment decisions in RRMS in order to minimize new lesion occurrence (Baecher-Allan, Kaskow, & Weiner, 2018). Findings of

gadolinium-enhancing lesions indicate active inflammation and a greater number and size of enhancing lesions is predictive of onset and severity of relapses. Grey matter atrophy, which has been correlated with cognitive dysfunction, may also be a useful biomarker for clinical severity (Housley et al., 2015). Traditional brain MRI findings at the time of diagnosis are found to be modestly predictive of long-term disability, suggesting the need for biomarkers with better prognostic potential (Fisniku et al., 2008).

An abnormal brain MRI is present in most patients with established MS and in more than 80% of patients with CIS who develop MS (Brownlee et al., 2017; Fisniku et al., 2008). The most common findings include multifocal T2-hyperintense white matter lesions in the perventricular, juxtacortical and infratentorial regions, which suggest progression from CIS to MS. As the element gadolinium can only cross the BBB at sites of damage or inflammation, gadolinium-enhancing lesions on MRI indicate the presence of active inflammation and disease burden, and predict onset and severity of relapses (Housley et al., 2015). Studies also suggest a positive correlation between grey matter atrophy and cognitive dysfunction and a positive association between grey matter atrophy and neuropsychological disability in MS patients, which suggests grey matter atrophy on MRI may hold potential as a biomarker for clinical severity (Geurts, Calabrese, Fisher, & Rudick, 2012).

Discovered in the 1960s, CSF IgG OCB is the most established diagnostic CSF biomarker in MS and continues to be routinely used. The presence of OCBs is highly sensitive and is present in approximately 80%-95% of MS patients, but OCBs can also be detected in other neurological conditions (Comabella & Montalban, 2014; Raphael et al., 2015). Since the 2010 revision of the McDonald criteria, evidence of CSF OCBs is no

longer required for the diagnosis of MS, but continues to be used to support the diagnosis. The presence of CSF IgG OCBs has been found to be predictive of CIS conversion to clinically-definite MS (Comabella & Montalban, 2014). The IgG index is also used and provides information on intrathecal synthesis of IgG, with a higher level being associated with greater activity of the disease (Fitzner, Hecker, & Zettl, 2015).

Serum anti-JCV antibodies are primarily used in MS as a biomarker in the context of natalizumab treatment. Natalizumab is a monoclonal antibody of the α4 integrin resulting in reduced T-cell trafficking to the CNS and effectively reducing relapses in MS (Comabella & Montalban, 2014). Progressive multifocal leukoencephalopathy (PML) is a rare, potentially fatal adverse event occurring in 3.7/1000 patients on natalizumab treatment, resulting from reactivation of the latent JCV in immune-compromised individuals (Housley et al., 2015). JCV antibody index in seropositive patients is a useful biomarker in assessing and stratifying risk of PML during natalizumab therapy (El Ayoubi & Khoury, 2017).

Serum anti-aquaporin 4 antibodies are highly specific biomarkers used to differentiate MS from neuromyelitis optica (NMO), an autoimmune inflammatory disease that selectively affects the optic nerves and spinal cord (D'Ambrosio et al., 2015). Seropositive anti-aquaporin 4 antibodies are present in 75-90% of patients with NMO and are close to absent in MS patients, making it a useful biomarker in differentiating MS and NMO, two conditions that may present similarly but have differing pathophysiology, prognosis and treatment (Comabella & Montalban, 2014).

Numerous potential diagnostic and disease activity biomarkers of MS have been explored and identified as promising; however, they have not been consistently replicated

or validated. Current potential biomarkers of interest for identifying conversion from CIS to MS that have been validated in at least two independent cohorts include IgM OCBs, neurofilament light chains, MRZ-specific IgG, kappa free light chains, C-X-C motif chemokine 13(CXCL13), chitinase-3-like protein 1 (CHI3L1), miR-20a-5p and miR22-5p (Teunissen, Malekzadeh, Leurs, Bridel, & Killestein, 2015; Brownlee et al., 2017). Additional exploratory biomarkers include epidermal and hepatocyte growth factors, eotaxin (CCL11), macrophage inflammatory protein-1β (CCL4), anti-myelin MOG antibodies, CSF tau protein levels, anti-KIR4.1 potassium channel antibodies, serum 24-hydroxycholesterol, apoptotic serum Fas molecule, Fas and Fas ligand, TNF-α, serum interleukin-17 concentration, B-cell activating factor (BAFF), anti-neurofilament antibodies, NO, metalloproteases, adhesion molecules, neurotrophins such as brain-derived neurotrophic factor (BDNF) and ciliary neutrotrophic factor and tryptophan metabolism alterations via the kynurenine pathway (El Ayoubi & Khoury, 2017; Housley et al., 2015). Currently, there are a limited number of validated CSF-based biomarkers used in clinical practice and there are no established blood-based biomarkers of MS.

1.7. Present study aims

This study aims to identify potential plasma biomarkers that will assist with the prediction of patients who may develop MS, and differentiate between controls with no known disease, CIS patients, and RRMS patients. In addition, the aim of this study is to look at potential predictors and statistical models of disease progression. Predictive biomarkers of conversion from CIS to clinical MS, and of disease activity and severity are of interest. A literature review was first conducted to determine the role of specific

amino acids and neuroactive steroids in MS pathology and potential as biomarkers. In this study, plasma levels of candidate amino acid levels, including glutamate, glutamine, aspartate, aspargine, glycine, D-serine, L-serine, L-serine-O-phosphate, arginine, alanine, and taurine, and candidate neuroactive steroid levels, including pregnenolone (PREGNEN), pregnanalone (PREG), allopregnanolone (ALLO), epiallopregnanolone, DHEA, and THDOC, were measured in healthy controls, CIS, and RRMS patients in an attempt to identify trends with progression and severity of symptoms and potential biomarkers of MS.

A retrospective chart review was conducted to identify any associations or correlations between patient demographic and clinical variables, such as age, sex, comorbidities, and disease-related dynamic variables such as relapse rate, initial and final EDSS scores, and treatment with DMTs. Clinical measures of disease severity and progression were calculated using the change in EDSS score during the study period, original MSSS, updated MSSS and ARMSS scoring systems. Measured biomarker data along with clinical measures of disease progression were combined to generate a regression model to predict disease progression in this population. Individuals with CIS at the time of study recruitment who converted to late RRMS were also compared to those who did not convert and had no further disease progression. The identification of plasma biomarkers of disease conversion and progression in MS may result in better prediction of disease course, targeted therapies directed towards preventing progression, and improved patient outcomes.

2. Literature review - neurochemical and clinical studies of amino acids and neuroactive steroids in MS

2.1. Overview of amino acids and neuroactive steroids

MS is a heterogeneous and complex disease with numerous documented and associated systemic neurochemical changes. Patterns and changes in one or more neurochemicals, such as amino acids and neuroactive steroids, hold potential as biomarkers of disease process and progression. Measurement of amino acids and neuroactive steroid levels in brain tissue, CSF, plasma, urine and via magnetic resonance spectroscopy (MRS) quantification have been published. CSF and tissue sampling remain invasive and difficult to obtain, MRS quantification is costly and not easily accessible, and urinary studies remain in their infancy. Thus, measurement of plasma neurochemical levels presents a less invasive, cost-effective, time-efficient, accessible and promising option.

Humans and animals need to ingest dietary protein composed of twenty different L-amino acids. In humans, there are nine essential amino acids, which either cannot be synthesized, or whose synthesis is minimal. For each amino acid, a strict homeostasis is maintained in the blood as well as brain (Tsurugizawa, Uneyama, & Torii, 2014). Amino acids play an essential role in synaptic neurotransmission and are involved in activities including learning, memory, sleep, and movement, and sensorimotor function, with abnormal levels of certain amino acids being associated with psychiatric and neurological diseases such as schizophrenia, depression, epilepsy, MS, Alzheimer's diseases (AD) and ALS (Xiong, Guo, Ge, Wang, & Zhang, 2013). Some amino acids function as neurotransmitters and play major excitatory and inhibitory roles in the regulation and control of various functions in the central and peripheral nervous system,

with the most studied amino acid neurotransmitters being glutamic acid, GABA, glycine and taurine.

Glutamic acid is present in over half of all CNS synapses and is the major excitatory neurotransmitter in the mammalian central nervous system and has been linked to several neuropsychiatric disorders whereas GABA, glycine in lower brain areas and taurine play an inhibitory neurotransmitter role in the CNS (Şanlı, Tague, & Lunte, 2015). Taurine has also been reported to be neuroprotective (Lee & Kang, 2017). Glycine and D-serine are co-agonists at the NMDA glutamate receptor. Arginine also acts as an excitatory neurotransmitter and has been reported to be involved in the etiology of MDD and schizophrenia (Musgrave, Tenorio, Rauw, Baker, & Kerr, 2011). In the EAE animal model of MS, Musgrave et al. identified strong correlations between levels of different amino acids and biogenic amines in brain tissue that fit into two distinct groups: Group 1 increased at EAE onset and peaked but decreased in the chronic phase, whereas Group II was composed of amino acids and biogenic amines that change in a progressive manner during EAE. These findings suggest that patterns in neurochemical levels, such as in CSF and plasma, may hold potential as biomarkers for MS disease (Musgrave et al., 2011).

Neuroactive steroids (NASs) are rapid acting steroids synthesized locally from precursors in the central or peripheral nervous system and exert region-specific effects via interacting with neurotransmitter receptors to influence specific neural cells (Belelli & Lambert, 2005; Noorbakhsh et al., 2014). NASs have been found to modulate multiple inhibitory and excitatory signaling pathways in the CNS, including those involving $GABA_A$ and NMDA glutamate receptors, and play a role in processes such as neurotransmission, neurotoxicity, synaptic plasticity and cell migration (Blanco et al.,

2018; MacKenzie et al., 2007; Yilmaz et al., 2019). ALLO is one of the most studied NASs and has been found to have neuroprotective and CNS disease modifying effects through its growth and differentiation promoting effects on neurons and glial cells, with ALLO dysregulation found to play a role in neurodegenerative disorders such as AD, Parkinson's disease and MS via its interactions with $GABA_A$ receptors in the CNS (Noorbakhsh et al., 2014; Tuem & Atey, 2017). NASs such as ALLO, PREGNEN and DHEA have been shown to have anti-inflammatory and neuroprotective properties, which is particularly relevant in MS where the underlying pathology is suspected to involve neuroinflammation and neurodegenerative processes (Orefice et al., 2016). NASs have been implicated in learning and memory, hippocampal information processing and synaptic plasticity and have been found to be associated with neuropsychiatric disorders such as schizophrenia, MDD and anxiety disorders (MacKenzie et al., 2007; Ratner, Kumaresan, & Farb, 2019). Sex differences in the synthesis and levels of NASs under physiological conditions and in various disease states such as AD, Parkinson's disease, MS, traumatic brain injury, stroke, diabetic encephalopathy, and affective and psychiatric disorders have also been shown (Giatti et al., 2019). Giatti et al. published a detailed review of sex differences in NAS levels and steroidogenesis in healthy and disease states (Giatti et al., 2019).

The following section will provide an overview of amino acids and NASs relevant to the study described in this thesis.

2.2. Amino acid abnormalities and MS

In this section, the current understanding of and evidence for the role of specific promising amino acids in MS disease pathology and progression will be provided.

Neurochemical and clinical studies will be summarized. The amino acids discussed are glutamate, arginine, taurine, D-serine, L-serine, glycine and aspartate.

2.2.1. Glutamate

Glutamate is the principal excitatory neurotransmitter in the CNS and its actions are mediated by ionotropic and metabotropic glutamate receptors that are expressed by most cells in the nervous system, non-neural cells in peripheral organs and tissues, and by immune cells including T cells and dendritic cells (Reiner & Levitz, 2018). Glutamate is essential for normal neurodevelopment and function of the CNS, where it exerts rapid and potent effects and plays a major role in cognition, learning, memory, cell migration and cell differentiation (Levite, 2017). Glutamate has been found to activate resting T cells, and T cells can further produce and release glutamate to affect other cells. While glutamate is essential for ongoing CNS function, glutamate excess can result in excitotoxicity which has been identified as an integral mechanism in the pathogenesis of neurodegenerative disease and brain injury, including MS pathology (Kostic et al., 2017). In MS and EAE models, excess glutamate has been found to result in over-activation of glutamate receptors and subsequent excitotoxicity, tissue damage, neuronal death and loss of brain function.

Glutamate and MS - Neurochemical and clinical studies

The role of glutamate in MS and the EAE model has been of great interest, and glutamate is the most widely studied amino acid with regard to contributions to the disease process. Neurochemical and clinical studies of glutamate and MS will be briefly summarized. In *in vitro* murine brain tissue culture, Piani et al. found that brain

macrophages secreted glutamate that resulted in NMDA receptor-mediated neurotoxicity which could subsequently be reduced by exposure to astrocytes or enzymatic degradation and proposed that macrophage-derived glutamate could play a pathological role in MS (Piani, Spranger, Frei, Schaffner, & Fontana, 1992). In EAE mice, Hardin-Pouzet et al. found a significant and dramatic reduction in the glutamate degrading enzymes glutamine synthetase (GS) and glutamate dehydrogenase (GDH), resulting in subsequent pathologic elevation of glutamate (Hardin-Pouzet et al., 1997). An NMDA-receptor-dependent increase in glutamate release, dysfunction of mitochondrial activity and marked astroglia activation was found in EAE mice by Grasselli et al. In a subsequent study, pharmacological blockade of NMDA receptors *in vivo* reversed both synaptic transmission defects and clinical disease course of EAE, suggesting a role for presynaptic NMDA receptor overactivity and increased glutamate release as a mechanism for glutamate-mediated excitotoxicity in EAE and MS (Grasselli et al., 2013). Further to receptor activity, Mandolesi et al. found that in the EAE model, interleukin-1beta (IL-1β) altered glutamate transmission at Purkinje synapses and that IL-1β plays a crucial role as a triggering molecule in EAE pathology (Mandolesi, Gentile, Musella, & Centonze, 2015).

In clinical populations, several studies have reported glutamate levels in the CSF (Kostic et al., 2014; Sarchielli et al., 2003; Stover et al., 1997), plasma (Pampliega et al., 2008) and brain tissue (Azevedo et al., 2014; Werner, Pitt, & Raine, 2001) of MS patients and, overall, found elevated glutamate levels.

Stover et al. found that CSF glutamate levels were significantly elevated in patients with acute MS, compared with known but silent MS (Stover et al., 1997).

Sarchielli et al. measured glutamate and aspartate levels in the CSF of patients with stable RRMS (n=25), acute relapse RRMS (n=30) and secondary progressive form of MS (n=25) compared to controls (n=20) to explore the contributions of excitotoxic insults to the pathological process of MS, and found significantly higher CSF glutamate levels in patients with RRMS compared to controls, with this increase more evident in patients with RRMS during relapse versus stable RRMS ($p<0.001$). Among patients with stable RRMS, significantly higher levels of glutamate were found in those with 1 or more gadolinium (Gd)-enhancing lesions on MRI scan ($p<0.001$). Patients with SPMS with an increase of at least 1 point in EDSS score over the past 6 months showed glutamate levels that were significantly greater than in patients with SPMS without significant changes in EDSS score in the same period ($p<0.001$) In RRMS patients examined within 72 hours of onset of relapse, the number of Gd-enhancing lesions was positively correlated with CSF levels of aspartate, a related excitatory amino acid ($r=0.49$, $p<0.005$) (Sarchielli et al., 2003). These findings suggest that altered glutamate homeostasis plays a role in MS pathology and that antagonizing glutamate-mediated excitotoxicity may have therapeutic implications for MS patients. Kostic et al. measured IL-17A and glutamate levels in active MS patients (n=39) compared to controls (n=40) and although IL-17A levels were significantly higher in MS patients and a direct correlation between IL-17A and glutamate levels was found, unlike other studies, no statistically significant changes in glutamate concentrations were found (Kostic et al., 2014).

Pampliega et al. found elevated plasma glutamate levels in MS patients (n=121) compared to controls (n= 88), and the presence of a polymorphism in the promoter of the glutamate transporter excitatory amino acid transporter (EAAT) 2 was associated with

higher plasma glutamate levels during relapse and may contribute to alterations in glutamate homeostasis (Pampliega et al., 2008).

In a study examining the contribution of imbalanced glutamate homeostasis to axonal and oligodendroglial pathology in MS, Werner et al. compared human CNS tissue obtained by autopsy of MS patients at different stages of disease (n=6) to CNS tissue samples from patients with other neurological conditions (n=10). Active MS lesions showed high-level expression of glutaminase, a marker for glutamate production, in macrophages and microglia, proximal to dystrophic axons. Correlation between glutaminase expression and axonal damage was further confirmed experimentally in EAE mice. In addition, the glutamate metabolizing enzymes GS and GDH were absent from oligodendrocytes in both active and chronic silent MS lesions, suggesting lasting metabolic impediments (Werner et al., 2001). Vercellino et al. evaluated the role of excitotoxicity in MS cortical pathology via immunohistochemistry by examining the pattern of expression of glutamate transporters in MS cortex in 10 autopsied MS brains compared to 3 controls by evaluating EAAT expression. EAATs play an essential role in maintaining lower extracellular glutamate concentrations and preventing excitotoxicity. Vercellino et al. found that in cortical lesions, activated microglia infiltration correlated with focal loss of EAAT1 and EAAT2 and with neuronal immunostaining for pJNK, a protein involved in response to excitotoxic injury, supporting the role of excitotoxicity in demyelination in MS pathology. No reduction in EAATs was observed in demyelinated cortex in the absence of activated microglia (Vercellino et al., 2007). In a study of the post-mortem CNS tissue from 19 cases of clinical MS to investigate the role of glutamate excitotoxicity in MS pathogenesis, Newcombe et al. found that although reactive

astrocytes in MS white matter lesions are equipped to play a protective role in sequestering and metabolizing extracellular glutamate, they may be unable to maintain glutamate at levels low enough to protect oligodendrocytes rendered vulnerable to excitotoxic damage because of glutamate receptor 1 (GluR1) up-regulation (Newcombe et al., 2008). In a prospective cohort study, Azevedo et al. evaluated the in vivo effects of excess brain glutamate on neuronal integrity by measuring N-acetylaspartate (NAA), brain volume, and clinical outcomes, and used multivoxel spectroscopy at 3T to estimate glutamate and NAA concentrations in NAWM and gray matter of MS patients (n=343). The results showed that higher glutamate concentrations increased the rate of NAA decline and each 10% increase in glutamate/$NAA_{[NAWM]}$ ratio was associated with loss of 0.33% of brain volume per year ($p<0.001$), as well as a decline in clinical outcomes on the MSFC and PASAT. Collectively, these findings suggests a relationship between brain glutamate and markers of disease progression in MS (Azevedo et al., 2014).

In summary, glutamate remains the most studied amino acid in the context of MS and EAE to date, with elevated glutamate levels reported most consistently and for decades (Barkhatova, Zavalishin, Askarova, Shavratskii, & Demina, 1998; Westall, Hawkins, Ellison, & Myers, 1980). In addition to increased glutamate levels, various glutamate-related abnormalities including dysregulation in glutamate degradation, transport and signaling have been reported. Numerous review articles have been published including a review paper by Levite which discusses seventeen studies involving glutamate abnormalities in MS and EAE (Levite, 2017) and a review paper by Stojanovic et al. which discusses the role of glutamate and its receptors in MS (Stojanovic, Kostic, & Ljubisavljevic, 2014).

2.2.2. Arginine

Arginine is a dibasic amino acid that is present in mammals in both D- and L-enantiomer forms. Although arginine is considered semi-essential in healthy adults, it is an essential amino acid during human development and under stress conditions such as trauma and disease (Canteros, 2014). Arginine is derived from glutamate or proline to which it is metabolically interconvertible. Its production is restricted to the liver and intestinal mucosa where it is converted to citrulline and is transformed directly into arginine or released in the blood circulation as citrulline which is taken up by the kidney and subsequently transformed into arginine and supplied to other tissues (Grillo & Colombatto, 2004). Arginine metabolism results in the production of NO, creatinine, urea, polyamines, agmatine, glutamate, proline and homoarginine (Morris, 2016). Arginine also acts a regulatory signal of protein uptake, with changes in arginine concentration resulting in regulation of cellular metabolism and function. In the brain, arginine is available through two sources: 1. the L-citrulline cycle and 2. through the BBB via transport by carrier proteins (Canteros, 2014). Arginine metabolism is noted to be complex and versatile with multiple interactions between enzymes using arginine as a substrate and unequal access by enzymes within cells and cell types to arginine reserves (Morris, 2007, 2016). At this time, the role of arginine metabolism in EAE and MS is not clearly understood.

L-arginine is catabolized into NO by nitric oxide synthases (NOS) (Grillo & Colombatto, 2004). NO has a neuroprotective function at physiologic levels and regulates CNS processes including the promotion of optimal cerebral blood flow, consolidation of memory, long-term potentiation, maintenance of sleep-wake cycles and modulation of

immunological function (Virarkar, Alappat, Bradford, & Awad, 2013; Willenborg, Staykova, & Cowden, 1999). However, excessive and pathological levels of NO results in reactive oxygen species (ROS) overproduction, neurotoxicity and neurodegeneration via NO-mediated tissue damage (Willenborg et al., 1999). There is some evidence for the effects of L-arginine regulation and elevated NO in several neurodegenerative and CNS disorders including AD, Parkinson's disease, MS, migraine, seizures, and subarachnoid hemorrhage (Mollace & Nisticò, 1995; Virarkar et al., 2013).

Less is known about D-arginine function, metabolism and transport in the CNS. There is some evidence that D-arginine may play a protective role and is produced when excessive glucocorticoids are present to counteract the neurotoxic effects of glucocorticoids by competing with L-arginine as an inactive stereoisomer in NO production, decreasing its availability for ROS overproduction (Canteros, 2011, 2014). Alternatively, a pathological mechanism has also been proposed such that in EAE and MS, D-arginine may convert to L-arginine, resulting in overexpression in infiltrated macrophages, microglia and astrocytes, leading to elevated NO production and resulting inflammation (Mangalam et al., 2013).

Arginine and MS – Neurochemical and clinical studies

Limited published neurochemical and clinical studies exist on the direct role of arginine in MS, however several studies have looked at NO and citrulline as surrogate markers of arginine in the context of EAE and MS with mixed findings. In a study by Noga et al. of CSF metabolomics in EAE rats at disease onset and disease peak, arginine levels were decreased at disease onset but not at disease peak relative to controls,

suggesting that changes in CSF metabolism occur throughout the disease course (Noga et al., 2012). NO has been found to play both a neurotoxic and neuroprotective role in MS. Ruuls et al. noted that macrophages isolated from the CNS of Lewis rats with clinical EAE produced elevated amounts of NO, which acted as an immune suppressor in EAE (Ruuls, Van Der Linden, Sontrop, Huitinga, & Dijkstra, 1996). Inhibition of the NO pathway by intrathecal and systemic administration of the NOS inhibitor N-nitro-methyl-L-arginine ester to Dark Agouti rats with MOG-induced EAE resulted in direct inhibition of the NO pathway in the CNS and protective effects on EAE severity, extent of CNS inflammation and demyelination, with intrathecal administration being more effective than systemic administration (Danilov, Jagodic, Wiklund, Olsson, & Brundin, 2005). In contrast, in a study investigating the effects of increasing CNS levels of NO via L-arginine administration in EAE, Scott and Bolton found that L-arginine administration significantly delayed disease onset ($p<0.05$), with reduced severity of neurological ($p<0.05$), and histological ($p<0.001$) signs and reduced ROS production, suggesting a protective role of NO during the development of EAE (Scott & Bolton, 2000).

Clinically, Calabrese et al. found the presence of inducible NOS in the CSF of MS patients, which was absent in the CSF of controls. Total NOS activity was increased by 24% and CSF NO levels were elevated in MS patients compared to matched controls (Calabrese et al., 2002). Danilov et al. also found elevated levels of NO in the CSF of MS patients (n=61) compared to controls and found a correlation between CSF nitrite levels and disease activity (Danilov et al., 2003). Haghikia et al. explored the role of L-arginine and the NO pathway in MS and neuromyelitis optica (NMO) by measuring the concentrations of key substrates and enzymes including NO metabolites (nitrate and

nitrite), NOS, asymmetric dimethylarginine (ADMA) – an endogenous inhibitor of NOS activity, and L-arginine levels in the CSF and serum of patients with MS, NMO and other neurological diseases (Haghikia et al., 2015). MS and NMO patients were found to have higher ADMA concentrations in serum when compared to healthy controls and for the MS group, this finding was confirmed in CSF. Serum concentrations of L-arginine and NO metabolites did not differ between groups.

Studies have also assessed the role of citrulline in MS. In a study looking at metabolic alterations associated with MS progression, Mangalam et al. identified D-arginine metabolism pathways, including its conversion to L-arginine, as one of the six pathways significantly altered in chronic EAE (Mangalam et al., 2013). Arginine levels were unchanged and a significant decrease in citrulline levels was noted in the plasma of EAE mice. The authors hypothesized that citrulline may have been extracted from the circulation and converted to arginine and later returned to the circulation, resulting in decreased citrulline and unaffected arginine levels (Mangalam et al., 2013). Conversion of arginine to citrulline is catalyzed by the enzyme peptidyl arginine deiminase (PAD) 2, and in a study by Moscarello et al., PAD2 levels in the brain were elevated in MS normal-appearing white matter, which may be a result of hypomethylation of the promoter region in the PAD2 gene in MS (Moscarello, Mastronardi, & Wood, 2007). Vande Vyer et al. assessed citrulline levels in the plasma of patients with MS (n-25), controls without neurological disease (n=25), and individuals with non-MS cerebral white matter lesions (n=25) and found that median peripheral vein citrulline levels were elevated in patients with MS, which suggests that increased plasma citrulline may be a promising biomarker in MS (Vande Vyver et al., 2018). The administration of citrulline

as a indirect alternative to arginine, has also been explored to control NO production in autoimmune diseases and MS with citrullination dysfunction of myelin basic proteins as the hypothesized mechanism (Curis et al., 2005). In contrast, Faigle et al. explored the role of brain citrullination patterns and T cell reactivity of CSF- derived $CD4^+$ T cells in MS and found that citrullination did not appear to be an important activating factor of T cell response, but may be the consequence of immune- or inflammatory response (Faigle et al., 2019).

2.2.3. Taurine

Taurine is a β-amino acid, derived from cysteine, found in high concentrations in most human cells, including brain, retina, muscle tissue and organ tissue, with notable cytoprotective effects, and is one of the few amino acids not utilized in protein synthesis in humans (Ripps & Shen, 2012). Taurine plays a role in multiple fundamental functions including the regulation of antioxidation, neuromodulation, neurotransmission, brain development, calcium homeostasis, energy metabolism, gene expression, maintaining membrane structural integrity, and the formation of bile salts (Schaffer & Kim, 2018). Growing evidence implicates the depletion of taurine in an array of pathological conditions including diabetes, cardiomyopathy, renal dysfunction, inflammatory disorders, seizures, strokes and neurodegeneration (Ripps & Shen, 2012).

Involvement in antioxidation processes by taurine contributes to its cytoprotective effects. Studies have shown that taurine decreases superoxide generation by mitochondria and reduces oxidative stress by preventing mitochondrial permeability transition and mitochondria-dependent apoptosis (Schaffer & Kim, 2018). Taurine is also an anti-

inflammatory agent that has been found to neutralize hypochlorous, a toxic neutrophil oxidant, to produce a less toxic and more stable anti-inflammatory mediator, taurine chloramine (Marcinkiewicz & Kontny, 2014). The activity of some antioxidant enzymes is highly sensitive to oxidative damage and by limiting oxidative stress and reducing the formation of ROS, taurine may support further antioxidant activity (Schaffer & Kim, 2018).

Taurine has also been shown to prevent glutamate-induced neurotoxicity in cultured neurons, and exerts its neuroprotective effects by reducing glutamate-induced increases in intracellular free calcium by inhibiting calcium influx from various calcium channels and reducing release from internal storage pools, by shifting the ratio of Bcl-2, an anti-apoptic protein, and Bad, a pro-apoptic protein, towards cell survival and by reducing endoplasmic reticulum stress induced by H_2O_2 which can impair neuronal signaling (Chen et al., 2001; Kumari, Prentice, & Wu, 2013; Lee & Kang, 2017; Leon et al., 2009; J.-Y. Wu & Prentice, 2010). Taurine also functions as an inhibitory neuromodulator by acting as a weak agonist of the $GABA_A$, glycine, and NMDA receptors, resulting in inhibition of neuronal excitability. Interestingly, in mice, acute taurine administration has been found to activate the $GABA_A$ receptor, whereas chronic taurine administration results in a functional alteration in the GABAergic system, and downregulates the $GABA_A$ receptor (L'Amoreaux, Marsillo, & El Idrissi, 2010).

Taurine and MS - Neurochemical and clinical studies

In a study comparing chronic EAE diseased and healthy SJL mice, Mangalam et al. identified 44 metabolite signatures which correlated with severity of EAE disease and provide support for metabolic changes as potential biomarkers for EAE/MS progression.

Taurine metabolism was found to be significantly altered and taurine levels elevated in the plasma of EAE mice, which also supports the potential for plasma taurine levels as a potential biomarker for EAE/MS (Mangalam et al., 2013). Using nuclear magnetic resonance (NMR)-based metabolomics and partial least squares discriminant analysis on plasma samples of EAE mice, Dickens et al. were able to identify distinct metabolic profiles between animals with clinically silent disease and animals with active disease via key metabolites including fatty acids, glucose and taurine (Dickens et al., 2015). Levels of taurine were found to be significantly elevated by approximately 20-fold during oligodendrocyte differentiation and maturation in a study using global metabolomics with targeted analyses to identify altered metabolite pools associated with oligodendrocyte differentiation and maturation, a limiting process during progressive stages of demyelinating diseases like MS (Beyer et al., 2018). This study also found that exogenous taurine addition resulted in large increases in pools of serine and cysteine, with serine being a required primary substrate for myelin production via glycosphingolipid (GSL) biosynthesis (Beyer et al., 2018). In a pilot study of the ability to reduce pathological symptoms of EAE by acamprosate (N-acetylhomotaurine), a synthetic acetylated taurine derivative that is able to cross the BBB more readily than taurine, Sternberg et al. found that neurological scores at disease peak were reduced by 21, 64 and 9% respectively in the 20, 100 and 500mg/kg groups, which suggests a potential role for acamprosate and other taurine analogs in the future treatment of MS (Sternberg et al., 2012). Using *in vivo* localized proton magnetic resonance spectroscopy (^{1}H-MRS) in cuprizone-fed mice, metabolic changes were monitored in the corpus collosum, and after 4 and 6 weeks of cuprizone treatment a significant increase in

taurine/total creatine levels was seen compared to mice under normal diet, with no significant metabolic differences seen between cuprizone and control groups after full remyelination 12 weeks later (Orije et al., 2015). This study further supports the potential of metabolic changes during acute demyelination, such as exhibited with cuprizone-induced demyelination and inflammation, to be sensitive markers of disease activity.

CSF taurine levels were found to be significantly increased in patients with acute MS, when compared to controls, and taurine levels appeared unchanged in silent MS (Stover et al., 1997). In addition, Stover et al. found that glutamate significantly increased taurine levels in patients suffering from acute MS, when correlating CSF taurine and glutamate concentrations. Santoro et al. studied the potential of long non-coding RNAs (lncRNAs) as predictive biomarkers of RRMS disease activity in patients (n=12). Taurine up-regulated 1 (TUG1), a lncRNA upregulated by taurine, was found to be upregulated in RRMS patients compared to controls (Santoro et al., 2016). In contrast, a study by Launes et al. comparing patients with acute encephalitis, stroke, MS and controls, found CSF taurine concentrations were significantly lower in those with encephalitis and multiple sclerosis compared to patients in the stroke or controls group (Launes, Sirén, Viinikka, Hokkanen, & Lindsberg, 1998). In a study aiming to use CSF amino acids to differentiate between RRMS and chronic progressive forms, little difference was found between MS patients and controls and a modest 15% increase was observed in taurine in the MS group compared to controls, which though significant, the authors questioned whether this was a chance occurrence (Gårseth, White, & Aasly, 2001). As taurine is an osmotic regulator of cell volume in the brain, this increase in

taurine release in the CSF may result from swelling of damaged neuronal cells in MS pathology.

Taurine levels are largely elevated in the presence of MS pathology in both basic and clinical studies to date, suggesting that there is potential for taurine as a biomarker for MS disease activity.

2.2.4. D-Serine

D-Serine is a D-amino acid that is found in high levels in mammalian brain tissue, notably in the forebrain, cerebral cortex, hippocampus, striatum and limbic forebrain (Armagan, Kanit, & Yalcin, 2012). D-Serine is heterogeneously distributed in the CNS, with the cerebrum containing the highest levels, and may reflect the functional diversity of glutamatergic neurons in humans (Suzuki et al., 2017). D-Serine is an endogenous and potent co-agonist at the NMDA glutamate receptor, increasing receptor affinity for glutamate and decreasing sensitization. D-Serine formation from L-serine is catalyzed by the enzyme serine racemase (SR) and its degradation by the enzyme D-amino acid oxidase (DAAO) (MacKay et al., 2019). D-Serine also plays a role in learning, memory, neuronal migration at developmental stages, synaptic plasticity and cell-death signaling (Armagan, Kanıt, & Yalcin, 2011).

There has been significant interest in recent years in D-serine as a potential treatment and biomarker in schizophrenia and MDD, with clinical studies showing that D-serine may have antidepressant effects and acts as a biomarker for antidepressant response to ketamine (MacKay et al., 2019). D-Serine metabolism deficiency has also been hypothesized to contribute to the underlying etiology of schizophrenia via NMDA

receptor hypo-function. In a 6-week double-blind, placebo controlled trial of D-serine 30mg/kg/day alongside conventional neuroleptics, patients who received D-serine treatment showed significant improvements in positive, negative and cognitive symptoms, and D-serine was well tolerated (Tsai, Yang, Chung, Lange, & Coyle, 1998). Subsequent clinical studies using higher dosages of D-serine also support these findings (Kantrowitz et al., 2010).

Elevated levels of D-serine have been associated with neurodegeneration, oxidative stress and neurotoxicity, with the potential to trigger NMDA-receptor mediated neuronal death via over-activation of NMDA receptors, increased Ca^{2+} and ROS production (Bardaweel, Alzweiri, & Ishaqat, 2014). Neurotoxic levels of D-serine have been linked to other neurodegenerative conditions such as AD and ALS. In AD, the role of excitotoxicity and neuroinflammation via pro-inflammatory stimuli and release of glutamate from microglia has been proposed (Beltrán-Castillo, Eugenín, & von Bernhardi, 2018). Wu et al. exposed cultured microglial cells to amyloid β-peptide (Aβ), a proinflammatory stimuli, and the cultured medium was assayed for levels of D-serine and for effects on primary cultures of rat hippocampal neurons. Elevated levels of D-serine in Aβ-treated microglia were found, suggesting that pro-inflammatory stimuli such as Aβ can contribute to neurodegeneration through the release of excitatory amino acids such as D-serine (S.-Z. Wu et al., 2004). D-Serine levels have also been found to be elevated in sporadic ALS patients and in the G93A SOD1 mouse model of ALS, and a pathogenic mutation (R199W) in DAAO has been found to co-segregate with disease in familial ALS (Paul & de Belleroche, 2014).

D-Serine and MS – Neurochemical and clinical studies

Musgrave et al. characterized changes in tissue concentrations of amino acids and biogenic amines in the CNS, including D-serine which had not previously been measured, in mice with MOG_{35-55} induced EAE in five anatomical regions of the CNS at onset, peak and chronic phase. Findings suggest strong correlations between different neurochemicals appearing to change in two distinct groups, with D-serine being in Group II where changes occur slower, and in a progressive manner and did not subside in the chronic phase of EAE. Spinal cord concentrations of D-serine were also found to be increased at the peak and chronic phases and the authors propose that this increase may be related to upregulation of the converting enzyme SR in activated microglia in the context of neuroinflammation (Musgrave et al., 2011).

In the rat brain *in vivo*, D-serine has been found to induce oxidative stress – increasing lipid peroxidation, protein oxidation, DNA damage and mitochondrial dysfunction and decreasing glutathione levels and antioxidant enzyme activity (Armagam et al, 2011). In a subsequent *in vitro* study, Armagan et al. investigated the use of nonsteroidal anti-inflammatory drugs (NSAIDs) against D-serine-induced oxidative stress in the rat brain and found that when NSAIDs were incubated with D-serine and ROS such as malondialdehyde, NSAID treatment significantly reduced ROS production, lipid peroxidation, and protein oxidation resulting from D-serine treatment. This suggests that controlling D-serine levels may be neuroprotective and in addition to stimulating ROS production via neuronal influx of Ca^{2+} and activation of the NMDA receptor, the authors hypothesize that D-serine may also stimulate ROS formation through a COX-dependent pathway (Armagan et al., 2012).

In a clinical study comparing amino acid levels in CSF of neurological patients with viral meningitis, silent (n=14) and acute (n=21) MS, myelopathy, epilepsy, normal pressure hydrocephalus, stroke and controls with peripheral facial nerve palsy, CSF serine levels (combination of both enantiomers) remained unchanged in all subgroups (Stover et al., 1997). To date, there are no published clinical studies specifically exploring the role of D-serine in MS. Elevated levels of D-serine have been associated with neurodegeneration, oxidative stress and neurotoxicity in other neurological disorders such as AD and ALS, and elevated levels of D-serine have been observed in the spinal cord in peak and chronic phase of the EAE model, suggesting that D-serine may have potential as a biomarker for neurodegeneration and neurotoxicity in MS.

2.2.5. L-Serine

L-Serine is derived from diet, glycine, protein degradation and *de novo* biosynthesis via phosphorylation initiated by 3-phosphoglycerate dehydrogenase (Phgdh) from the glycolytic intermediate 3-phosphoglycerate (3-PG) (Furuya, 2008). Evidence suggests that L-serine is neuroprotective and *de novo* synthesis of L-serine is linked to the development and function of the CNS. L-Serine is essential for mice and human embryo viability and development, and concentrations of L-serine in human CSF and plasma have been shown to decrease with age, suggesting changing needs for L-serine throughout the lifespan. (van der Crabben et al., 2013). In addition to its role in protein synthesis and cell signaling, primarily as a phosphorylation site in proteins, L-serine is a versatile metabolic intermediate necessary for several biosynthetic pathways including direct involvement in the synthesis of D-serine and glycine (both of which both function

as co-agonists at the NMDA glutamate receptor), L-cysteine, phosphatidylserine (PS) and sphingolipids (both of which are components of the cell membrane and cell membrane microdomains); it is also involved indirectly in the biosynthesis of purines and pyrimidines via methylene group transfer (Metcalf, Dunlop, Powell, Banack, & Cox, 2018). L-Serine also acts as a trophic factor for neurons and has been found to enhance hippocampal and cerebellar Purkinje neuron survival and neuritogenesis, along with maturation of membrane voltage responses of Purkinje neurons in cultures. Once differentiated, neurons have a reduced capacity to synthesize L-serine independently and depend primarily on radial glial/astrocyte lineages for exogenous L-serine supply in the CNS (Furuya & Watanabe, 2003).

Altered L-serine synthesis and dysregulation in phosphorylation have been implicated in several human diseases including progressive neurodegenerative conditions such as AD, ALS, and subtypes of frontal temporal lobar degeneration (FTLD) (Metcalf et al., 2018). Dietary supplementation with L-serine as a therapeutic agent is currently being explored in clinical trials for conditions including ALS, AD, hereditary sensory neuropathy Type 1, non-alcoholic fatty liver disease, alcoholic fatty liver disease, and ichthyosis/polyneuropathy (Metcalf et al., 2018). Human L-serine deficiency disorders, particularly in the context of Phgdh deficiency, result in dysmyelination and white matter attenuations on MRI pathologically, and severe neurodevelopmental defects in newborns and children clinically including congenital microcephaly, feeding difficulties, seizures and growth and psychomotor retardation, and progressive polyneuropathy in adults (van der Crabben et al., 2013). Supplementation with L-serine during pregnancy has been shown to prevent the development of neurological symptoms whereas supplementation

after one year of age has been shown to treat seizures and reverse white matter attenuations on MRI but resulted in no improvement in psychomotor development (Furuya, 2008).

L-serine and MS – Neurochemical and clinical studies

Limited published neurochemical and clinical studies exist on L-serine in MS. In EAE mice brain tissue, L-serine levels were significantly elevated at onset and peak phases of disease in the spinal cord, decreased at EAE onset in the brainstem, and elevated at peak and the chronic phase in the cerebellum (Musgrave et al., 2011). There are currently no clinical studies specifically looking at L-serine as a biomarker in MS. As MS pathology has been associated with glutamate excitotoxity, and L-serine is involved in the biosynthesis of co-agonists of the NMDA receptors such as D-serine and glycine, L-serine may be a potential candidate as a surrogate biomarker for MS.

2.2.6. Glycine

Glycine, the simplest amino acid in nature, has anti-inflammatory, immune-modulatory and cytoprotective effects. In addition to its function in protein synthesis, glycine is the precursor for the biosynthesis of bile acids, creatine, glutathione, heme, nucleic acids, uric acid, porphyrins and purines in humans (Furuya, 2008). Glycine is synthesized from serine, threonine, choline and hydroxyproline, primarily in the liver and kidneys (W. Wang et al., 2013). Systolic and mitochondrial serine hydroxylmethyl transferase (SHMT) catalyzes the interconversion between serine and glycine.

Glycine is in substantial metabolic demand in healthy individuals, and plays a key role in: 1. neurotransmission as an inhibitory neurotransmitter via postsynaptic inhibition

at the glycine receptor in the brainstem and spinal cord and as an excitatory neurotransmitter and co-agonist to glutamate at the NMDA receptor in other brain regions; 2. anti-oxidative reactions with anti-inflammatory, immunomodulatory and cytoprotective roles; 3. sensorimotor function; and 4. metabolic regulation. It is also a major constituent in extracellular structural proteins such as collagen and elastin, comprising 1/3 of amino acids (Şanlı et al., 2015; W. Wang et al., 2013). In leukocytes and macrophages, glycine modulates intracellular Ca^{2+} levels via glycine-gated chloride channels and regulates immune function, cytokine production, and the generation of superoxide (Zhong et al., 2003). Glycine levels have been previously found to be elevated in several neuroinflammatory disorders including ALS and MS. One mechanism by which this may occur is through modulation of macrophage effector functions via stimulation of myelin phagocytosis and production of pro-inflammatory mediators such as NO and activation of neutral amino acid transporters (Carmans et al., 2010). Dietary glycine supplementation has been used with some effect in the treatment of metabolic disorders, diabetes, cardiovascular disease, ischemia-reperfusion injuries, inflammatory diseases, and cancers, and to improve neurological function and sleep (Bannai & Kawai, 2012; W. Wang et al., 2013).

Glycine and MS – Neurochemical and clinical studies

In an early study, Tureky et al. identified an increase in glycine concentration in the spinal cord but not in brain tissue of guinea pigs with EAE which was linked with animal paralysis and differed significantly within the spinal cord, with the greatest increase in the lumbosacral cord and at the time of appearance of clinical manifestations of disease, which suggests that glycine plays a role in altered spinal cord transmission in

EAE animals (Turecký, Líška, & Pecháň, 1980). In the EAE mouse model, compared to controls, glycine levels were significantly increased during onset and peak of disease in the spinal cord, decreased during onset in the brainstem and increased during the peak and chronic phase in the cerebellum (Musgrave et al., 2011).

In an early clinical study, Westall et al. compared serum amino acid levels of MS patients (n=99) to controls (n=50) and found glycine levels to be higher than controls in the lower disability states but decreased with greater disability. In comparing RRMS and progressive MS types, the authors did not note any statistical difference from controls in glycine levels. Previously this group also compared urinary amine-containing compunds from MS patients with those of controls and found elevated concentrations of glycine in MS populations (Westall et al., 1980). In another clinical study, glycine levels in the CSF of MS patients were found to be significantly increased in patients presenting with acute MS compared to controls and patients with silent MS (Stover et al., 1997). In a study of blood and CSF catecholamine and amino acid levels in MS patients (n=30) compared to controls (n=20), Barkhatova et al. found significantly elevated blood levels of glycine, although further details were not provided (Barkhatova et al., 1998). Launes et al. studied CSF amino acids in acute encephalitis where patients with MS (n=10) were used as a positive control in the study, and found that levels of glycine were significantly higher in the encephalitis group than controls, and high concentrations of glycine correlated to more favourable outcomes compared to disease-free controls and positive controls (Launes et al., 1998). In a more recent study, Durfinova et al. compared glycine levels in the CSF of 85 MS patients with controls (n=26) and found significantly higher concentrations of glycine in the CSF of all MS patients compared to controls (p=0.0104),

and also in the subset of patients with RRMS (p=0.0133). The authors also noted an increasing trend during the relapse phase compared to remission phase but this was not significant. Collectively, the authors speculated that elevations in glycine levels in the CSF could be a result of impaired glycinergic transmission, glutamate imbalance, or oxidative stress on glycine transporters, leading to reduction in ability to remove glycine from synapses and modulate extracellular concentration as a result of MS pathology (Ďurfinová et al., 2018).

As elevated levels of anti-Epstein-Barr nuclear antigen antibodies, especially Epstein-Barr nuclear antigen-1 (EBNA-1), is currently the strongest non-genetic risk factor for MS, using a peptide microarray, Ruprecht et al. identified 39 significantly higher EBV-peptides in the serum of MS patients (n=29) compared to controls (n=22), of which 17 of those peptides were from EBNA-1 and 13 (76%) were located within the glycine-alanine repeat, suggesting this region may be the primary target and hotspot of altered EBV-antibody response in MS (Ruprecht et al., 2014). In a study looking at pediatric MS patients (n=10) compared to controls (n=10), Lunemann et al. also found that antibody specificities were primarily directed at the glycine-alanine domain of EBNA-1 (Lünemann et al., 2008).

Although changes have been noted in glycine levels in EAE and MS pathology, the implications and relevance of these changes and consideration of glycine as a potential biomarker for MS require further study.

2.2.7. Aspartate

Aspartate is an endogenous amino acid that plays a key role in CNS development, neurotransmission and neuromodulation, reproduction, hormone synthesis and regulation, and cell-to-cell signaling (Ota, Shi, & Sweedler, 2012). Like glutamate, aspartate is present in large amounts in the CNS and though largely recognized to play an excitatory role, more recently aspartate is believed to act as a neuropeptide-like co-transmitter by pathways that release either glutamate or GABA (Nadler, 2011).

Like serine, aspartate occurs in mammalian tissue in significant concentrations as the D-enantiomer in addition to the L-enantiomer (Errico, Napolitano, Nisticò, Centonze, & Usiello, 2009). D-Aspartate occurs particularly in CNS, endocrine, and neuroendocrine tissues, and is involved in the synthesis and release of glucocorticoids, prolactin, oxytocin and steroids (D'Aniello, 2007; Katane & Homma, 2011). In the brain, it is localized to the olfactory bulb, frontal cortex, hippocampus and cerebellum (Afraei et al., 2017). D-Aspartate is also a regulator of neurogenesis and neuronal dendritic growth (Kim et al., 2010). It is abundant during embryonic nervous system development and decreases significantly after birth due to postnatal expression of D-aspartate oxidase (DDO), the only known enzyme that tightly controls endogenous D-aspartate levels (Errico et al., 2009).

D-Aspartate is best known as an endogenous NMDA receptor agonist, directly activating postsynaptic glutamate receptors, and indirectly inducing significant glutamate release in specific brain areas through presynaptic activation of NMDA, metabotropic glutamate receptor 5, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors (Errico, Nuzzo, Carella, Bertolino, & Usiello, 2018).

Currently, D-aspartate is hypothesized to exert an effect in neurological and psychiatric illnesses such as in MS via the potentiation of NMDA receptor-mediated neurotransmission (Katane & Homma, 2011). Through its impact on D-aspartate concentrations, DDO has also been shown to regulate homeostasis of the glutamatergic system and may play a role in suppression of neurodegenerative processes (Li, Han, Yin, Li, & Yin, 2018). In animal models, oral D-aspartate administration has been found to enhance learning and memory in rats and to prevent depression in mice (Errico et al., 2008; Topo et al., 2010).

Aspartate and MS – Neurochemical and clinical studies

Very limited neurochemical and clinical studies currently exist studying the role of aspartate in EAE and MS beyond its role in NMDA receptor activation and glutamate-induced neurotoxicity. Clinically, aspartate has consistently been found to be elevated in the CSF of MS patients, especially in patients with acute neurological impairment (Barkhatova et al., 1998; Sarchielli et al., 2003; Stover et al., 1997). In addition, Sarchielli et al. found that aspartate levels were significantly higher in patients with RRMS during relapse ($p<0.001$) and among patients with stable RRMS with 1 or more Gd-enhancing lesions on MRI scan ($P<0.001$), when compared to stable RRMS patients without Gd-enhancing lesions. Patients with SPMS who had significant changes in EDSS score over a 6 month period also had significantly higher aspartate levels than SPMS patients without significant changes in EDSS score in the same period ($P<0.002$) (Sarchielli et al., 2003). These findings suggest that aspartate levels may vary depending on MS disease phase and activity.

There has been some evidence to suggest a role for exogenous D-aspartate administration in EAE and MS disease progression. Afraei et al. administered D-aspartate to EAE mice to examine its therapeutic efficacy in reducing EAE symptoms and found that D-aspartate may be correlated with disease activity and may have beneficial effects by attenuating the severity and delaying the onset of EAE. In addition, histological analysis showed a reduction in inflammation with D-aspartate administration with significantly lower serum levels of interleukin-6 and higher total antioxidant capacity than control animals, suggesting a neuroprotective effect of D-aspartate (Afraei et al., 2017). Oral D-aspartate may exert its neuroprotective effect by increasing the synthesis of neuroactive steroids that are essential to myelin sheath health (Afraei et al., 2017). Reduced synaptic plasticity reserve has also been linked to progression of disability in MS (Nicoletti et al., 2020). In a study looking at the impact of daily oral D-aspartate intake on synaptic plasticity reserve in progressive MS patients (n=31), Nicoletti et al. found that daily supplementation over a 4 week period increased synaptic plasticity reserve and increased trans-synaptic glutamatergic transmission, suggesting that oral D-aspartate may have a clinical effect on disability progression (Nicoletti et al., 2020).

Aspartate-related molecules and MS

N-Acetylaspartate (NAA) is a derivative of aspartate and a CNS specific metabolite that is well documented to be decreased in patients with MS (Battini et al., 2018; Duan et al., 2017; Orije et al., 2015). Asparagine (ASN) is an α-amino acid formed via the asparagine synthetase reaction where adenosine triphosphate activates aspartate forming β-aspartyl-adenosine monophosphate, which then reacts with glutamine to release free adenosine monophosphate, glutamate and ASN (Poddighe et al., 2017).

Poddighe et al. used metabolomics to analyze the plasma of MS patients (n=32) compared to controls, and L-asparagine was one of the discriminant metabolites of MS identified. Pathway analysis further indicated that asparagine alongside citrulline biosynthesis were key pathways involved in MS (Poddighe et al., 2017).

2.3. Neuroactive steroid abnormalities and MS

The following section will review current understanding and evidence for the role of specific promising neuroactive steroids in MS disease pathology and progression. Neurochemical and clinical studies will be summarized. The neuroactive steroids (NASs) discussed are allopregnanolone, pregnenolone, DHEA, THDOC, pregnanolone, and epiallopregnanolone.

2.3.1. Allopregnanolone (ALLO)

ALLO is an endogenous progesterone derivative that has been widely studied among NASs, with known neuroprotective and neuroregenerative effects alongside an extensive role in neural physiology including cell proliferation, differentiation, survival, migration, and gene expression (Noorbakhsh et al., 2014). Additionally, ALLO modulates the release of several neurotransmitters including glutamate, GABA, acetylcholine, norepinephrine, dopamine, and serotonin (Tuem & Atey, 2017; Zheng, 2009). Progesterone and ALLO are associated with adaptation to stress and injury in the nervous system and with axonal regeneration and myelin repair (Schumacher et al., 2014; 2012). ALLO has largely been studied as a mediator of progesterone's effect and primarily as a potent positive allosteric modulator of $GABA_A$ receptors (Schumacher et

al., 2014). In MS, ALLO is believed to reduce demyelination, axonal injury, microglial reactivity, and lymphocyte infiltration in EAE mice (Noorbakhsh et al., 2014).

ALLO has been shown to reduce inflammation in several animal models of neurodegenerative diseases including MS, AD, Parkinson's disease, Niemann-Pick disease, traumatic brain and spinal cord injury, and epilepsy (Brinton, 2013; Tuem & Atey, 2017). In an AD mouse model, ALLO was found to induce neurogenesis, oligodendrogenesis, white matter generation and cholesterol homeostasis and to reduce β-amyloid and neuroinflammatory burden (Irwin, Solinsky, & Brinton, 2014). ALLO has also been shown to be protective in animal models of neuropathic pain, ischemia, alcoholism, MDD, anxiety, and schizophrenia (Balan, Beattie, O'Buckley, Aurelian, & Morrow, 2019; Melcangi & Panzica, 2014). To date, ALLO and its analog ganaxolone have been tested in preclinical studies for their anxiolytic and antidepressive properties with promising results (Luchetti, Huitinga, & Swaab, 2011). Ganaxolone, a synthetic analogue of ALLO, has been shown to regulate GABAergic transport through GABA transporter 2 induction during neuroinflammation and axonal injury in EAE models, suggesting that ALLO my hold therapeutic potential in MS due to its anti-inflammatory and promyelinating effects and its impact on GABA transport (Paul et al., 2014).

ALLO and MS – Neurochemical and clinical studies

Noorbakhsh et al. compared ALLO levels in autopsy brain tissues of MS patients (n=16) to controls, and found that ALLO levels were significantly reduced, alongside two crucial upstream enzymes (5-α-reductase and akr1c1/c2) in the white matter of MS patients. Analysis of EAE mice spinal cord tissue also showed decreased levels of ALLO, alongside diminished RNA and protein levels of murine isoforms of the akr1 enzymes. In

addition, EAE mice treated with ALLO at the onset of neurological signs showed reduced disease severity and reduced myelin and axonal injury, along with lower lymphocyte infiltration and microglial activation in subsequent neuropathological analysis compared to controls (Noorbakhsh et al., 2011).

Mitochondrial translocator protein (TSPO) is upregulated in microglia and astroglia during neural inflammation and is responsible for the rate-limiting step in the conversion of cholesterol to pregnenolone (PREGNEN) and its derivatives (Daugherty et al., 2013). Daugherty et al. examined the effects of etiofoxine, a pharmacological ligand with ability to modulate TSPO activity and initiate production of NASs locally in the CNS, in the development and progression of EAE in the mouse model. The results suggest that etifoxine attenuated EAE severity when administered before the development of clinical signs and reduced disease severity when administered at disease peak. These findings correlate with diminished inflammation in the lumbar spinal cord and TSPO activation was also associated with upregulation of akr1c14, an enzyme responsible for ALLO synthesis. Modulation of TSPO activity by etifoxine increased oligodendroglial regeneration after demyelination in EAE with reduced peripheral immune cell infiltration and may hold potential as a therapeutic option in MS (Daugherty et al., 2013). Emapunil (XBD173) is another TSPO ligand that has been shown to ameliorate clinical signs and neuropathology in EAE mice. A study by Leva et al. showed that emapunil increased ALLO concentration in the spinal and brain tissue of EAE mice (Leva et al., 2017).

Balan et al. examined the effects of ALLO on proinflammatory signaling through toll-like receptors (TLRs), particularly TLR4 activation in the periphery and CNS and

found that both ALLO and PREGNEN inhibited TLR4 activation and proinflammatory signaling in LPS-activated macrophages and ventral tegmental area in rats, suggesting that inhibition of proinflammatory neuroimmune signaling underlies the protective effect of ALLO in immune cells and in the brain (Balan et al., 2019).

Sex and anatomic differences with several NASs including ALLO levels were studied in the acute EAE (Giatti et al., 2010) and chronic EAE (Caruso et al., 2010) rat models in the spinal cord, cerebellum, cerebral cortex and plasma. In the acute EAE model, Giatti et al. found that ALLO levels were significantly higher in the spinal cord and cerebellum of female control animals compared to males, and acute EAE resulted in a significant increase in ALLO levels in male but not female animals in the spinal cord, and reduced ALLO levels in the female cerebellum. ALLO levels were also increased in the cerebral cortex of female compared to male animals, and EAE resulted in an increase in ALLO in male EAE rats. In the plasma of control animals, ALLO was higher in female animals, and in acute EAE rats ALLO levels were decreased in female animals (Giatti et al., 2010). In the chronic EAE rat model, Caruso et al. found that ALLO was unchanged in both sexes in the spinal cord. In the cerebellum, ALLO levels were higher in control females compared to control males, and significantly decreased in EAE females but not in EAE males. In the cerebral cortex, ALLO levels were significantly higher in control females than in control male animals, but EAE did not induce significant changes in ALLO levels in this region. In plasma, ALLO levels were significantly higher in control females than in males, and also increased in EAE males but not in EAE females (Caruso et al., 2010).

Mifflin et al. explored the role of NASs as mediators of the beneficial effects of exercise in MS using the EAE model. The effect of voluntary wheel running on NAS levels was measured, and male EAE mice had significantly higher ALLO levels compared to female EAE mice, regardless of exercise (Mifflin, Baker, & Kerr, 2018). The authors postulated that the increase in ALLO levels in male mice with EAE may reflect a neuroprotective mechanism in males during chronic EAE.

Clinically, Caruso et al. measured the levels of several NASs in the plasma and CSF of male MS patients (n=26) compared to age-matched controls, and found a significant decrease in ALLO levels in both CSF and plasma (Caruso et al., 2014). Orefice et al. found that ALLO was significantly lower in female RRMS patients than male RRMS patients and controls without neurological disorder. In addition, ALLO levels of female RRMS patients in clinical relapse were significantly decreased compared to male RRMS patients in the same group. ALLO levels were also elevated when male and female RRMS patients with Gd-enhanced lesions were compared to respective gender groups without Gd-enhancing lesions (Orefice et al., 2016).

In summary, ALLO appears to be neuroprotective, with reduced ALLO levels found in both animal models of MS and clinical MS patients. Early studies exploring the treatment of neurological diseases with ALLO have been promising and suggest that ALLO holds the potential to be a biomarker and therapeutic agent in MS.

2.3.2. Pregnenolone (PREGNEN)

PREGNEN is the first steroid formed from cholesterol by P450 side chain cleavage enzyme CYP11A1 in the mitochondria and is the precursor to progesterone

(Payne & Hales, 2004). PREGNEN is synthesized from three main sources: gonads, adrenal glands and brain (Vallée, 2016). PREGNEN is often studied as the precursor to progesterone or in tandem with other NASs, rather than as the primary NAS of interest, and few studies exist studying PREGNEN in MS. Concentrations of PREGNEN are low in the systemic circulation, but much higher in the brain (Weng & Chung, 2016). PREGNEN is required in embryogenesis, with levels decreased with aging and reduced in neurological disease such as with spinal cord injury and neurodegenerative diseases (Weng & Chung, 2016). PREGNEN has been shown to regulate microtubule assembly, neurite outgrowth and cell migration via microtubule-associated protein 2 (MAP2) and CAP-Gly domain containing linker protein 1 (CLIP1, or CLIP-170), by stabilizing and promoting microtubule polymerization and promoting conformational change and activation of CLIP-1 respectively (Fontaine-Lenoir et al., 2006; Weng et al., 2013). PREGNEN has also been found to inhibit NMDA receptor-mediated dopamine release via sigma receptors in the striatum (Nuwayhid & Werling, 2003). Other studies, however, suggest that PREGNEN itself is ineffective and rather that it exerts its effects through metabolites such as PREGNEN sulfate (PREGNENS) and ALLO (Zheng, 2009).

PREGNENS is synthesized from PREGNEN by the enzyme sulfotransferase and is an active steroid within the brain with high affinity for $GABA_A$ and NMDA glutamate receptors (Vallée, 2016). PREGNENS has a significant regulatory effect on the release of neurotransmitters including glutamate, GABA, acetylcholine, norepinephrine and dopamine (Zheng, 2009). PREGNENS is a modulator of synaptic plasticity and has been found to have cognitive enhancing, promnesic, antistress and antidepressant effects (Smith, Gibbs, & Farb, 2014; Zheng, 2009).

PREGNEN and some of its derivatives are known to promote neuronal activity and recovery, enhance learning and memory, reduce depressive symptoms, modulate neuronal excitability, and increase synaptic plasticity (Weng & Chung, 2016). PREGNEN has been proposed to play a role in and/or have therapeutic potential in AD, Parkinson's disease, MS, and peripheral neuropathy (Vallée, 2016). PREGNEN has also been reported to be promising as a treatment for schizophrenia and as an augmentation for antipsychotic treatment (Ritsner, 2010).

PREGNEN and MS – Neurochemical and clinical studies

Sex and regional differences in levels of NASs levels including PREGNEN were studied in the acute EAE (Giatti et al., 2010) and chronic EAE (Caruso et al., 2010) rat models. In the spinal cord, cerebellum, and cerebral cortex, Giatti et al. found that acute EAE resulted in a significant decrease in PREGNEN levels in male but not female animals, indicating that sex dimorphic changes in PREGNEN levels exist (Giatti et al., 2010). In plasma, PREGNEN levels were significantly higher in female animals. In the chronic EAE rat model, Caruso et al. found that PREGNEN levels were significantly affected in the spinal cord and cerebellum in a sex-dimorphic way such that PREGNEN levels were once again decreased in males but unchanged in female animals (Caruso et al., 2010). In the same study, PREGNEN levels were decreased in the cerebral cortex of EAE males compared to control males, and increased in EAE females compared to control females. Plasma PREGNEN levels were also significantly higher in female compared to male rats. In a subsequent clinical study, Caruso et al. found significantly elevated PREGNEN levels in the CSF and plasma of male MS patients compared to age matched controls (Caruso et al., 2014).

Mifflin et al. found that female EAE mice with daily access to a running wheel had increased levels of PREGNEN compared to male mice with EAE given the same access to exercise, suggesting that elevated PREGNEN levels may play a role in promoting disease progression and/or severity (Mifflin et al., 2018). As previously mentioned, Balan et al. studied TLR4 activation in the periphery and CNS and found that both PREGNEN and ALLO inhibited TLR4 activation and proinflammatory signaling in LPS-activated macrophages and the ventral tegmental area in rats (Balan et al., 2019).

In a clinical study by Orefice et al., PREGNEN was found to be increased in the CSF of RRMS patients compared to control groups. During clinical relapse, female RRMS patients also had significantly increased PREGNEN levels compared to stable female RRMS patients. In addition, male RRMS patients with Gd-enhancing lesions had elevated PREGNEN levels compared to female RRMS patients with Gd-enhancing lesions (Orefice et al., 2016)

In summary, studies on the role of PREGNEN in MS are limited. There does appear to be preliminary evidence to suggest that sex, regional and temporal (acute versus chronic) differences exist in PREGNEN levels in EAE and MS. In humans with MS, PREGNEN levels appear to be elevated in CSF and plasma, and this increase is greater in males compared to females. In EAE rats, males appear to exhibit decreased PREGNEN levels in brain tissue, while females appear to have increased PREGNEN levels in plasma. In chronic EAE, elevated plasma PREGNEN levels appear higher in female compared to male rats. PREGNEN levels may also play a role in promoting disease progression.

2.3.3. Dehydroepiandrosterone (DHEA)

DHEA is an endogenous steroid hormone synthesized in the CNS, adrenal glands and gonadal tissue and has known immunomodulating properties (Kipper-Galperin, Galilly, Danenberg, & Brenner, 1999). DHEA is the most abundant circulating steroid in humans and is known to provide protection from several viral, bacterial and parasitic infections (Kipper-Galperin et al., 1999). DHEA is the precursor of androgens and estrogens. DHEA and its sulfated ester DHEA-S, are known to have immunomodulatory and neuroprotective properties, including the promotion of myelination, brain plasticity, and neuronal survival (Aggelakopoulou et al., 2016; Orefice et al., 2016). DHEA-S is the predominant form found in plasma circulation and is converted to the bioactive form DHEA by intracellular sulfatases, which are present in several cell types including monocytes and macrophages ("DHEA. Monograph," 2001). In humans, DHEA concentrations are 4-6.5 times higher in the brain than in plasma, and due to its hypophillic nature, circulating DHEA can cross the BBB readily (Alexaki et al., 2018). The enzyme CYP171 in oligodendrocytes is responsible for DHEA synthesis from precursor PREGNEN (Boghozian et al., 2017).

DHEA is known to bind to numerous receptors, including $GABA_A$, NMDA, G protein-coupled receptors, sigma 1 receptors, and estrogen receptors α and β and to activate several pathways (Alexaki et al., 2018). One pathway by which DHEA has been shown to exert neuroprotective effects is through binding to tropomyosin-related kinase A, a nerve growth factor receptor (Lazaridis et al., 2011). DHEA has also been shown to reduce microglia-mediated neuroinflammation *in vitro* and *in vivo* produced in LPS-induced brain inflammation (Alexaki et al., 2018).

Levels of circulating DHEA decline progressively with age, and have been associated with several diseases associated with aging including AD, cardiovascular disease, cancer, diabetes, and autoimmune diseases such as systemic lupus erythematous and rheumatoid arthritis ("DHEA. Monograph," 2001). There is also growing evidence to support the role of decreased DHEA levels in neurodegenerative conditions including MS, AD, Parkinson's disease, and traumatic brain injury (Yilmaz et al., 2019).

DHEA and MS – Neurochemical and clinical studies

DHEA administration has been shown by Du et al. to prevent development of EAE in SJL/L mice through the reduction of Th1 immune responses on the CNS (Du et al., 2001). Du et al. further examined the effects of supraphysiologic levels of DHEA on the proliferative response and cytokine profile of T cells and found that elevated DHEA favoured Th2 immune responses *in vitro* through inhibition of interleukin-12 production from antigen-presenting cells and/or stimulation of Th2 proliferation during interactions between T cell and antigen-presenting cells, with elevated interleukin-4 and inhibition of interleukin-12-mediated T-cell proliferation and production (Du et al., 2001). Kipper-Galperin et al. examined the ability of DHEA to inhibit inflammatory mediator production in mycoplasma-stimulated glial cells and the impact on acute EAE *in vivo* and found that DHEA markedly inhibited TNFα and interleukin-6 production by glial cells. However, the daily administration of DHEA to mice and rats did not change the clinical outcome of EAE in this study (Kipper-Galperin et al., 1999).

Aggelakopoulou et al. studied the effects of DHEA on CNS autoimmunity and Th17 response in EAE mice and MS patients (n=8) and found that DHEA administration treated established acute EAE in four different mouse models and that estrogen receptor

β expression in $CD4^+$ T cells is required for disease suppression by DHEA. In MS patients, DHEA directly suppressed MS patient immune response and inhibited human Th17 polarization and maintenance, suggesting that compounds targeting estrogen receptor β expression in $CD4^+$ T cells may be beneficial in the treatment of MS (Aggelakopoulou et al., 2016).

Boghozian et al. investigated the expression and action of DHEA and DHEA-S in the CNS tissues of both EAE mice and MS patients (n=10), and found reduced DHEA and CYP17A1 transcript levels in the white matter of both MS patients and EAE mice, compared to controls (Boghozian et al., 2017). This reduction was further associated with increased expression of inflammatory genes including those for interferon-γ and interleukin-1β. Subsequent treatment with DHEA-S in EAE mice ameliorated EAE severity, reduced neurobehavioural deficits, preserved myelin basic protein immunoreactivity, reduced axonal loss, and reduced expression of inflammatory genes including interleukin-1β transcript levels, suggesting a potential role of DHEA-S in the treatment of neuroinflammatory diseases (Boghozian et al., 2017).

Clinically, Orifice et al. assessed the levels of several NASs in the CSF of RRMS patients and found increased DHEA levels in both male and female RRMS patients compared to two control groups (non-inflammatory neurological disease patients and patients without neurological disorders). In addition, male RRMS patients with Gd-enhanced lesions had higher DHEA values than male RRMS patients without Gd-enhanced lesions and higher DHEA levels than female RRMS patients with Gd-enhanced lesions. As DHEA is believed to be neuroprotective, the authors hypothesize that the gender difference in DHEA levels observed is in line with lower MS prevalence in men

than in women. The elevated DHEA values in patients with Gd-enhanced lesions may also suggest that NASs are transported from brain to the systemic circulation across a damaged BBB (Orefice et al., 2016). Ramsaransing et al. attempted to identify factors associated with progression of MS by investigating serum levels of DHEA-S and other molecules in SPMS, PPMS, benign MS and controls, and found that mean DHEA-S levels were lower in MS patients compared to controls though no significant differences between clinical subgroups of MS was found (Ramsaransing, Heersema, & De Keyser, 2005). Kümpfel et al., also found decreased plasma DHEA levels in active MS patients (n=24) after dexamethasone and corticotrophin-releasing hormone test administration compared to age and sex matched controls, suggesting a dysfunction in DHEA secretion in patients with MS (Kümpfel et al., 1999).

In contrast, a study on HPA axis activity in MS patient subgroups by Ysrraelit et al. measured plasma DHEA-S levels alongside cortisol and ACTH levels and found that MS patients had significantly higher cortisol, ACTH and DHEA-S plasma concentrations than controls, suggesting HPA axis hyperactivity in MS (Ysrraelit, Gaitán, Lopez, & Correale, 2008). Heidbrink et al. determined cortisol and DHEA levels in the serum and CSF of MS patients with stable disease or acute relapse compared to patients with other inflammatory and non-inflammatory neurological diseases, and found no significant difference between groups in serum and CSF DHEA levels, although CSF cortisol was decreased and there was a trend in acute MS patients towards elevated CSF DHEA levels, which may be a result of insufficient compensatory mechanisms (Heidbrink et al., 2010).

Téllez et al. found that serum DHEA and DHEA-S levels appear to be significantly lower in progressive MS patients with fatigue at baseline and over time, in comparison to MS patients without fatigue (Téllez et al., 2006). There is also some support for the role of sex hormones in the modulation of MS disease pathology. In a cohort study evaluating sex hormone levels during menstrual cycle phase and correlation with MS disease severity, Foroughipour et al. found that patients with MS had significantly lower DHEA-S, testosterone, and prolactin levels compared to controls in the follicular and luteal phases (Foroughipour et al., 2012).

In summary, most studies suggest that DHEA and DHEA-S concentrations are decreased in EAE and MS pathology. Administration of DHEA has been shown to be neuroprotective and promising in the EAE model. However, its use as a treatment for MS in human subjects has yet to be studied (Yilmaz et al., 2019).

2.3.4. Tetrahydrodeoxycorticosterone (THDOC)

Currently the physiological importance of THDOC is not fully understood. THDOC is a metabolite of the mineralocorticoid deoxycorticosterone and is derived primarily from the adrenal cortex, with conversion of deoxycorticosterone to THDOC occurring in both peripheral tissues and in the brain (Reddy, 2003). THDOC is a positive allosteric modulator of the $GABA_A$ receptor with several documented effects including neuronal membrane hyperpolarization, suppression of gonadotropin-releasing hormone release, and blockage of seizure propagation in the hippocampus (Tuem & Atey, 2017). THDOC also has known sedative, anxiolytic, anesthetic, anticonvulsant, and neuroprotective effects (Tuem & Atey, 2017). Under normal physiological conditions,

THDOC potentiates the inhibitory effects of GABA on corticotrophin-releasing hormone (CRH) neurons, resulting in decreased HPA axis activity. Under stress, THDOC activates the HPA axis, resulting in excitatory GABAergic transmission (Sarkar, Wakefield, MacKenzie, Moss, & Maguire, 2011).

The ability of physiological stress to induce an increase in THDOC and subsequently activate $GABA_A$ receptors makes THDOC a promising biomarker of interest in stress-sensitive conditions such as MDD, epilepsy, panic disorder, and post-traumatic stress disorder (Reddy, 2006). THDOC has been identified as a potential anticonvulsant and may play a role in catamenial epilepsy, where serum THDOC concentrations are reduced, and may modulate seizure frequency (Tuveri et al., 2008). THDOC has also been found to be reduced in AD and is believed to have neuroprotective effects (Saleh & Sadeghi, 2019). Studies suggest that THDOC is an endogenous regulator of acetylcholinesterase and inhibits plaque deposition in AD (Saleh & Sadeghi, 2019). There are currently no published neurochemical or clinical studies of THDOC levels in MS or EAE.

2.3.5. Pregnanolone (PREG)

PREG is a progesterone-derived NAS that is closely related to and shares similar properties with ALLO including being a potent enhancer of $GABA_A$ receptor response and having anticonvulsant, anxiolytic and sedative properties (Kokate et al., 1998). PREG has been termed the equipotent stereoisomer to ALLO and is believed to exert similar effects (Rasmusson et al., 2017). ALLO is one of the most studied NASs in MS and has been found to be neuroprotective. There are currently no specific studies on and a

limited understanding of the role of PREG in animal models of MS and in clinical patients with MS.

PREG has been identified as a GABA-potentiating NAS with anticonvulsant effects that do not appear to result in anticonvulsant tolerance on repeated treatment (Kokate et al., 1998). PREG may also affect nociceptive sensory processing under physiological and pathological conditions via modulation of glycinergic inhibitory transmission (Jiang, Yang, Wang, & Xu, 2006). Further study is required to better understand the role of PREG in neuroinflammatory and neurodegenerative disorders.

2.3.6. Epiallopregnanolone (3β,5α-THP)

The current body of evidence regarding epiallopregnanolone (3β,5α-THP) is limited and its neurobiological role largely unknown. 3β,5α-THP is the 3beta-isomer of ALLO, but it does not appear to have the ability to exert the same effects on $GABA_A$ receptors as ALLO (He, Hoffman, & Stein, 2004). In fact, studies have suggested that 3β,5α-THP can selectively antagonize the positive modulation of ALLO and PREG on GABAergic inhibition (Wang et al., 2000). 3β,5α-THP has also been postulated as a by-product during ALLO synthesis (Higashi, Takido, & Shimada, 2005).

There are currently no studies of the role of 3β5α in animal models of MS or in clinical studies with MS patients. 3β,5α-THP levels have been measured in several other disease processes. In an experimental model of Parkinson's disease, an increase in 3β,5α –THP was found to occur in the striatum and cerebral cortex alongside a significant decrease in PREGNEN in the striatum, supporting a link between changes in the levels of NASs and neurodegenerative diseases (Melcangi, Panzica, & Garcia-Segura, 2011).

3β,5α-THP sulfate has been found to play a role in intrahepatic cholestasis of pregnancy and is elevated in the serum of patients with this condition (Ren et al., 2019). Elevated 3β,5α-THP levels have also been found in women with chronic fatigue syndrome (n=20) (Murphy et al., 2004). In a study looking at levels of NASs in the brain after traumatic brain injury, 3β,5α-THP was one of the NASs found to be decreased at twenty-four hours, seventy-two hours, and two weeks after traumatic brain injury (Lopez-Rodriguez et al., 2015). In a study looking at the influence of 3β,5α-THP on the development of rapid tolerance to the anxiolytic effect of ethanol in mice, pretreatment with 3β,5α-THP was shown to interfere with the development of rapid tolerance to the anxiolytic effect of ethanol (Debatin & Barbosa, 2006). 3β,5α-THP levels were also measured alongside the levels of several NASs in the orbital frontal cortex brain tissue of individuals with PTSD (n=18) (Cruz et al., 2019) and in individuals with migraine and cluster headaches (Koverech et al., 2019), but no significant differences in levels were found compared to controls in either study.

3. Methods and materials

3.1. Study population

Patients for this study were enrolled from the Northern Alberta Multiple Sclerosis (NAMS) outpatient clinic located at the University of Alberta Hospital in Edmonton, Alberta, Canada as part of the blood biobanking study at the University of Alberta MS Centre. This study was conducted with the approval of the University of Alberta Health Research Ethics Board. All participants were diagnosed with CIS or RRMS by a NAMS clinic staff neurologist and followed as an outpatient at the NAMS clinic by a multidisciplinary team. Study controls were voluntary participants recruited with no known neurological disease. NAMS research nurses provided study information verbally and via an information sheet regarding the research study to patients in person following an outpatient clinical visit. The nature and purpose of the study, risks associated with the study participation including blood sample collection, participant confidentiality, and access to patient health information were explained. Potential participants were encouraged to ask questions and given a copy of the study information sheet and consent form for their records. Participants were not compensated for participation in this study and were clearly made aware that study participation was optional and voluntary, and that consent could be withdrawn at any time and for any reason. Potential participants were made aware that their decision as to whether to take part would not affect medical care received. Informed and written consent was obtained and blood draws were performed by NAMS clinic nurses. Patients were recruited on a voluntary basis and informed consent was obtained from all participants. A total of 81 participants were enrolled as follows: 33 RRMS patients, 31 CIS patients, and 17 healthy controls. Blood samples were drawn

between September 16, 2014 and July 7, 2016 and stored at -80°C in a biobank at the BrainPowerLab at the University of Alberta, Edmonton, Alberta. The study period for clinical data collection was from September 16, 2014 to June 30, 2018.

3.2. Analysis of plasma levels of amino acids

A modification of the high-pressure liquid chromatography (HPLC) method of Grant et al. (2006) was used. Protein was precipitated by adding 200 µl of ice-cold methanol to 100 µl of plasma and centrifuging at 12,000 x g for 5 min. The supernatants were then added to vials and derivatized with a mixture of o-phthaldialdehyde (OPA) and the chiral derivatizing reagent N-isobutyl-L-cysteine to produce fluorescent derivatives (structure shown in Figure 1). The samples were then injected on a Waters Alliance 2690XE HPLC system with a fluorescence detector with an excitation wavelength of 344 nm and an emission wavelength of 433 nm. A Waters SYMMETRY C18 precolumn was used and the column was a Waters 4.6 x 150mm SYMMETRY C18 column (3.5um). Calibration curves consisting of varying amounts of each of the amino acids of interest were run in parallel with each assay run. Amino acids measured included alanine, arginine, asparagine, aspartate, D-serine, L-serine, L-serine-O-phosphate (LSOP), glutamine, glutamate, glycine, and taurine. The ratio of glutamate to glutamine, and the ratio of D-serine to L-serine were also determined.

3.3. Analysis of plasma levels of neuroactive steroids

Levels of NASs were determined using gas chromatography-mass spectrometry analysis (GC-MS). A modification of the procedure of Noorbakhsh et al. (2011) was

used. Protein was precipitated from the plasma samples (1ml) by the addition of methanol (500µl) and centrifuging at 6.000 x g for 5 min. The supernatants were retained and the NASs were isolated using solid-phase extraction with Oasis 30mg HLB plates. Extraction from the plates was done using 500µl of methanol:methylene dichloride , 10:90 v/v and the eluates were taken to dryness in a Savant evaporator. The residues were derivatized with heptafluorobutyrylimidazole and the resultant derivatives were analyzed on an Agilent gas chromatograph-mass spectrometer using negative ion chemical ionization. Alafaxolone was carried through the entire procedure as an internal standard, and standard calibration curves consisting of the same fixed amount of internal standard as added to the plasma samples and varying amounts of each of the steroids were run through the procedure in parallel. A schematic of the dervatization procedure is shown in Figure 2. NASs measured included 3β,5α-THP, ALLO, DHEA, PREG, PREGNEN and THDOC.

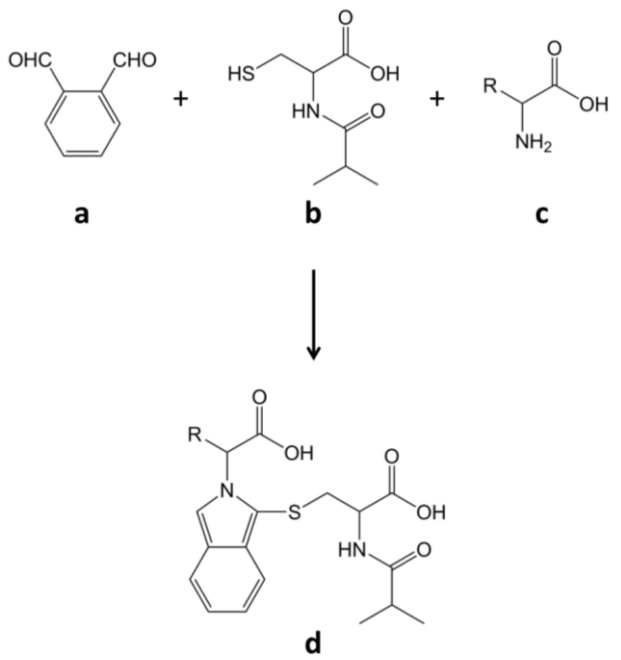

Figure 1: Derivatization of amino acids to form fluorescent derivatives for HPLC analysis.
a = o-phthaldialdehyde; b = N-isobutyl-L-cysteine, c = generic structure of amino acids and d = isoindole derivative of an amino acid.

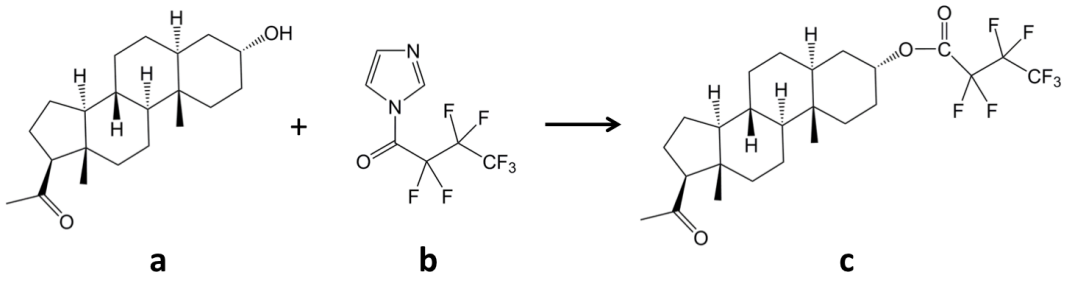

Figure 2: Derivatization of neuroactive steroids to form derivatives for GC-MS analysis.
a = allopregnanolone, b = heptafluorobutyrylimidazole and c = HFP derivative of allopregnanolone.

3.4. Clinical data collection

A retrospective electronic chart review was conducted to extract clinical and demographic variables using a combination of eClinician and Alberta Netcare provincial patient databases for all study participants. Information reviewed included clinical notes, hospital admission reports, discharge reports, diagnostic imaging reports, and medication prescription records. Data extracted included demographic variables including gender, age, disease duration at recruitment, disease duration at final EDSS, and length of study participation. Treatment variables included whether patients were on DMTs at time of blood draw and during the study period, corticosteroid use during the study period, reported fatigue at blood draw, medication prescribed for fatigue during the study period, reported neuropathic pain at blood draw, and medication prescribed for neuropathic pain during the study period. Comorbidities studied included depression, cardiovascular disease, metabolic risk factors (hypertension, dyslipidemia, type 2 diabetes, and documented obesity), headache, malignancy, thyroid disorder, respiratory condition and inflammatory bowel disease.

Initial and final clinical measures of disease severity were determined from clinical data collected. Initial and final EDSS scores were obtained from documentation in clinical visit notes. For participants where EDSS was not documented and sufficient information was available, clinic visit notes were reviewed and scored retrospectively by two independent clinicians. Consensus was obtained in rare instances of discrepancy. Initial and final MSSS scores were determined based on protocols detailed in Roxburgh et al. (2005). Initial and final updated MSSS and ARMSS scores were determined based on protocols detailed in Manouchehrinia et al. (2017). Participant data were excluded

from analysis in cases where clinical scores or variables could not be determined or information was insufficient or unavailable.

3.5. Statistical analysis

Analysis was carried out using IBM SPSS Statistics for Windows, Version 24. The statistical analyses were carried out in three parts. First, differences in demographic variables were assessed via χ^2-tests for categorical variables, and t-tests and ANOVA with Bonferroni *post-hoc* corrections tests for continuous variables. Differences between levels of NASs and amino acids were assessed via Kruskal-Wallis tests. Second, t-tests were used to evaluate the differences in disability scores between CIS and RRMS groups. To assess correlates of change in disease severity, Pearson correlations were conducted to identify the biochemical correlates of the change in disability scores. Lastly, a three-step hierarchical regression was conducted with change in disability score as the dependent variable. In the first step age, sex, and disease duration were entered to control for demographic differences. NASs that correlated with disease progression (at $p < 0.10$) were added in the second block. Depression was added at the last stage. All non-parametric biochemical variables were log transformed to conduct correlation and regression analyses. As indicated in the figure legends, $p < 0.05$ was considered significant.

4. Results

4.1. Clinical Cohort

Three patient groups were recruited to the study, namely healthy controls (n=17), CIS (n=31) and RRMS (n=33). Demographic and clinical features of the groups are summarized in Table 1.

Table 1. Demographic and clinical features of the groups (N = 81)

Variables (last 4 variables expressed as mean +/- SD)	**HC (*n* = 17)**	**CIS (*n* = 31)**	**RRMS (*n* = 33)**
Gender (%female)	9 (52.94%)	21 (67.74%)	24 (72.73%)
Age (in years)	38.44 (11.72)	38.65 (10.81)	35.91 (10.65)
Disease duration at recruitment (in years)	-	0.85 (2.42)	5.22 (6.31)$^{+}$
Disease duration at final EDSS (in years)	-	2.68 (2.68)	7.52 (6.83)$^{+}$
Length of study participation (in years)	-	1.85 (0.93)	2.30 (0.97)
Treatment Variables			
DMTs at blood draw	-	0	9 (27.27%)$^{+}$
DMTs during study period	-	14 (45.16%)	26 (78.79%)$^{+}$
Corticosteroids during study period	-	5 (16.13%)	14 (42.42%)$^{+}$
Fatigue reported at blood draw	-	4 (12.90%)	11 (33.33%)
Medication prescribed for fatigue during study period	-	1 (3.23%)	2 (6.06%)
Neuropathic pain reported at blood draw	-	11 (35.48%)	15 (45.45%)
Medication prescribed for neuropathic pain during study period	-	9 (29.03%)	9 (27.27%)
Comorbidities			
Depression	0	7 (22.58%)	14 (42.42%)*
Cardiovascular disease	0	1 (3.23%)	3 (9.09%)
Metabolic risk factors (Hypertension, Dyslipidemia, Type 2 Diabetes, Documented obesity)	2 (11.76%)	6 (19.35%)	6 (18.18%)
Headache	0	4 (12.90%)	3 (9.09%)
Malignancy	0	0	1 (3.03%)
Thyroid disorder	0	4 (12.90%)	4 (12.12%)
Respiratory condition	0	3 (9.68%)	2 (6.06%)
Inflammatory bowel disease	1 (5.88%)	2 (6.45%)	0
Clinical measures of disease severity (values expressed as mean +/- SD)			
Mean initial EDSS	-	1.84 (0.98)	2.52 (1.28)$^{+}$

Mean final EDSS	-	1.93 (0.99)	2.42 (1.36)
Mean initial Original MSSS	-	4.43 (2.85)	4.16 (2.35)
Mean final Original MSSS	-	4.39 (1.96)	3.83 (2.11)
Mean initial Updated MSSS	-	4.39 (2.69)	4.69 (2.22)
Mean final Updated MSSS	-	4.73 (1.76)	4.48 (2.03)
Mean initial ARMSS	-	3.73 (2.05)	5.18 (2.21)$^{+}$
Mean Final ARMSS	-	3.62 (1.77)	4.65 (2.09)$^{+}$
Conversion to RRMS during study period	-	16 (51.61%)	-

Note. HC =healthy controls; CIS = clinically isolated syndrome; RRMS = relapsing-remitting multiple sclerosis; DMTs = disease modifying therapies; EDSS = Expanded Disability Status Scale; MSSS= Multiple Sclerosis Severity Score; ARMSS = Age Related Multiple Sclerosis Severity Score

*Significant difference from controls, $p < 0.05$; $^{+}$Significant difference from CIS to RRMS, $p < 0.05$

Groups were similar in terms of demographic and clinical features including age, sex distribution, reported fatigue, medication prescribed for fatigue during the study period, reported neuropathic pain, and medication prescribed for neuropathic pain during the study period. The patient groups were also similar in terms of medical comorbidities including cardiovascular disease, metabolic risk factors, headache, malignancy, thyroid disorder, respiratory conditions, and inflammatory bowel disease. Of interest, the likelihood of depression was significantly greater in the RRMS group ($p = 0.005$) than in the control group. As expected, disease durations at the study onset and completion were significantly shorter in the CIS versus RRMS groups, and CIS patients were less likely to receive DMTs or corticosteroids during the study period. In terms of clinical measures of disease severity, initial EDSS was significantly lower in the CIS group compared to the RRMS group ($p = 0.021$). Both initial ARMSS ($p = 0.009$) and final ARMSS ($p = 0.04$) were found to be significantly higher in the RRMS group. Initial and final original MSSS and updated MSSS scores did not differ between the patient groups and did not change significantly during the study period. Over half (51.61%) of the CIS patients in the study

converted to RRMS over a median period of 1.85 years. Thus, the present cohort resembled prior studies of CIS in terms of disease conversion to clinical MS.

4.2. Plasma amino acid and neuroactive steroid levels

Previous studies have shown that NAS and amino acid metabolism and/or concentrations are affected in several neuropsychiatric and neurodegenerative conditions, including MS pathology. Thus, the plasma concentrations of specific amino acids and NASs of interest were investigated in the present cohort using blood samples collected as part of the Blood Biobank at the MS Centre study. Table 2 summarizes the median plasma levels of specific amino acids and NASs in healthy controls and CIS and RRMS patient cohorts.

Median concentration of 3β,5α-THP ($p = 0.021$) was significantly lower and allopregnanolone ($p = 0.031$) was significantly higher in RRMS patients compared to CIS patients (Table 2). No significant differences in plasma concentrations were found with all other NASs measured in this study between patient cohorts including DHEA, PREG, PREGNEN, and THDOC. Ratios of ALLO:PREGNEN, DHEA:PREGNEN, and THDOC:PREGNEN concentrations did not differ amongst groups.

Among amino acids measured, alanine ($p =0.002$), arginine ($p = 0.027$) and glutamine ($p = 0.039$) concentrations were significantly lower in the RRMS group than in healthy controls while LSOP ($p = 0.008$) and taurine ($p = 0.017$) concentrations were higher in the RRMS than the HC group. Aspartate ($p = 0.009$) and taurine ($p = 0.012$) concentrations were also found to be significantly higher in the RRMS group compared to CIS group. Alanine ($p = 0.027$) and arginine ($p = 0.024$) concentrations were

Table 2. Median plasma concentrations of amino acids and neuroactive steroids in patients with CIS and RRMS compared with controls (N = 81)

BIOCHEMICAL	HC (n = 17)	CIS (n = 31)	RRMS (n = 33)
Neuroactive steroid (pg/mL)			
Epiallopregnanolone (3β5α-THP)	73 (18-274)	146 (0-560)	63 (0-253)$^{+}$
Allopregnanolone (ALLO)	98 (25-550)	65 (24-169)	104 (10-553)$^{+}$
DHEA	3291 (1530-8319)	4533 (1294-8929)	4051 (200-10576)
Pregnanolone (PREG)	213 (74-453)	198 (25-415)	180 (25-503)
Pregnenolone (PREGNEN)	3078 (1109-5409)	3176 (200-8531)	3023 (200-8092)
THDOC	15 (9-38)	21 (6-99)	21 (7-50)
ALLO:PREGNEN ratio	0.03 (0.01-0.16)	0.02 (0.0-0.33)	0.03 (0.0-0.41)
DHEA:PREGNEN ratio	1.32 (0.50-4.92)	1.39 (0.40-40.17)	1.21 (0.06-52.88)
THDOC:PREGNEN ratio	0.006 (0.002-0.02)	0.007 (0.001-0.19)	0.006 (0.002-0.05)
Amino acid (μG/mL)			
Alanine (ALA)	31.88 (13.08-49.62)	25.73 (0.56-35.87)*	23.90 (0.56-39.34)*
Arginine (ARG)	8.27 (5.26-14.44)	6.83 (4.33-10.63)*	6.96 (0.30-12.29)*
Asparagine (ASN)	5.19 (0.03-7.65)	4.77 (3.17-7.48)	4.39 (0.03-7.11)
Aspartate (ASP)	0.45 (0.02-0.70)	0.40 (0.19-0.98)	0.54 (0.02-1.10)$^{+}$
D-Serine (D-SER)	0.11 (0.06-0.19)	0.13 (0.08-0.22)	0.13 (0.02-0.33)
L-Serine (L-SER)	10.41 (5.44-15.07)	9.96 (5.54-15.12)	9.19 (0.20-13.52)
L-Serine-O-phosphate (LSOP)	0.04 (0-0.11)	0.08 (0-0.14)	0.08 (0-0.12)*
Glutamine (GLN)	74.71 (57.84-93.21)	71.73 (1-96.69)	63.19 (1-100.91)*
Glutamate (GLU)	2.80 (1.63-8.99)	2.64 (0.80-9.19)	3.12 (0.14-7.53)
Glycine (GLY)	16.29 (12.03-26.23)	15.73 (9.29-38.64)	17.10 (9.81-35.68)
Taurine (TAUR)	5.52 (3.45-12.52)	5.83 (3.07-18.28)	9.53 (3.71-26.15)$^{*,+}$
GLU:GLN ratio	0.04 (0.02-0.15)	0.04 (0.01-3.70)	0.05 (0-4.71)
D-SER:L-SER ratio	0.01 (0.01-0.02)	0.01 (0.01-0.02)	0.01 (0.01-1.65)

Note. *Significant difference from controls, $p<0.05$; $^{+}$Significant difference from CIS to RRMS, $p<0.05$

significantly lower in the CIS group compared to healthy controls. No significant differences in plasma concentrations were found among the remaining amino acid

concentrations measured including plasma concentrations of asparagine, D-serine, L-serine, glutamate and glycine. Ratios of glutamate:glutamine and D-serine:L-serine concentrations did not differ among groups. In summary, the plasma levels of several specific NASs and amino acids differed significantly between groups.

4.3. Correlates of change in disease disability scores

Correlates to changes in disease disability and severity over the study period were of interest, particularly with regard to NASs and amino acid levels. Thus, initial and final EDSS, original MSSS, updated MSSS and ARMSS values were obtained or determined from clinical chart review. EDSS and ARMSS data were available for n=64 (CIS and RRMS; missing 2) whereas original and updated MSSS scores were missing or undeterminable for half of the CIS and RRMS patients (n = 34 available, n = 31 missing). To maximize data retention, the disability analyses focused on the EDSS and ARMSS values. Corresponding EDSS and ARMSS mean (+/- SD) values in the initial and final assessments are in Table 1. To assess the correlates of the change in disability scores, both CIS and RRMS groups were combined. The change in EDSS (Figure 3) and ARMSS (Figure 4) between the initiation and completion of the study was computed for each CIS and RRMS patient revealing a wide distribution of values for each group. An increase in EDSS scores across both groups was significantly and inversely correlated with plasma PREG concentrations ($r = -0.267$; $p = 0.041$) and positively correlated with disease duration at final EDSS assessment ($r = 0.310$; $p = 0.013$), accompanied by a non-significant positive correlation with THDOC concentrations in plasma ($r = 0.237$; $p = 0.068$), depression ($r = 0.231$; $p = 0.071$) and cardiovascular disease ($r = 0.236$; $p =$

0.062). Changes in ARMSS scores were not significantly correlated with any NAS or amino acid, and were only non-significantly correlated with THDOC concentrations ($r = 0.225$; $p = 0.084$) and disease duration at final EDSS assessment ($r = 0.244$; $p = 0.053$).

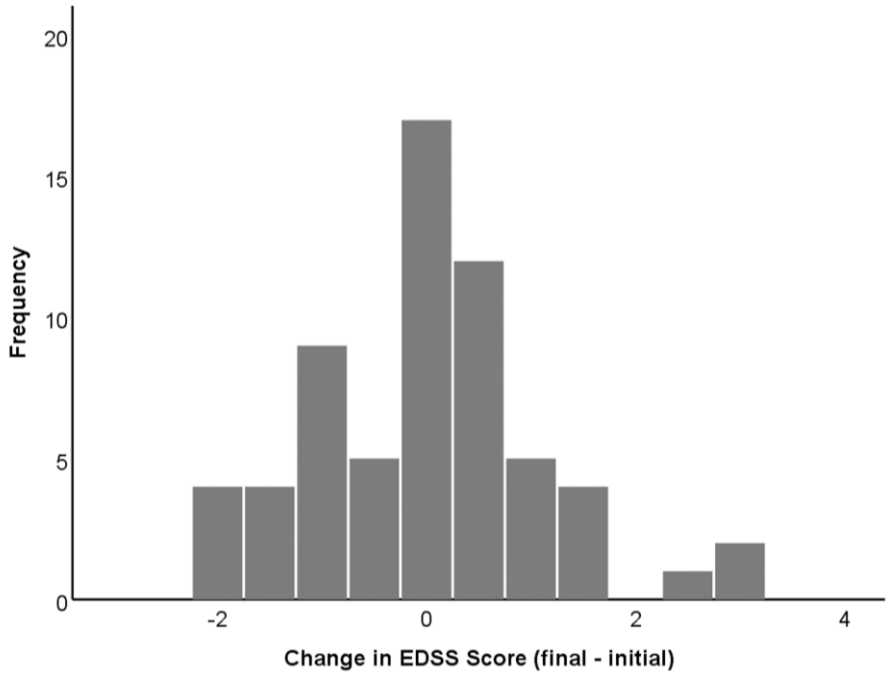

Figure 3. Change in EDSS scores between initiation and completion of study.
Distribution of change in EDSS scores among patients with MS and CIS during the study period.

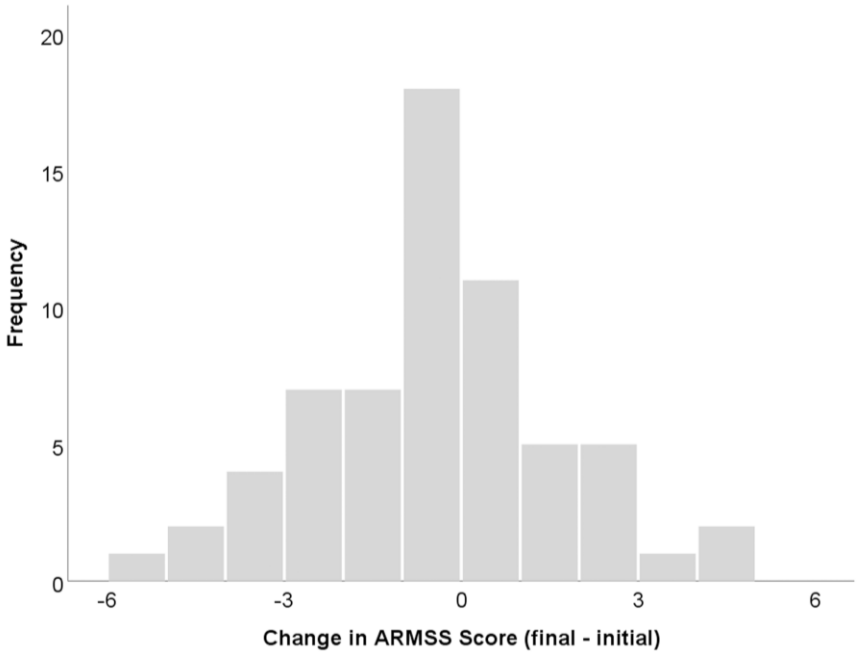

Figure 4. Change in ARMSS scores between initiation and completion of study.
Distribution of change in ARMSS scores among patients with MS and CIS during the study period.

4.4. Predictors of change in disability scores

A three-step hierarchical regression was carried out to assess predictors of EDSS change scores given the few correlations found previously. Given that the few correlations with the change in ARMSS replicated some of the correlations found with the EDSS score, only the latter was used as a dependent variable. Variables in the first block accounted for 14.3% of variance, and only disease duration at the final EDSS assessment was significantly associated with an increase in change scores (Table 3). Adding PREG and THDOC to the model further explained 11.0% of the variance. With the addition of the NASs, disease duration was no longer a significant predictor. Changes in EDSS scores were significantly positively associated with changes in plasma concentrations of THDOC, whereas PREG concentrations were inversely associated but not significant (Table 3; Figure 5 & 6). Adding depression to the model did not significantly increase the explained variance and was a not found to be significantly associated with change in EDSS.

Table 3. Three-step hierarchical regression model variables and variances.

Variable		β	*p*-value	R^2	ΔR^2
Step 1				.14	--
	Age	.05	.70		
	Sex	-.10	.46		
	Disease duration	.35	.01		
Step 2				.24	.11
	Age	-.06	.68		
	Sex	-.05	.72		
	Disease duration	.25	.08		
	Pregnanolone	-.30	.04		
	THDOC	.29	.03		
Step 3				.28	.04
	Age	-.07	.63		
	Sex	-.05	.70		
	Disease duration	.26	.06		
	Pregnanolone	-.26	.08		
	THDOC	.28	.04		
	Depression	.20	.12		

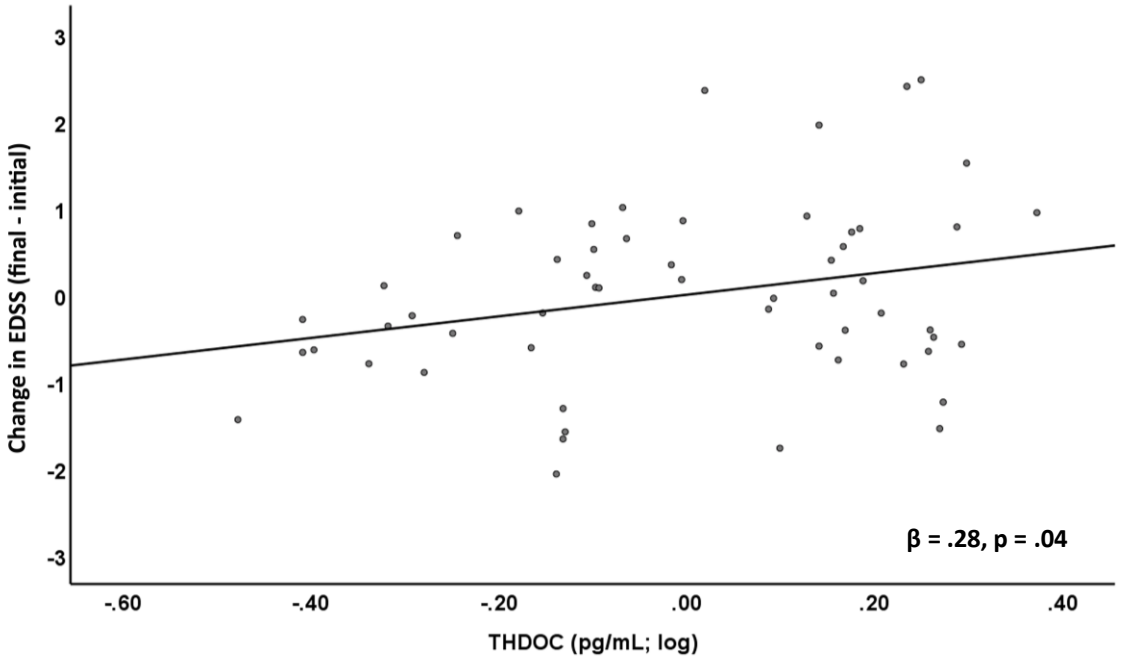

Figure 5. THDOC concentration with change in EDSS.
Plasma THDOC concentrations were positively correlated with EDSS changes in a three-step hierarchical regression model.

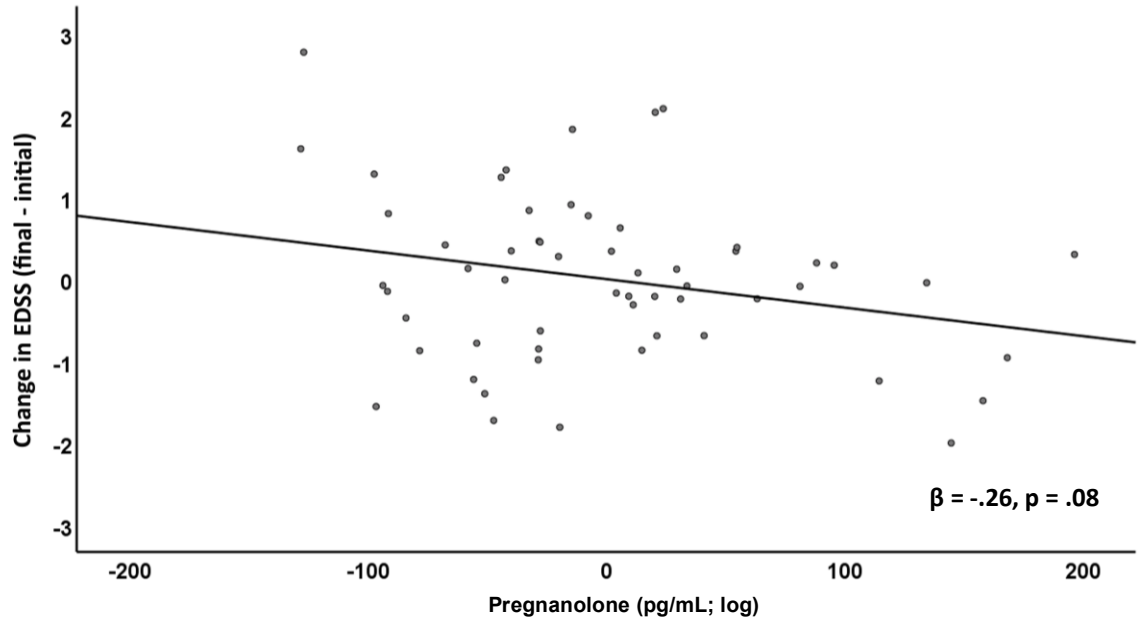

Figure 6. Pregnanolone concentration with change in EDSS.
Plasma pregnanolone concentrations were negatively correlated with EDSS changes in a three-step hierarchical regression model.

5. General discussion

5.1. Plasma amino acid and NAS levels

The results of this study suggest that there is a potential role for plasma amino acids and NASs as biomarkers of MS disease conversion and progression. Of interest, this study found that among the individual amino acids studied, glutamine, alanine, and arginine concentrations were significantly lower in the RRMS group compared to healthy controls. Alanine and arginine were also significantly lower in the CIS group compared to controls.

These findings support the current understanding of MS pathogenesis, in which neuroexcitotoxicity via overproduction of glutamate plays a central role. In MS and EAE, excess in glutamate has been found to result in over activation of glutamate receptors and subsequent excitotoxicity, tissue damage, neuronal death, and loss of brain function.

Glutamine and arginine both belong to the "glutamate family" of amino acids, that are interconvertible with and disposed of through conversion to glutamate (Tapiero et al, 2002). The decrease in glutamine and arginine concentrations in the RRMS group and arginine in the CIS group may result from the depletion of glutamine and arginine used as substrates as a result of conversion and overproduction of glutamate. In our study, the level of plasma glutamate was not found to be significantly elevated which may be a function of small sample size, complex mechanisms and function of the glutamate-glutamine cycle, and measurement in the periphery using plasma samples as opposed to CSF in which prior studies have reported elevated CSF glutamate levels.

L-Arginine is also catabolized into NO, which at physiological levels is neuroprotective but, in excess and at pathological levels, arginine leads to ROS

overproduction in macrophages, microglia and astrocytes, and results in inflammation, neurotoxicity, and neurodegeneration. As previously discussed in chapter 2.2.2, NO overproduction is one mechanism linked to neurodegeneration in several CNS disorders including AD, Parkinson's disease, and MS, and thus, a decrease in arginine concentration in the RRMS and CIS groups may also be a result of its use as a substrate in the overproduction of NO. Interestingly, Noga et al. previously found that in EAE rats, CSF arginine levels were decreased at disease onset but not at disease peak relative to controls, suggesting that temporal changes in arginine metabolism occur throughout EAE disease course (Noga et al., 2012). If this change in metabolism is reflected in plasma, then arginine concentration may be a useful biomarker for MS disease course and stage, including identification of the acute disease peak.

Plasma alanine concentrations were also found in this study to be significantly lower in both RRMS and CIS groups compared to healthy controls. Alanine has not previously been studied specifically in MS and EAE and thus little is known in terms of the significance of this finding. However, alanine is also known to play a role in immune system regulation, which is disrupted in MS pathology (Cruzat, Krause, & Newsholme, 2014).

In this study, LSOP and taurine concentrations were significantly higher in the RRMS cohort than in healthy controls. Aspartate and taurine concentrations were also found to be significantly higher in the RRMS group compared to the CIS group.

Taurine has been shown to decrease superoxide generation and act as an anti-inflammatory agent, with the ability to dampen glutamate-induced neurotoxicity (Schaffer & Kim, 2018). As discussed in chapter 2.2.3, acute taurine administration has

been found to activate the $GABA_A$ receptor, whereas chronic taurine administration results in a functional alteration in the GABAergic system and downregulation of the $GABA_A$ receptor (L'Amoreaux et al., 2010). Findings in our study of elevated plasma taurine levels in CIS and RRMS cohorts are in keeping with previous evidence in the EAE model, where taurine metabolism was found to be significantly elevated in the plasma of EAE mice (Mangalam et al., 2013). Clinically, taurine levels have been found to be significantly increased in the CSF of patients with acute MS and unchanged in silent MS, suggesting a temporal relationship in taurine concentrations depending on phase of disease (Stover et al., 1997). One limitation of the current study is that information on the phase of disease at the time of blood draw is unavailable, making it difficult to explore temporal relationships in disease course and whether plasma concentrations varied in our study population depending on disease phase. To our knowledge, the majority of clinical studies have measured taurine levels in CSF samples, and this study is one of the first to show elevated taurine levels in the plasma of MS patients. This finding is also promising in that should distinct biomarkers of MS disease be present in plasma, this would present a more accessible, cost-effective, and less invasive method of detection in comparison to obtaining CSF samples. Taurine levels are largely elevated in the presence of MS pathology in both basic and clinical studies to date, and our current study continues to support this finding. Collectively, this suggests that there is potential for taurine as a biomarker for MS disease activity.

In this current study, aspartate concentrations were found to be significantly higher in the RRMS group compared to the CIS group. As discussed in chapter 2.2.7, D-aspartate is known to be an endogenous NMDA receptor agonist, directly activating

postsynaptic glutamate receptors and indirectly inducing significant glutamate release in specific brain areas. D-Aspartate is hypothesized to exert an effect in neurological and psychiatric illnesses, such as in MS, via the potentiation of dysfunctional NMDA receptor-mediated neurotransmission (Katane & Homma, 2011). D- and L-aspartate were not separated in our study (and in most studies on aspartate reported in the literature), but it appears that in future studies examination of the individual isomers would be warranted. Clinically, aspartate has consistently been found to be elevated in the CSF of MS patients, especially in patients with acute neurological impairment and during relapse compared to stable disease (Barkhatova et al., 1998; Sarchielli et al., 2003; Stover et al., 1997). We have found that this elevation in concentration appears to also be reflected in the systemic circulation via plasma samples. One limitation of our study is that this increase in aspartate levels observed in RRMS patients compared to CIS patients was not significant when RRMS patients were compared to healthy controls. This may be a result of small sample size. A significant change in aspartate levels may also be masked due to acute relapse RRMS and stable RRMS patients being aggregated in our study and the lack of ability to distinguish between phases of disease which has been shown to affect aspartate levels in previous studies (Sarchielli et al., 2003).

LSOP concentrations were also found to be significantly higher in the RRMS group compared to controls in our study. Little is known about LSOP in EAE and MS disease pathology and the implications of this result. LSOP is the immediate precursor to L-serine and is also an agonist at the Group III metabotropic glutamate receptors (Antflick et al., 2009). In looking at prior studies, in EAE mice brain tissue, L-serine levels were significantly elevated at onset and peak phases of disease in the spinal cord,

decreased at EAE onset in the brainstem, and elevated at peak and chronic phase in the cerebellum (Musgrave et al., 2011). As MS pathology has been associated with glutamate excitotoxity, and LSOP is a precursor to L-serine, which is involved in the biosynthesis of co-agonists of the NMDA receptors such as D-serine and glycine, LSOP may have potential as a surrogate biomarker for L-serine in MS; however, greater understanding and research is required at this time.

Among the individual NASs studied, median concentration of epiallopregnanolone was significantly lower and ALLO was significantly higher in RRMS patients compared to CIS patients. The significance of decreased epiallopregnanolone is unclear at this time as the body of evidence regarding epiallopregnanolone is limited. Epiallopregnanolone is the 3beta-isomer of ALLO but it does not have the ability to exert the same effects on $GABA_A$ receptors as ALLO. Interestingly, studies have suggested that epiallopregnanolone can selectively antagonize the positive modulation of ALLO and PREG on GABAergic inhibition (Wang et al., 2000).

As no prior studies have found significant differences when comparing ALLO levels in CIS to RRMS directly, the finding of elevated ALLO levels in RRMS patients compared to CIS in this study may represent a novel finding where ALLO levels can assist in the differentiation between CIS and MS progression. However, further study would be required before such a conclusion could be drawn. This finding may also potentially contradict prior studies where ALLO has been found to be neuroprotective and reduced in both animal models and clinical MS patients compared to healthy controls. As discussed in chapter 2.3.1, ALLO is believed to reduce demyelination,

axonal injury, microglial reactivity, and lymphocyte infiltration in EAE mice (Noorbakhsh et al., 2014). As CIS is a demyelinating event isolated in time, due to the comparatively short duration of disease and isolated nature of CIS compared to RRMS, elevated ALLO levels in the RRMS cohort compared to the CIS cohort may reflect study design and recruitment limitations relating to the phase of disease at which time plasma samples were drawn. For example, if recruitment and blood draw for the CIS cohort is in close proximity to the initial acute demyelinating event and initial visit, ALLO would likely be decreased in the context of acute demyelination, whereas participants in the RRMS cohort are a mix of acute, stable and chronic RRMS patients where changes in individual ALLO concentrations would likely be varied depending on acuity, activity and chronicity of disease. NAS levels have also been shown to vary throughout the menstrual cycle (Foroughipour et al., 2012), and as this information was not collected in our present study, this presents a potential future direction in order to better understand the role of ALLO and other NASs in CIS and MS pathology. ALLO has been previously found to be elevated when male and female RRMS patients with Gd-enhanced lesions were compared to respective gender groups without Gd-enhancing lesions (Orefice et al., 2016). This would be another notable area that warrants future study and may assist in understanding the role of ALLO in CIS and MS.

Overall, our study did not find any significant changes in the remaining NASs levels measured in RRMS, CIS and healthy controls. This lack of significant change from healthy controls may be a result of a variety of factors including small sample size, variations in detectable concentrations of NASs in peripheral circulation compared to CNS where they are synthesized, the complex and nonspecific action of NASs which act

on multiple neurotransmitter receptors, and other factors including but not limited to phase of disease at recruitment, the role of sex hormones in NAS production, and variations in hormone levels during different phases of the menstrual cycle in reproductive age women. Further investigation would be required.

5.2. Correlates of change in disease disability scores

Disability analyses focused on the EDSS and ARMSS values of CIS and RRMS groups. The two cohorts were combined in this analysis to address the main research question of determining correlates of progression in disease score, which occur in both cohorts and occur irrespective of the cohort category. In addition, combining the two cohorts allowed for greater power in this study. An increase in EDSS across both groups was significantly and inversely correlated with plasma PREG concentrations ($r = -0.267$; $p = 0.041$). There is currently a limited understanding of the role of PREG in MS. However, PREG is a progesterone-derived NAS that is closely related to and shares similar properties with ALLO. ALLO is one of the most studied NASs in MS and has been found to be neuroprotective. Previous studies suggest that the concentration of ALLO is reduced in EAE and MS (Noorbakhsh et al., 2011) and the inverse correlation we found in our study with PREG may reflect a similar mechanism of action and effect. In our study, ALLO was not significantly correlated to change in disability scores. Further study would be required to draw any definitive conclusions. An increase in EDSS scores was also significantly and positively correlated with disease duration at final EDSS assessment ($r = 0.310$; $p = 0.013$). This result is in keeping with current understanding of MS clinical pathology in that the longer the disease duration, the greater

potential for increase in disease severity, morbidity, and accumulation or residual disability between acute relapses.

These findings were accompanied by a non-significant positive correlation with THDOC concentrations in plasma ($r = 0.237$; $p = 0.068$), depression ($r = 0.231$; $p = 0.071$) and cardiovascular disease ($r = 0.236$; $p = 0.062$). In addition to change in EDSS scores, a nonsignificant positive correlation was also found in this study with change in ARMSS scores and THDOC concentration. Although not well studied, THDOC is a positive allosteric modulator of the $GABA_A$ receptor and under physiologic stress, THDOC activates the HPA axis, resulting in increased GABAergic transmission. Though not significant, this positive trend in correlation would be in keeping with current understanding of CIS and RRMS where the body is under physiologic stress in the case of an acute demyelinating event, and subsequent HPA axis activation and elevation in THDOC could occur. Studies have also shown that physiological stress has the ability to induce an increase in THDOC and subsequently activate $GABA_A$ receptors, making THDOC a promising biomarker of interest in stress-sensitive conditions such as MS and warranting further study. To our knowledge, THDOC has not been previously been studied in EAE and MS. The non-significant positive correlation with depression found in our study could represent the underlying role of inflammation in both MS and MDD pathology or may reflect the fact that depression is more common in individuals with chronic diseases, such as in the case of RRMS. The non-significant positive trend of change in EDSS scores with cardiovascular disease may reflect the decreased ability to exercise with increasing disability, or may reflect a state of increased inflammation in the body with cardiovascular plaque buildup and associated conditions such as metabolic

syndrome. More research and a higher-powered study would be necessary to make any conclusions relating to these trends.

Changes in ARMSS scores were not significantly correlated with levels of any NAS or amino acid in this study, but were non-significantly associated with THDOC concentrations and disease duration at final EDSS assessment which are congruent with correlates of change findings in relation to change in EDSS discussed in this section.

5.3. Predictors of change in disability scores

Variables in the first block accounted for 14.3% of variance, and only disease duration at the final EDSS assessment was significantly associated with an increase in change scores. This finding is in keeping with the current understanding of MS disease pathology in that disability scores increase over time. Adding PREG and THDOC to the model further explained 11.0% of the variance. Plasma concentrations of THDOC were significantly positively associated with increases in EDSS, whereas PREG was inversely associated but not significant. As previously discussed, under normal physiological conditions, THDOC potentiates the inhibitory effects of GABA on corticotropin releasing hormone (CRH) neurons, resulting in decreased HPA axis activity. However, under physiologic stress, such as in an acute MS flare or relapse, THDOC activates the HPA axis, resulting in excitatory GABAergic transmission and likely may play a role in the observed neurotoxicity and neurodegeneration seen in MS. In terms of the inverse trend seen in step two of this model with PREG, there is limited study into the role of PREG in MS and a greater understanding is necessary to draw any conclusions. As previously discussed, PREG is produced from progesterone and, like ALLO, is a positive allosteric

modulator of the $GABA_A$ receptor. These findings suggest some potential for PREG and THDOC to be further explored as potential plasma biomarkers in MS. Adding depression to the model did not significantly increase the explained variance, and was not found to be significantly associated to change in EDSS scores. A limitation of this current study is our sample size in which a larger study population would allow for greater analysis and modeling of potential predictors of change in disability scores. Replication of this study and modeling with a larger sample size would be of interest to determine if these findings are reproducible.

5.4. Clinical Cohort

The patient groups were similar in terms of demographic and clinical features including comorbidities and treatment course. As expected, disease durations at the study onset and completion were significantly shorter in the CIS versus RRMS groups, CIS patients were less likely to receive DMTs or corticosteroids during the study period, initial EDSS scores were significantly lower in the CIS group compared to the RRMS group, and both initial and final ARMSS scores were found to be significantly higher in the RRMS group. A study result of interest is that the likelihood of depression was significantly greater in the RRMS group than in the control group. This is in keeping with previous findings by Marrie et al. of increased incidence and prevalence of MDD in MS populations (Marrie et al., 2015). In the current study, initial and final original MSSS scores and updated MSSS scores did not differ between groups and did not change significantly during the study period. A lack of change in MSSS scores may be a result of a limitation in our study as we were unable to calculate MSSS scores for a portion of the patients due to missing information, or may reflect that a study period of longer duration

may be required in order for differences to be reflected, particularly as one key variable in the algorithm is the years since onset of MS.

Over half (51.61%) of CIS patients in the study converted to RRMS over a median period of 1.85 years. This finding is in keeping with previous literature where, depending on the localization of the original lesion, previous studies have suggested that 10-85% of CIS converts to MS pathology (Miller et al., 2012).

6. Conclusion

Currently no established plasma biomarkers of MS disease and progression exist. In this study, plasma amino acid and NAS levels were measured in controls (n=17) and CIS (n=31) and RRMS (n=33) patient cohorts and patient demographic and clinical variables were collected in an attempt to identify correlates of change and trends with progression and severity in disability scores. The results of this study provide evidence that concentrations of specific plasma amino acids and NASs do vary between controls, CIS, and RRMS patients. Though the exact mechanisms of MS pathogenesis are not completely understood, amino acids and NASs hold promise as biomarkers of MS disease progression in the context of neuroinflammation and immunomodulation, and warrant further investigation.

Limitations of the current study include the availability of patient information regarding phase of disease at blood draw, information regarding the last menstrual period in reproductive women (which may have an effect on NAS levels), and generalizability due to sample size. Although this study has identified several significant changes in amino acid and NAS levels that are reflected in plasma in MS pathology, changes in concentrations of amino acids and NASs may become subtle, mitigated or not reflected in plasma in comparison to the CNS where pathologic processes initiate. Future directions of interest include replication of this study with a larger patient cohort to allow for greater subgroup analysis, concurrent plasma and CSF measurements and comparisons, the inclusion of neuroimaging data, and modification of the study design to ensure that phase of disease and sex differences in steroid levels are taken into account. Identification of

neuroinflammatory biomarkers in MS is fundamental to the diagnosis, prognostication, and treatment of this debilitating condition.

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